

Splenda Alters Gut Microflora and Increases Intestinal P-Glycoprotein and Cytochrome P-450 in Male Rats

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Splenda is comprised of the high-potency artificial sweetener sucralose (1.1%) and the fillers maltodextrin and glucose. Splenda was administered by oral gavage at 100, 300, 500, or 1000 mg/kg to male Sprague-Dawley rats for 12-wk, during which fecal samples were collected weekly for bacterial analysis and measurement of fecal pH. After 12-wk, half of the animals from each treatment group were sacrificed to determine the intestinal expression of the membrane efflux transporter P-glycoprotein (P-gp) and the cytochrome P-450 (CYP) metabolism system by Western blot. The remaining animals were allowed to recover for an additional 12-wk, and further assessments of fecal microflora, fecal pH, and expression of P-gp and CYP were determined. At the end of the 12-wk treatment period, the numbers of total anaerobes, bifidobacteria, lactobacilli, *Bacteroides*, clostridia, and total aerobic bacteria were significantly decreased; however, there was no significant treatment effect on enterobacteria. Splenda also increased fecal pH and enhanced the expression of P-gp by 2.43-fold, CYP3A4 by 2.51-fold, and CYP2D1 by 3.49-fold. Following the 12-wk recovery period, only the total anaerobes and bifidobacteria remained significantly depressed, whereas pH values, P-gp, and CYP3A4 and CYP2D1 remained elevated. These changes occurred at Splenda dosages that contained sucralose at 1.1–11 mg/kg (the US FDA Acceptable Daily Intake for sucralose is 5 mg/kg). Evidence indicates that a 12-wk administration of Splenda exerted numerous adverse effects, including (1) reduction in beneficial fecal microflora, (2) increased fecal pH, and (3) enhanced expression levels of P-gp, CYP3A4, and CYP2D1, which are known to limit the bioavailability of orally administered drugs.

The artificial high-potency sweetening compound sucralose is a chlorinated disaccharide with the chemical formula

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1,6-dichloro-1,6-dideoxy- β -D-fructofuranosyl-4-chloro-4-deoxy- α -D-galactopyranoside (*Federal Register*, 1998). Sucralose is ubiquitous in the world food supply as an ingredient in over 4000 products, including tabletop sweeteners and sugar substitutes (e.g., Splenda), baked goods, beverages such as soft drinks, coffee, and tea, breakfast cereals, chewing gum, desserts, and pharmaceutical products (International Food Information Council Foundation, 2004). Because sucralose is approximately 600 times sweeter than sucrose by weight (Schiffman & Gatlin, 1993; Schiffman et al., 2008), sucralose formulations such as Splenda utilize fillers including maltodextrin and glucose for volume. In acidic environments and at elevated temperatures, sucralose hydrolyzes over time to its constituent monosaccharides 1,6-dichloro-1,6-dideoxyfructose (1,6-DCF) and 4-chloro-4-deoxy-galactose (4-CG) (Grice & Goldsmith, 2000).

Pharmacokinetics and metabolism studies of sucralose have shown that the majority of ingested sucralose (approximately 65–95% depending on the species) is not absorbed from the gastrointestinal tract (GIT) but rather was excreted in the feces (Sims et al., 2000; Roberts et al., 2000; *Federal Register*, 1998). The low absorption of sucralose from the GIT is surprising, because this sweetener is an organochlorine molecule with appreciable lipid solubility (Miller, 1991; Wallis, 1993; Yotka et al., 1992). The low bioavailability of sucralose suggests that it is likely extruded back into the intestinal lumen during first-pass metabolism in the GIT. The concentrations of many orally consumed compounds including drugs and nutrients are reduced during first-pass metabolism in the small intestine by the membrane efflux transporter P-glycoprotein (P-gp) and the cytochrome P-450 (CYP) metabolism system. P-gp extrudes these compounds from the intestinal walls back to the lumen and/or CYP enzymes metabolize the compounds. P-gp and CYP are both involved in xenobiotic detoxification in the intestine and liver of many diverse chemicals, including organochlorine compounds (Lanning et al., 1996; Bain & LeBlanc, 1996; Abou-Donia et al., 2001; Poet et al., 2003; El-Masry & Abou-Donia, 2003, 2006; Abu-Qare et al., 2003; Leslie et al., 2005).

Adverse consequences from the elevated presence of sucralose in the GIT were reported in animal models including cecal enlargement in rats (Goldsmith, 2000) and GIT distress in rabbits which was at times extensive (Kille et al., 2000). In a subsequent study, sucralose in a Comet test was found to induce DNA damage in the GIT tract of mice (Sasaki et al., 2002). Bacteria in culture do not utilize sucralose as a carbon source (Young & Bowen, 1990; Labare & Alexander, 1994), and this finding raises the question of whether the presence of unabsorbed sucralose in the GIT might potentially affect the intestinal microbial milieu. Gut microflora carry out many important functions, including (1) fermentation of dietary carbohydrates, (2) salvage of energy as short-chain fatty acids (primarily acetate, propionate, and butyrate), (3) production of vitamins, (4) maintenance of normal immune system functioning, (5) GIT mobility, (6) inhibition of pathogens, and (7) metabolism of drugs (Cummings & Macfarlane, 1997; Holzapfel et al., 1998; Hart et al., 2002; Teitelbaum & Walker, 2002).

The objective of this study was to determine the effects of orally administered Splenda on the composition and number of the major microbial population groups of fecal microflora in the GIT of male Sprague-Dawley rats. The subsequent effects of Splenda treatment were also investigated on body weight, fecal pH, the integrity of the epithelium of the colon, the expression of intestinal membrane P-gp, and the expression of two members of the CYP protein family (CYP 3A4 and CYP2D1).

METHODS

Chemicals

Test Material

“Splenda[®] No Calorie Sweetener, Granular” (McNeil Nutritionals, LLC, Fort Washington, PA) was purchased from the supermarket. The contents were analyzed by Northland Laboratories (Northbrook, IL), using high-performance liquid chromatography (HPLC) for the concentration of the high-potency sweetener sucralose as well as glucose. Moisture content was determined by a vacuum oven (70°C) whereas maltodextrin was estimated by calculation. The analytical tests on Splenda indicated that its contents were: sucralose (1.10%), glucose (1.08%), moisture (4.23%), and maltodextrin (93.59%).

Culture Media

Peptone yeast extract glucose agar (PYG), Rogosa agar, Reinforced Clostridial agar, Brucella agar supplemented with hemin, vitamin K₁, and fluid thioglycollate were obtained from Sigma-Aldrich (St. Louis, MO); defibrinated horse blood, from HemoStat Laboratories (Dixon, CA); MacConkey agar no. 3, from Oxoid (Remel, Inc., Lenexa, KS); and Bacteroides Bile Esculin agar, from Becton Dickinson and Company (Sparks,

MD). All other materials and culture media were of analytical or molecular biology grade and obtained from commercial sources.

Other Chemicals

The polyvinylidene difluoride (PVDF) membrane (Hybond-P) and enhanced chemiluminescence reagents were obtained from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal antibody against P-gp was from Chemicon (Temecula, CA). Rabbit polyclonal antibodies against CYP3A4 or CYP2D1 were from Abcam (Cambridge, MA). The secondary antibodies, goat anti-mouse (for P-gp) or anti-rabbit (for CYP) horseradish peroxidase-conjugated immunoglobulin IgG, were obtained from Amersham Biosciences. β -actin polyclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO). All electrophoresis reagents were from Bio-Rad (Hercules, CA). Hematoxylin and eosin (H&E) stain was obtained from Shandon, Inc. (Pittsburgh, PA).

Animals

Adult male Sprague-Dawley rats weighing 200–240 g were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed at the Duke University Medical Center Vivarium. The rat model was used for this study because it has been studied extensively in investigations of the microbial ecology of the gut. The animals were randomly assigned to control and treatment groups and housed at 21–23°C with a 12-h light/dark cycle. The animals were supplied with feed (Purina certified rodent chow, Ralston Purina, St. Louis, MO) and water ad libitum. The rats were allowed to adjust to their environment for a week before starting the treatment. The protocol for these studies was approved by the Duke University Institutional Animal Care and Use Committee (IACUC).

Treatment Protocol

Five groups ($n = 10$ per group) of rats received water or freshly prepared solutions of Splenda (1 ml/kg) in water by oral gavage for 12-wk at the following dosages (mg/kg body weight/d):

Group 1, Control: water.

Group 2, Splenda: 100 mg/kg/d in water (1.1 mg/kg/d sucralose).

Group 3, Splenda: 300 mg/kg/d in water (3.3 mg/kg/d sucralose).

Group 4, Splenda: 500 mg/kg/d in water (5.5 mg/kg/d sucralose).

Group 5, Splenda: 1000 mg/kg/d in water (11 mg/kg/d sucralose).

These dosage levels were selected because they span the range of values below and above the accepted daily intake (ADI) for sucralose of 5 mg/kg/d established by the U.S. Food and Drug Administration (FDA) (*Federal Register*, 1998). Fresh fecal pellets were collected aseptically using sterile forceps from each rat on the day before beginning of treatment, and every week thereafter, for determination of fecal pH and

bacterial analysis. In addition, the physical state of the fecal samples was classified into one of three categories: formed, unformed, and soft. Body weight was recorded weekly for every animal.

After the 12-wk treatment period, half of the animals from each of the 5 groups were anesthetized by CO₂ followed by decapitation. Subsequently, the small and large intestines were collected. The small intestine was used to evaluate the effect of Splenda on the levels of P-glycoprotein (P-gp) and cytochrome P-450 (CYP). The large intestine was used for determining the histopathological effect of Splenda on colon tissue. The other half of the animals in each group was kept for observation for an additional 12-wk (recovery period), and further assessments of bacteria, body weight, fecal pH, P-gp, and CYP were performed.

Bacteriological Analysis of Fecal Specimens

Fresh fecal samples (approximately 0.5–1g) from individual control and Splenda-treated rats were transferred aseptically into preweighed Eppendorf tubes and transported in anaerobic jars within 20 min after collection to the microbiological lab, where they were immediately processed. A 10% (w/v) suspension was made aseptically with cryoprotective broth (pre-reduced brain–heart infusions broth containing 20% glycerol) and immediately frozen and stored at –80°C until analyzed (Crowther, 1971; Kleessen et al., 1995, 1997). The cryoprotective broth maintains the viability of fecal organisms for a period of 3-wk (Kleessen et al., 1995).

To perform the bacterial counts, thawed samples were first homogenized with a pipette tip and serially diluted to 10⁻⁸ with half strength of pre-reduced fluid thioglycollate (Rath et al., 2001). Aliquots (100 µl) from each dilution were added to petri dishes containing the selective and non-selective agar plates in triplicate (Mevisen-Verhage et al., 1987; Kleessen et al., 1995). The media used for growing and counting different bacterial species were as follows: total aerobes, peptone yeast extract glucose agar (PYG) (Holdeman et al., 1977); total anaerobes, Brucella agar supplemented with hemin, vitamin K₁, and 5% (v/v) defibrinated horse blood (Sutter et al., 1985); lactobacilli, Rogosa agar (Rogosa et al., 1951; MacFaddin, 1985; Sharpe, 1960); enterobacteria, MacConkey no. 3 agar; clostridia, Reinforced Clostridial agar with neomycin sulfate (Hirsch & Grinsted, 1954); bifidobacteria, Beerens agar (Beerens, 1990; Tzortzis et al. 2005); *Bacteroides*, Bacteroides Bile Esculin agar (Macfarlane et al., 1992). Incubation of the inoculated media for anaerobic bacteria (Brucella agar, Rogosa agar, Reinforced Clostridial agar, Bacteroides Bile Esculin agar, and Beerens agar) was carried out at 37°C for 3 d under anaerobic conditions. Plates for the enumeration of aerobic and facultative aerobic bacteria (peptone yeast extract glucose agar and MacConkey agar) were incubated aerobically for 2 d at 37°C.

Colonies were counted using a fully automated colony counting system (aCOLyte colony counter, Synbiosis, USA). All data handling was automated to eliminate transcription errors. The viable counts were expressed as colony-forming units (CFU) per gram of wet weight feces.

Fecal pH Determination

Fresh fecal samples (approximately 0.5–1g) were immediately weighed and diluted with three weight volumes of ice-cold distilled water and homogenized with pipette tips. Samples were centrifuged (3000 × g, 15 min, 20°C), and the pH was measured by a pH electrode (Topping et al., 1993).

Histopathological Assessment of Colon

The colon was fixed in 4% paraformaldehyde (Sigma-Aldrich) for 24 h, and then processed and embedded in paraffin. Four micrometer-thick coronal sections were cut (*n* = 5) and stained with hematoxylin and eosin (H&E) for light microscopy. Sections of the distal colon were examined for the presence and preservation of surface epithelium, scar tissue, distribution of lymphocytes, glandular structure, muscularis mucosa, and muscularis externa. Pathological evaluation was blinded to the identity of the slides.

Determination of P-gp and CYP450 Isozymes in Intestinal Tissues

The effect of Splenda on P-gp and two CYP450 isozymes (CYP3A4 or CYP2D1) was determined by Western blot (Dürr et al., 2000) using homogenates of crude membranes of the distal regions of the small intestine (Iida et al., 2005), which were homogenized with a polytron tissue homogenizer. The Western blots were probed with appropriately diluted antibodies: mouse monoclonal antibody against P-gp and rabbit polyclonal antibodies against CYP3A4 or CYP2D1; CYP2D1 in rat is analogous to CYP2D6 in humans (Laurenzana et al., 1995). The secondary antibodies utilized were goat anti-mouse (for P-gp) or goat anti-rabbit (for CYP) horseradish peroxidase-conjugated IgG. β-Actin was used as a control. Integrated optical densities (IODs) were obtained using the Bio-Rad software program Quantity One (version 4.2).

Statistical Analysis

For each dependent variable, an analysis of variance was used to test whether there was any overall effect of group (all five groups, i.e., four Splenda treatment groups plus control group). When the overall effect of the analysis of variance was significant at *p* < .05, specific contrasts (*t*-tests) were used to determine whether each treatment group was significantly different from control. A *t*-test with a *p* value < .05 was considered significant.

RESULTS

Clinical Condition

No animals died during the treatment or the recovery period. There were no visual differences noted in general condition between Splenda-treated animals and controls.

Physical State of Feces

The majority of the feces in all treatment groups were formed. However, intermittent incidences of unformed or soft feces occurred during the treatment phase: control (none), 100 mg/kg (10.8%), 300 mg/kg (10.8%), 500 mg/kg (14.2%), and 1000 mg/kg (14.2%). Most of the unformed or soft feces in the Splenda groups occurred during the first weeks of treatment, and no overt diarrhea was observed in any animals during the study. By wk 12 of treatment, the feces for all groups were formed. No soft or unformed feces occurred during the recovery period.

Body Weight

During the 12-wk treatment period, all control and Splenda-treated animals gained weight. Body weight expressed as percent of initial weight (baseline) for each week of the treatment and recovery periods is given in Figure 1, a and b, respectively. At the lowest Splenda dose level of 100 mg/kg, rats showed a significant increase in body weight gain relative to controls; the changes at 300 mg/kg, 500 mg/kg, and 1000 mg/kg did not differ significantly from controls. The mean rise in body

weight from baseline to 12-wk was: control (93.1%), 100 mg/kg (104.0%), 300 mg/kg (100.7%), 500 mg/kg (101.5%), and 1000 mg/kg (88.5%). The mean increases in body weight from baseline to 24-wk (after recovery) were: control (116.6%), 100 mg/kg (133.7%), 300 mg/kg (123.5%), 500 mg/kg (137.9%), and 1000 mg/kg (124.5%). After the recovery from the 100 mg/kg dose, there continued to be a significant increase in body weight (17.1%) relative to controls. There was also a significant increase in body weight (21.3%) after recovery from consumption of the 500-mg/kg dose relative to controls.

Effect of Splenda on Major Fecal Bacteria

The means for the number of colony-forming units (CFU) for the control group and Splenda groups (10 rats per group) over the 12-wk treatment period are plotted in Figure 2 (a–g). The means for CFU for the 5 groups (5 rats per group) for the 12-wk recovery period are plotted in Figure 3 (a–g). Generally, fecal bacteria from control animals continued to increase throughout the treatment period and peaked at 6–8-wk following the onset of the experiment, after which they began to decrease. The number of total anaerobes and aerobic bacteria began to decrease immediately after the beginning of oral administration of Splenda. By the end of the 12-wk dosing period, at the lowest dose (100 mg/kg/d) of Splenda, the number of total anaerobes was reduced by 49.8% relative to control. In addition, the bacterial counts of bifidobacteria, lactobacilli, and *Bacteroides* were reduced by 36.9%, 39.1%, and 67.5% respectively (Table 1). Although the number of

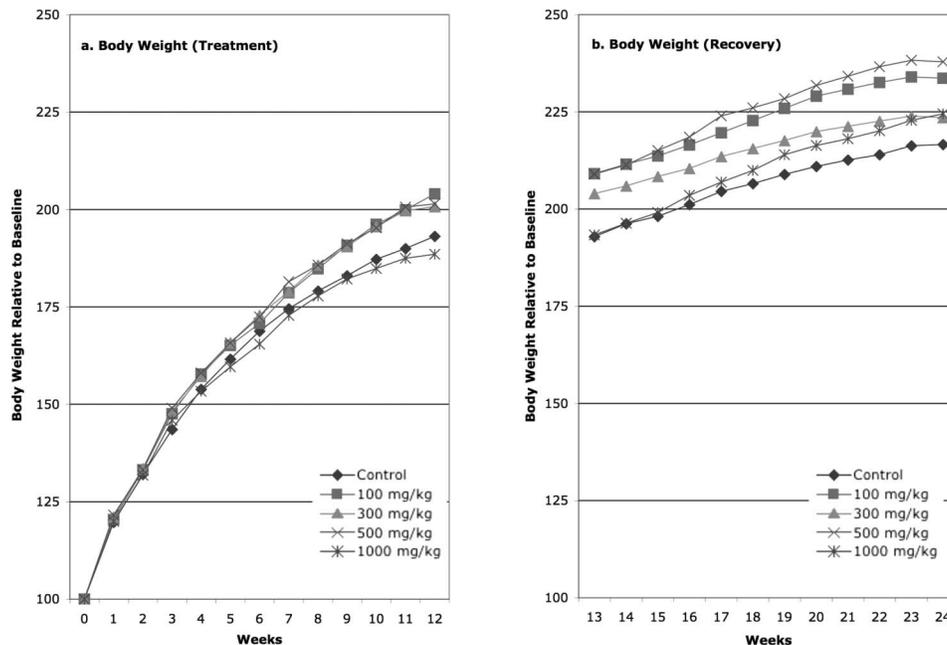


FIG. 1. The percent difference in body weight of rats over the 24-wk experimental period relative to initial weight (baseline, wk 0): (a) during 12-wk of treatment with Splenda and (b) during 12-wk after Splenda discontinuation (the recovery period).

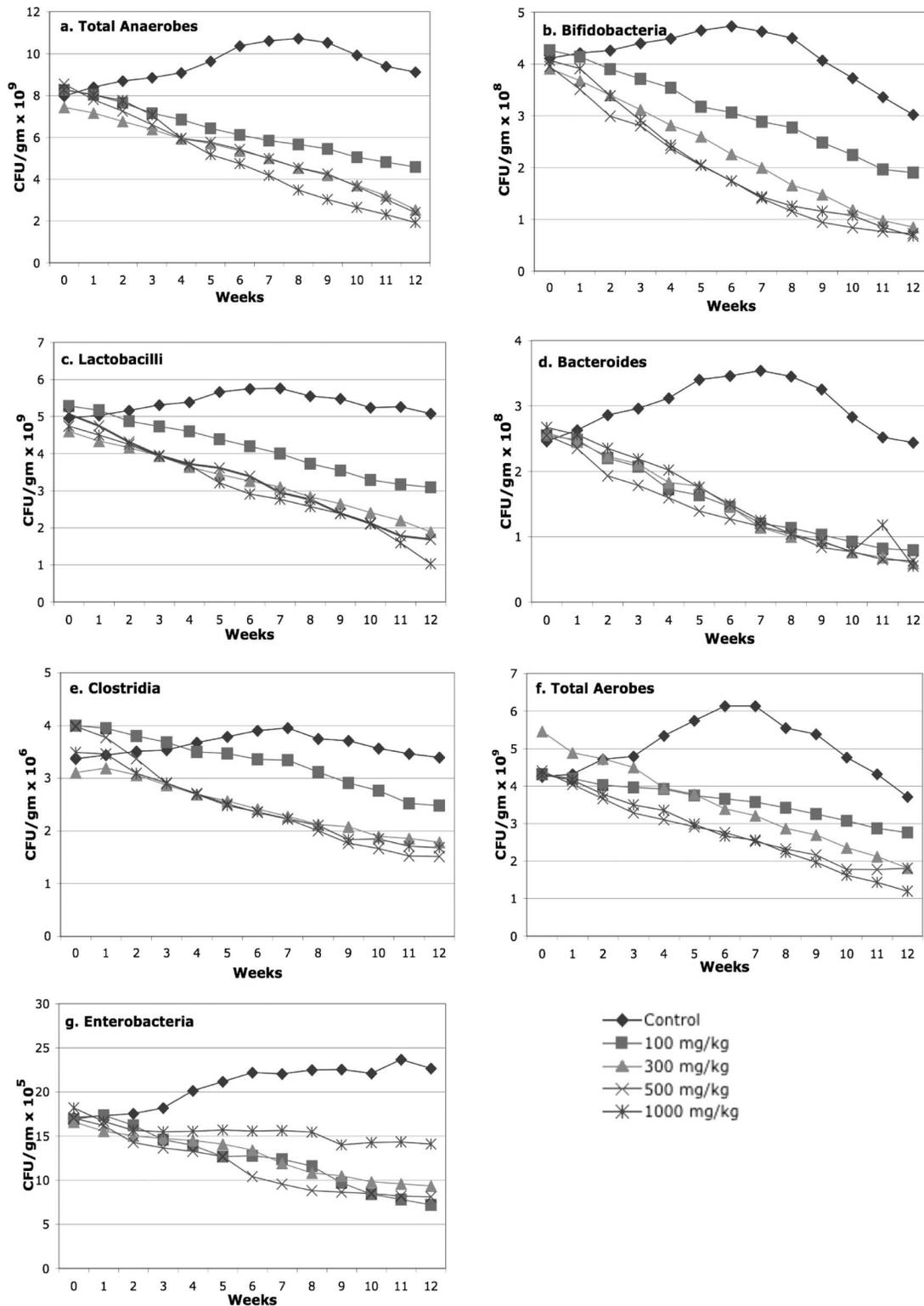


FIG. 2. Effect of Splenda on bacterial counts in rat feces over 12-wk with daily gavage at doses of 100, 300, 500, and 1000 mg/kg body weight: (a) total anaerobes, (b) bifidobacteria, (c) lactobacilli, (d) *Bacteroides*, (e) clostridia, (f) total aerobes, and (g) enterobacteria, respectively. The viable counts are expressed as colony-forming units (CFU) per gram of wet weight feces. Values are means for 10 rats. Splenda treatments for which the mean number of CFU for each bacterial type is statistically different from control at the end of the 12-wk treatment period are given in Table 1.

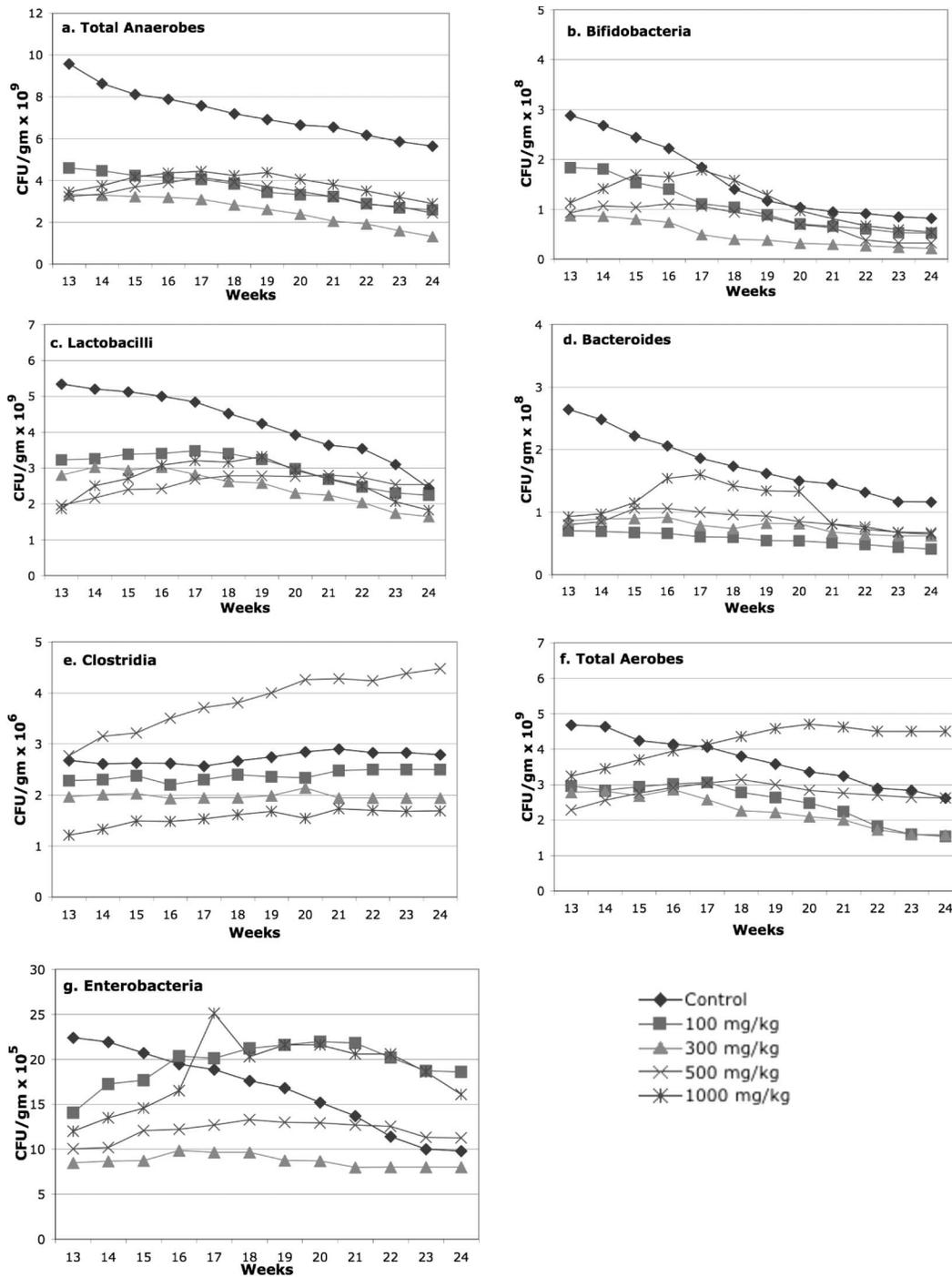


FIG. 3. Bacterial viable counts in rat feces determined after discontinuation of Splenda treatment over 12-wk (recovery period): (a) total anaerobes, (b) bifidobacteria, (c) lactobacilli, (d) *Bacteroides*, (e) clostridia, (f) total aerobes, and (g) enterobacteria, respectively. The viable counts are expressed as colony forming-units (CFU) per gram of wet weight feces. Values are means for five rats. Splenda treatments for which the mean number of CFU for each bacterial type is statistically different from control at the end of the recovery period are given in Table 1.

clostridia decreased throughout the treatment period, it was not significantly different from control at 100 mg/kg. Higher doses of Splenda (300, 500, and 1000 mg/kg/d) produced significant reduction in the number of total anaerobes and other anaerobic

bacteria, ranging from 47.4 to 79.7% of control, by the end of the 12-wk treatment period.

Oral administration of 100 mg/kg Splenda for 12-wk produced no significant change in the number of total

TABLE 1
Percent Difference in Bacterial Counts for Splenda-Treated Rats Relative to Untreated Control at the End of the 12-wk Treatment Period and at the End of the 12-wk Recovery, Followed by *p* Values

Treatment ^a	100 mg/kg		300 mg/kg		500 mg/kg		1000 mg/kg	
	Percent change ^b	<i>p</i> Value ^c	Percent change	<i>p</i> Value	Percent change	<i>p</i> Value	Percent change	<i>p</i> Value
End of treatment ^d								
Total anaerobes	-49.8	<0.001	-72.2	<0.00001	-73.7	<0.00001	-78.9	<0.00001
Bifidobacteria	-36.9	<0.05	-71.9	<0.0001	-76.0	<0.0001	-77.7	<0.0001
Lactobacilli	-39.1	<0.01	-62.8	<0.00001	-66.8	<0.00001	-79.7	<0.00001
<i>Bacteroides</i>	-67.5	<0.00001	-75.6	<0.00001	-74.1	<0.00001	-77.5	<0.00001
Clostridia	—	NS	-47.4	<0.05	-55.3	<0.05	-50.5	<0.05
Total aerobes	—	NS	-51.2	<0.05	-51.2	<0.05	-67.8	<0.01
Enterobacteria	—	NS	—	NS	—	NS	—	NS
End of recovery ^e								
Total anaerobes	-53.9	<0.05	-76.6	<0.0001	-56.7	<0.05	-48.6	<0.05
Bifidobacteria	—	NS	-74.6	<0.001	-61.1	<0.05	—	NS
Lactobacilli	—	NS	—	NS	—	NS	—	NS
<i>Bacteroides</i>	—	NS	—	NS	—	NS	—	NS
Clostridia	—	NS	—	NS	—	NS	—	NS
Total aerobes	—	NS	—	NS	—	NS	—	NS
Enterobacteria	—	NS	—	NS	—	NS	—	NS

^aRats treated by oral gavage with Splenda at doses of 100, 300, 500, and 1000 mg/kg body weight/d for 12-wk. Fecal samples were collected weekly over a 24-wk experimental period (12-wk of treatment and 12-wk of recovery).

^bPercent difference in the mean values of bacterial counts of Splenda-treated groups relative to untreated controls.

^cValues are significantly different from the control group according to Student's *t*-test. NS: not significantly different from the control group according to Student's *t*-test.

^dPercent difference after the 12-wk treatment.

^ePercent difference after 12-wk discontinuation of Splenda treatment.

aerobes compared to control. In contrast, higher doses of Splenda (300, 500, or 1000 mg/kg/d) resulted in significant reduction of the numbers of total aerobes, which ranged from 51.2 to 67.8% compared to control groups. The enterobacteria count was not significantly different from control at any treatment level with Splenda throughout the dosing period.

During the recovery period, control groups exhibited a general trend toward reduction of all bacterial types with increasing animal age that tended to begin toward the end of the treatment period and continued through the recovery period (Figure 3). In addition, at the end of the recovery period, total anaerobes continued to be significantly lower at all Splenda dosage levels, ranging from 48.6 to 76.6% reductions relative to control (Table 1), suggesting that, as a group, they did not recover. Bifidobacteria at 300 and 500 mg/kg continued to be reduced. Neither lactobacilli, *Bacteroides*, clostridia, total aerobes, nor enterobacteria were significantly different from controls during the recovery period.

Fecal pH

The mean fecal pH during each week of treatment and recovery is given in Figure 4 (a and b). Relative to control, at the end of 12-wk of Splenda treatment at dosages of 100, 300, 500, or 1000 mg/kg/d, there were significant increases in pH values of 5.24, 6.16, 6.32, and 7.4% respectively. At the end of the recovery period, the fecal pH values following treatment with 300, 500, or 1000 mg/kg/d remained significantly elevated by 3.54, 3.24, and 4.13% respectively compared with control.

Histological Assessments

Histological assessments of the colon after the 12-wk treatment with Splenda and after the 12-wk recovery period are given in Table 2. At the end of the 12-wk treatment with Splenda, numerous alterations were observed that did not occur in control animals, including lymphocytic infiltrates into epithelium, epithelial scarring, mild depletion of goblet cells, glandular disorganization, and focally dilated vessels stuffed with

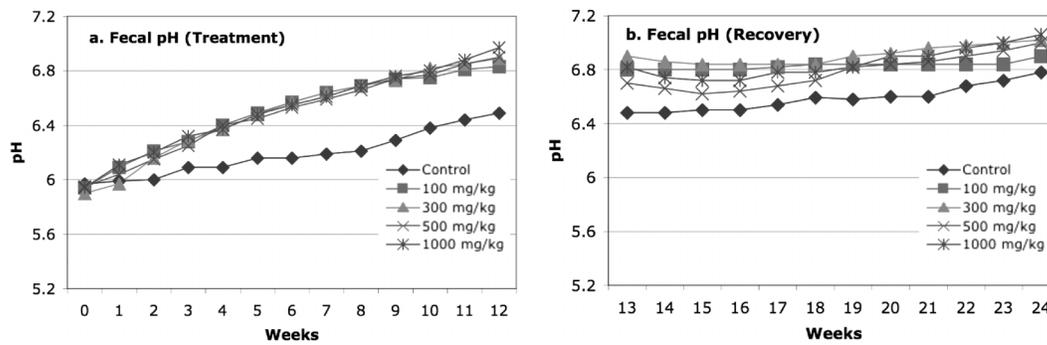


FIG. 4. Effect of Splenda on fecal pH of rats: (a) mean fecal pH at baseline (wk 0) and during 12-wk of treatment with Splenda; (b) mean fecal pH during the 12-wk recovery period.

intravascular lymphocytes (the last being an artifact that was almost always related to large nodular lymphoid aggregates within the submucosa and apparently related to a procurement effect at autopsy that occurred when the microtome cut the colon into thin sections). A range of apparently age-related histological changes (e.g., loss of superficial mucin) also occurred during the recovery in all animals including the controls.

Intestinal P-Glycoprotein

The Western blot analysis for P-gp and β -actin (control protein) is shown in Figure 5a; an immunoreactive band corresponding to P-gp with an apparent molecular mass of 170-kD was detected while β -actin is shown as an immunoreactive band with an apparent molecular mass of 45-kD. P-gp level was quantified by densitometry relative to β -actin. Figure 6a shows the relative P-glycoprotein expression. Relative to the control the 12-wk treatment with 100 mg/kg Splenda exerted no apparent effect on the expression of P-gp, whereas the expression of P-gp with 300 mg/kg Splenda increased markedly by 143.5% and with 500 mg/kg by 122.6%, while the expression of P-gp at 1000 mg/kg decreased significantly by 64% at the end of the 12-wk treatment. These results indicate that P-gp exhibited a 2.43-fold rise at 300 mg/kg and a 2.23-fold increase at 500 mg/kg at the end of the treatment period.

At the end of the 12-wk recovery from 100 mg/kg Splenda, there was no effect on P-gp expression, whereas P-gp was significantly elevated after recovery from 300 mg/kg Splenda by 16%, from 500 mg/kg by 56.8%, and from 1000 mg/kg by 82.2% relative to the control. Thus, after the 12-wk recovery period, P-gp exhibited a 1.16-fold rise at 300 mg/kg, a 1.57-fold increase at 500 mg/kg, and a 1.82-fold elevation at 1000 mg/kg.

Intestinal CYP3A4 and CYP2D1

The Western blot analyses for CYP3A4 and CYP2D1 are shown in Figure 5b. CYP3A4 is shown as a 46-kD protein

band and CYP2D1 is shown as a 57-kD protein band. CYP3A4 and CYP2D1 levels were quantified by densitometry relative to β -actin. The relative expression of CYP3A4 and CYP2D1 is shown in Figure 6, b and c, respectively.

Relative to control the 12-wk treatment with 100 mg/kg Splenda exerted no apparent effect on the expression of CYP3A4 and CYP2D1. The expression of CYP3A4 at 300 mg/kg increased markedly by 43.5%, at 500 mg/kg by 70%, and at 1000 mg/kg by 151.3% relative to control. The expression of CYP2D1 at 300 mg/kg increased markedly by 36.7%, at 500 mg/kg by 152.1%, and at 1000 mg/kg by 249.3%. Overall, the results show that CYP3A4 increased 1.43-fold at 300 mg/kg, 1.7-fold at 500 mg/kg, and 2.51-fold at 1000 mg/kg at the end of the 12-wk treatment period. For CYP2D1, there was a 1.37-fold rise at 300 mg/kg, a 2.52-fold elevation at 500 mg/kg, and a 3.49-fold increase at 1000 mg/kg at the end of the treatment period.

After recovery, the expression of CYP3A4 was significantly increased by 22.4% only at 1000 mg/kg, and the expression of CYP2D1 at 500 mg/kg was significantly increased by 32.9% and at 1000 mg/kg by 22.1% relative to control. Thus, at the end of the 12-wk recovery period, CYP3A4 exhibited a 1.22-fold rise at 1000 mg/kg relative to control; CYP2D1 exhibited a 1.33-fold rise at 500 mg/kg and a 1.22-fold increase at 1000 mg/kg.

DISCUSSION

This study showed that intake of Splenda for 12-wk exerted several adverse effects on the intestines of male Sprague-Dawley rats, including a significant decrease in beneficial intestinal bacteria, elevated fecal pH, histopathological changes in the colon, increased body weight, and enhanced intestinal expression of P-gp, CYP3A4, and CYP2D1. Furthermore, several parameters continued to differ from control values after a 12-wk discontinuation of Splenda, including decreased total anaerobes (at all dosages of Splenda), increased body weight (100 and 500 mg/kg/d), and enhanced

TABLE 2
Histological Assessments of Colon From Rats After 12-wk Treatment With Splenda and After 12-wk Recovery

Histological assessment of colon	Percent of animals showing histological changes									
	Treatment					Recovery				
	Control	100 mg/kg	300 mg/kg	500 mg/kg	1000 mg/kg	Control	100 mg/kg	300 mg/kg	500 mg/kg	1000 mg/kg
Lymphocytic infiltrates into epithelium	NC	NC	20%	20%	40%	NC	NC	NC	NC	NC
Epithelial scarring	NC	NC	NC	NC	20%	NC	NC	NC	NC	NC
Intravascular lymphocytes (a.k.a. vessels filled with lymphocytes)	NC	NC	20%	20%	40%	NC	NC	40%	40%	20%
Atrophy, disorganization, architectural disruption, or inflammation of glands	NC	NC	NC	20%	NC	20%	100%	80%	80%	20%
Submucosal (and/or lamina propria) lymphoid aggregates, lymphoid follicles	20%	NC	NC	20%	40%	40%	100%	60%	40%	20%
Mild depletion of goblet cells, loss of superficial mucin	NC	20%	20%	NC	NC	80%	100%	100%	80%	60%

Note. NC, no changes.

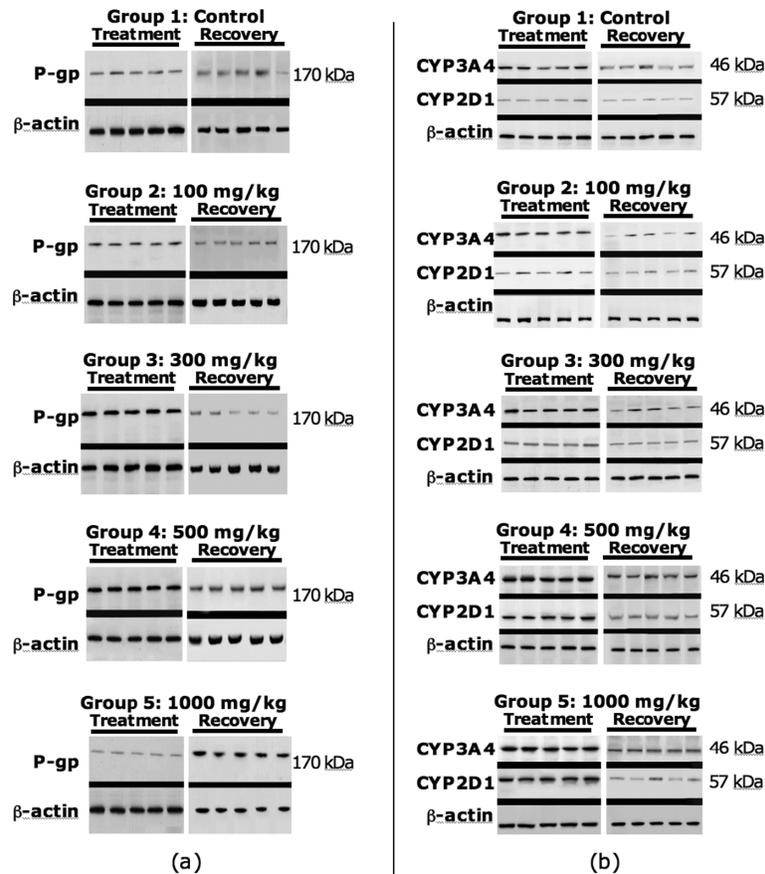


FIG. 5. Western immunoblot showing expression of P-gp, CYP3A4, and CYP2D1 in the small intestine from control and Splenda-treated rats at the end of 12-wk of treatment and after the recovery period. (a) An immunoreactive band corresponding to P-gp with an apparent molecular mass of 170-kDa was detected in the membrane protein along with a β -actin band with an apparent molecular mass of 45-kDa. (b) CYP3A4 appeared as an immunoreactive band with an apparent molecular mass of 46-kDa and CYP2D1 is shown as 57-kDa protein band. P-gp, CYP3A4, and CYP2D1 were quantified by densitometry relative to β -actin.

P-gp expression (300, 500, and 1000 mg/kg/d), CYP3A4 expression (1000 mg/kg/d), and CYP2D1 expression (500 and 1000 mg/kg/d). Table 3 summarizes these effects induced at each Splenda dosage.

The increase in body weight at the low dose of Splenda (100 mg/kg) is in agreement with the recent findings that composition of intestinal bacteria plays a major role in body weight regulation (Bäckhed et al., 2004; Ley et al., 2006; Turnbaugh et al., 2006). It is unlikely that the increased body weight at 100 mg/kg is due to increased food consumption because a previous 26-wk gavage study in rats (Goldsmith, 2000) found no statistically significant differences in food intake by sucralose-treated rats when compared with controls. Another potential cause of this body weight gain is the recent finding that sucralose modulates the Na^+ -glucose transporter (Margolskee et al., 2007) and glucagon-like peptide-1 (Jang et al., 2007) in the intestinal lumen. Thus, the elevated body weight after low-dose Splenda treatment as well as after recovery from Splenda appears to be a form of body weight dysregulation. The lack of a dose-response effect of Splenda

on body weight is likely due to the combined elevation of both intestinal P-gp and CYP that affected the bioavailability of Splenda. At the higher concentrations, less Splenda was absorbed due to the increase in the expression of both P-gp and CYP proteins.

The present finding that Splenda reduces the number of intestinal bacteria is consistent with the observation that bacteria in culture do not utilize sucralose as a carbon source (Young & Bowen, 1990; Labare & Alexander, 1994). Data also agree with a previous report that incorporation of sucralose into glucose agar medium produced total inhibition of growth of several *Streptococcus* spp. and of *Actinomyces viscosus* (Young & Bowen, 1990). In the current study, the intake of Splenda by rats significantly reduced the number of indigenous intestinal bacteria resident in the gut, with the greatest suppression for the generally beneficial anaerobes (e.g., bifidobacteria, lactobacilli, and *Bacteroides*) and with less inhibition for bacteria with mostly detrimental effects (e.g., enterobacteria). Disruption in the number and state of balance of intestinal microflora may potentially interfere with many essential gut functions,

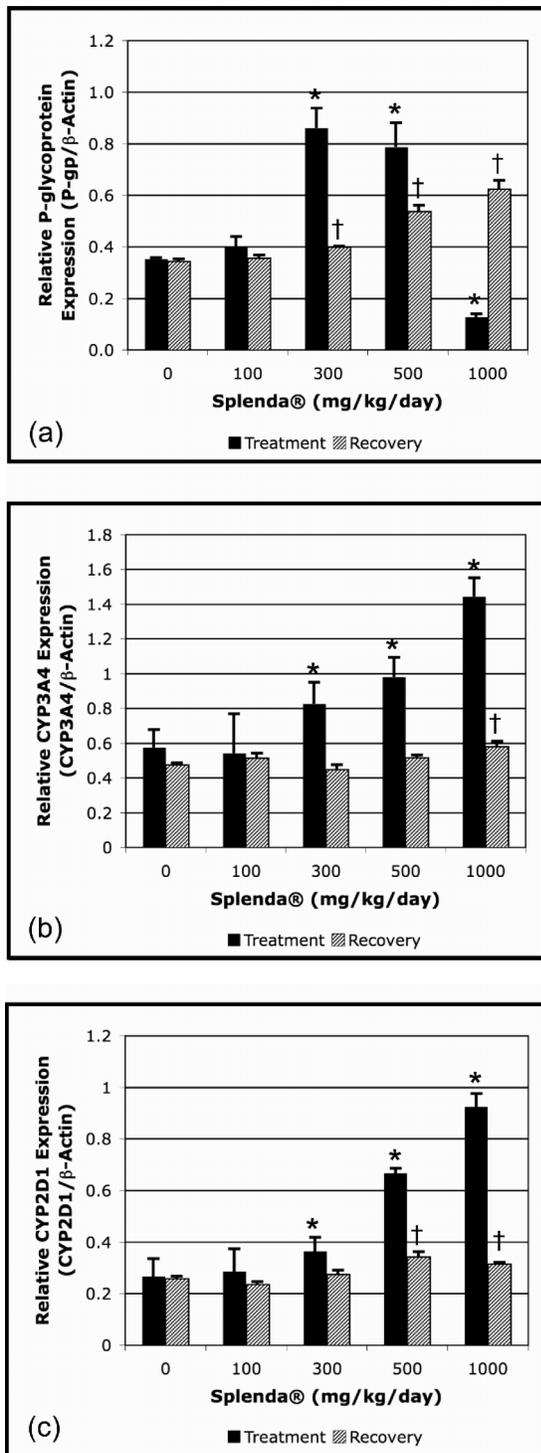


FIG. 6. Quantification of P-gp, CYP3A4, and CYP2D1 expression in the rat membrane protein from the small intestine analyzed by Western blot. Expression of P-gp, CYP3A4, and CYP2D1 was quantified by densitometry relative to β -actin. (a) Relative P-glycoprotein expression, (b) relative CYP3A4 expression, and (c) relative CYP2D1 expression. Values marked with an asterisk are significantly different from the control group at the end of the treatment period. The dagger indicates a significant difference from the control at the end of the recovery period.

including nutrient metabolism, normal immune system functioning, gastrointestinal mobility, inhibition of pathogens (Cummings & Macfarlane, 1997; Holzapfel et al., 1998; Hart et al., 2002), vitamin synthesis (B group and K) (Albert et al., 1980; Hill, 1997; Shearer, 1995), and metabolism of drugs (Bauer, 1998; Peppercorn & Goldman, 1972; Williams et al., 1971).

The reduction in intestinal bacteria in this study was accompanied by an increase in fecal pH that typically occurs when there is a decrease in the production of short-chain fatty acids (SCFA) by colonic bacteria consequent to fermentative metabolism of carbohydrates and protein that were not digested in the upper gut. SCFA decrease luminal pH and hence provide antagonistic properties against intestinal pathogens and invading organisms (Fooks & Gibson, 2002). Suppression of bacteria, alterations in microbial composition, and reduction in SCFA in the gut might have clinical significance for humans in the management of many medical conditions such as irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease, obesity, and cancer, in which gut flora play an important role (Cummings & Macfarlane, 1997; Wong et al., 2006; Hart et al., 2002; Fooks & Gibson, 2002; Ley et al., 2006; Turnbaugh et al., 2006). Furthermore, recent studies using probiotics (e.g., lactobacilli and bifidobacteria) that modify bacterial balance for therapeutic purposes further emphasize that even fairly small changes in gut flora may impact health and disease (Fukushima et al., 1998; Teitelbaum & Walker, 2002; Fooks & Gibson, 2002; Gill & Guarner, 2004). The increase in pH is also noteworthy because changes in intestinal pH are known to modify the absorption of nutrients and drugs from the GIT (Dudeja et al., 2001; Asghar & Chandran, 2006).

Splenda increased the expression of the intestinal chemical transporter P-glycoprotein (P-gp) and two CYP450 isozymes (CYP3A4 and CYP2D1) at levels that have been associated with reduced bioavailability of drugs and chemicals. In the present study, ingestion of 300 mg/kg/d Splenda (or 3.3 mg/kg/d sucralose, a level currently approved for use in foods by the U.S. FDA) produced a 2.43-fold and a 1.4-fold rise in the expression of P-gp and CYP3A4 respectively. The magnitudes of these increases for P-gp and CYP3A4 are greater than or comparable to those shown to reduce the bioavailability of many drugs. In a clinical study, for example, a 1.4-fold increase of P-gp subsequent to administration of St. John's wort (an herbal medicine commonly used as an antidepressant) for 14 d in healthy humans resulted in an 18% decrease in the absorption of digoxin, which is a P-gp substrate that undergoes minimal metabolism (Dürr et al., 2000). Furthermore, repeated administration of St. John's wort, which is known to reduce the bioavailability of many other drugs including cyclosporine, indinavir, and amitriptyline, produces increases in CYP3A4 expression similar in magnitude to that found here for Splenda (Dürr et al., 2000). Overexpression of P-gp is a major mechanistic contributor to the phenomenon of multidrug resistance that is associated with the chronic use of anticancer agents such as anthracyclines (doxorubicin and daunorubicin) and vinca

TABLE 3

Summary of the Effects of Splenda Observed at the End of the 12-wk Treatment Period and at the End of the 12-wk Recovery Period (No Splenda), With Sucralose Level Contained in the Splenda Product Given as Well

Dosage of Splenda (mg/kg/d)	Sucralose (mg/kg/d) ^a	Effects	
		After 12-wk treatment	After 12-wk recovery
100	1.1	Decrease of beneficial intestinal bacteria; increased fecal pH; increased body weight	Total anaerobic bacteria remained suppressed; body weight remained elevated
300	3.3	Decrease of beneficial intestinal bacteria; increased fecal pH; histopathological changes in the gut ^b ; increased P-gp, CYP3A4, and CYP2D1	Total anaerobes and bifidobacteria remained suppressed; fecal pH remained elevated; P-gp remained slightly elevated
500	5.5	Decrease of beneficial intestinal bacteria; increased fecal pH; histopathological changes in the gut; increased P-gp, CYP3A4, and CYP2D1	Total anaerobes and bifidobacteria remained suppressed; fecal pH remained elevated; body weight increased; P-gp and CYP2D1 remained elevated
1000	11	Decrease of beneficial intestinal bacteria; increased fecal pH; histopathological changes in the gut; decreased P-gp, increased CYP3A4 and CYP2D1	Total anaerobes remained suppressed; fecal pH remained elevated; P-gp rebounded beyond control; CYP3A4 and CYP2D1 remained elevated

^aThe sucralose concentration in the Splenda product utilized was 1.1%.

^bLymphocytic infiltrates in the epithelium not seen in controls.

alkaloids (vinblastine and vincristine); i.e., increased P-gp efflux lowers intracellular drug concentrations in neoplastic tissues (Gottesman et al., 2002). CYP3A4 and CYP2D6 (human analog of rat CYP2D1) are involved in the metabolism of a vast number of prescription medications (Rendic & DiCarlo, 1997; Michalets, 1998; Clarke & Jones, 2002), and increased expression of these CYP isozymes results in accelerated drug metabolism and hence reduced clinical efficacy.

The present finding of increased expression of P-gp and CYP proteins by Splenda at the low dosages used in this experiment is clinically important with regard to potential drug interactions. For example, it is known that P-gp becomes saturated when large doses of certain drugs are administered (Lin & Yamazaki, 2003). Splenda-enhanced expression of P-gp, however, makes saturation unlikely even in the presence of high doses of drugs. That is, if the results in rats are similar for humans, the magnitude of the elevation of P-gp consequent to consuming Splenda over time might lead to extrusion of high doses of drugs. Furthermore, in cases of drugs such as digoxin that are given at a low oral dose (0.25–1 mg), it is unlikely that P-gp becomes saturated even at basal level.

While the increase in the expression of CYP3A4 and CYP2D1 was linear and dose dependent, the expression of

P-gp was nonlinear. For P-gp, there was a 2.43-fold increase at 300 mg/kg/d Splenda (i.e., 3.3 mg/kg/d sucralose) and a 2.23-fold increase at 500 mg/kg/d Splenda (i.e., 5.5 mg/kg/d sucralose) followed by a precipitous suppression of P-gp relative to control at the 1000 mg/kg/d Splenda dose (i.e., 11 mg/kg/d sucralose). A possible explanation for the significant reduction (not increase) of P-gp expression at the 1000 mg/kg Splenda dose may be due to a marked increase in CYP isozyme expression at the highest dose, i.e., 2.51-fold for CYP3A4 and 3.49-fold for CYP2D1 expression. This enhanced expression of CYP isozymes likely metabolizes sucralose and decreases its bioavailability so that it no longer exists intact in the small intestine at levels sufficient to increase P-gp. That is, the effective dose of sucralose at the highest dosage of Splenda would actually be far lower at the intestinal level than the 11 mg/kg/d of sucralose consumed orally because it is degraded by CYP3A4 and/or CYP2D1. This explanation is supported by the observation that P-gp and CYP3A4 have common tissue distribution and are known to interact in a coordinated manner in enterocytes (Matheny et al., 2001; Eagling et al., 1999; Wacher et al., 1998). The effects on P-gp and CYP enzymes seen here cannot be due to the maltodextrin component of Splenda because it is hydrolyzed

in the duodenum to glucose and then rapidly absorbed (Engfer et al., 2000; Booth, 1994). In contrast, the sucralose portion of Splenda, for the most part, stays in the gut (*Federal Register*, 1998).

Our results show that the Splenda-induced increase in CYP450 proteins was linear and did not level off throughout the dosage range used here that was equivalent to 1.1–11 mg/kg sucralose. It is likely that higher doses of sucralose saturate CYP isozymes resulting in decreased metabolic activity of these enzymes. Prior studies examined the metabolic profile of sucralose in rats treated with chronic, nonphysiological “mega” doses (e.g., 2.2 g/kg/d sucralose) (Sims et al., 2000; Mann et al., 2000). This mega-dosage in rats is equivalent to 20 g/kg/d of Splenda or 1.4 kg/d for a 70-kg human. Rats given a diet containing a mega-dosage of sucralose for 18 mo reportedly excreted sucralose unchanged in the feces without metabolism (Sims et al., 2000). This high dose of sucralose may have saturated the CYP metabolism enzymes, thus impeding the body’s ability to metabolize sucralose. As a result, P-gp and/or other ABC transporters played a quantitatively significant role in the extrusion of sucralose and its subsequent excretion from the body, mostly in the feces as the parent compound. P-gp would continue to be induced at elevated sucralose concentrations if CYP is not removing sucralose from the GIT.

The impact of Splenda (and particularly the chlorocarbon sucralose) on both P-gp and CYP is consistent with previous reports that chlorinated hydrocarbons interact with efflux transporters and cytochrome P-450 isozymes (Lanning et al., 1996; Bois et al., 1998; Bain & LeBlanc, 1996; Leslie et al., 2005; Poet et al., 2003). The increased expression of P-gp and CYP3A4 may involve induction of gene expression via the nuclear pregnane X receptor (PXR). Several lines of evidence indicate that PXR regulates gene expression of CYP3A4 and other P-450 isozymes as well as the *MDR1* gene that encodes P-gp (Tompkins & Wallace, 2007; Geick et al., 2001). Furthermore, the PXR receptor was shown to be activated by organochlorine compounds (Kliwer, 2003; Medina-Diaz et al., 2007; Jacobs et al., 2005). The enhanced expression of P-gp and CYP isozymes found in this study may explain why 65–95% of the sucralose administered orally is reportedly not absorbed from the gastrointestinal tract (*Federal Register*, 1998). Data presented suggest that sucralose undergoes two processes during first-pass metabolism in the intestinal membranes: (1) P-gp extrudes sucralose from the intestinal walls back to the lumen and (2) CYP enzymes metabolize the compound. These results explain why sucralose, though lipid soluble (Miller, 1991; Wallis, 1993; Yotka et al., 1992), was reported to be poorly absorbed from the gastrointestinal tract. Overall, the elevated expression of P-gp and CYP explains the low bioavailability of sucralose (*Federal Register*, 1998); i.e., it invokes the same presystemic first-pass metabolism mechanisms as drugs and toxicants.

In conclusion, the findings of this study indicate that Splenda suppresses beneficial bacteria and directly affects the expression of the transporter P-gp and cytochrome P-450 isozymes that are known to interfere with the bioavailability of

drugs and nutrients. Furthermore, these effects occur at Splenda doses that contain sucralose levels that are approved by the FDA for use in the food supply.

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