Effects of Structured Triacylglycerols Containing Stearic, Acetic, and Propionic Acids on the Intestinal Microflora of Rats

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The structured triacylglycerols SALATRIM 23CA lot A014 and SALATRIM 32CA lot A015 exhibit lower caloric availability than corn oil. Acetate and stearate predominate in SALATRIM 23CA lot A014 and propionate and stearate in SALATRIM 32CA lot A015. Rats were exposed for 13 weeks to rodent chow or 10% (w/w) SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, or corn oil. Cecal contents were examined for changes in five variables associated with intestinal bacterial activity: (1) bacterial morphotypes, (2) cecal pH, (3) conversion of primary to secondary bile acids, (4) conversion of primary to secondary phytosterols, and (5) conversion of cholesterol to coprostanol. Fusiform rods (diet-sensitive bacteria) were not detectably altered by the SALATRIM fats. No major pH differences were detected. No biologically significant differences were seen in secondary bile acid levels as a percent of total, nor did the ratios of cholesterol to coprostanol vary significantly, indicating that bacterial conversions were unaffected. Whereas all four secondary phytosterols increased with corn oil, only one (24α-methylcoprostanol) increased with SALATRIM 32CA lot A015. SALATRIM fats had less effect, if any, on the intestinal microflora of rats than corn oil.

INTRODUCTION

The microflora of the human large intestine are part of a complex ecosystem involving over 400 species. Each animal species appears to have its own distinct flora. Therefore, results from studies using a specific animal model may not exactly mimic the potential changes in the human intestinal microflora. However, such studies are useful because any compound that causes major changes in the microflora of an animal model could have the potential to produce such changes in humans.

The intestinal bacterial flora depend, in part, on energy derived from food ingested by the host. A change in food patterns and/or composition may alter the species composition and/or metabolic profiles of the microflora. These changes can range from a decrease or increase in certain species to the alteration of the metabolic capabilities of a single species. Determining the potential for dietary changes to produce alterations in the microflora of animals is difficult. Direct studies of the cecal bacterial flora of animals can yield information that is misleading if extrapolated to humans. The species of bacteria that occur in common animal models are different from those found in humans, and it is currently not feasible to accurately identify the bacteria at the species level. For example, the fusiform rods that occur in the rat intestine are mainly unnamed clostridia. They are difficult to grow on culture media and have rarely been identified at the species level.

Since dietary fat may have the potential to alter the intestinal bacterial flora, the potential for the SALATRIM family of structured triacylglycerols to alter the flora has been investigated. SALATRIM fats are mixed triacylglycerols distinguished by their preponderance of short-chain fatty acids and stearic acid esterified to glycerol. The specific SALATRIM fats investigated here are SALATRIM 23CA lot A014 and SALATRIM 32CA lot A015. These SALATRIM fats are composed of triacylglycerols containing acetic, propionic, and stearic acids esterified to glycerol. They are distinguished by SALATRIM 23CA lot A014, containing a predominance of acetic acid, while SALATRIM 32CA lot A015 contains a predominance of propionic acid.

The approach taken in the present study was to determine if feeding rats each of the two SALATRIM fats for at least 13 weeks altered a battery of characteristics associated with intestinal bacteria and their metabolic activity. These characteristics, among others, can be termed microflora-associated characteristics (MACs). MACs have been used to investigate the effect of diet on the normal intestinal flora of humans (Mallet et al., 1988) and animal models (Treon et al., 1989; Felix et al., 1990; Rumney and Rowland, 1992). When mice fed laboratory chow were switched to a diet free of cellulose, the cellulolytic clostridia were eliminated (Wilkins, 1981). A similar change in the microflora was reported when rats were fed grain and meat diets (Weinstein et al., 1974). Conversion to a meat diet induced higher levels of certain bacterial enzyme activities, such as tryptophanase, in rats (Chung et al., 1975). Comprehensive discussions of the role of ingested compounds on the cecal and fecal ecology of animal models and humans can be found in the reviews of Hentges (1983), Hill (1986), Macfarlane and Cummings (1991), and Rumney and Rowland (1992).

The MACs investigated in this study were (1) bacterial morphotypes, (2) cecal pH, (3) bile acids and their secondary bacterial metabolites, (4) phytosterols and their secondary metabolites, and (5) the conversion of cholesterol to coprostanol. Considerations used in choosing these specific MACs included the following: (1) use of several MACs increases the chance of detecting potential changes; (2) previous use of a particular MAC in other studies; (3) availability of the required methodology. Monitoring the levels of bile acids and their bacterial metabolites and the

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Table 1. Total Fatty Acid Profile for SALATRIM 23CA Lot A014[a,b]

fatty acid name	designation	wt %
stearic (octadecanoic)	C18:0	57 ± 1
acetic	C2:0	21.1 ± 0.1
propionic	C3:0	2.58 ± 0.03
palmitic (hexadecanoic)	C16:0	2.37 ± 0.04
arachidic (eicosanoic)	C20:0	1.50 ± 0.03
behenic (docosanoic)	C22:0	0.668 ± 0.007
oleic (9-octadecenoic)	C18:1	0.572 ± 0.005
lignoceric (tetracosanoic)	C24:0	0.335 ± 0.001
linoleic (9,12-octadecadienoic)	C18:2	0.066 ± 0.001
lauric (dodecanoic)	C12:0	0.007 ± 0.000

[a] Data represent the mean ± standard deviation for triplicate determinations. [b] Data developed by EPL Bio-Analytical Services, Inc.

Table 2. Total Fatty Acid Profile for SALATRIM 32CA Lot A015[a,b]

fatty acid name	designation	wt %
stearic (octadecanoic)	C18:0	51.6 ± 0.4
propionic	C3:0	21 ± 1
palmitic (hexadecanoic)	C16:0	3.39 ± 0.01
acetic	C2:0	1.70 ± 0.07
oleic (9-octadecenoic)	C18:1	1.55 ± 0.03
arachidic (eicosanoic)	C20:0	1.43 ± 0.01
behenic (docosanoic)	C22:0	0.583 ± 0.003
linoleic (9,12-octadecadienoic)	C18:2	0.35 ± 0.01
lignoceric (tetracosanoic)	C24:0	0.306 ± 0.003
lauric (dodecanoic)	C12:0	0.013 ± 0.000

[a] Data represent the mean ± standard deviation for triplicate determinations. [b] Data developed by EPL Bio-Analytical Services, Inc.

conversion of cholesterol to coprostanol seemed particularly important because dietary fats may influence the host's steroid metabolism. High dietary concentrations of triacylglycerols conceivably could result in higher levels of bile acids, their metabolites, and coprostanol reaching the colon. Such a result could be deleterious because high concentrations of bile acids and secondary bacterial metabolites have been associated with higher rates of colonic cancer (Reddy et al., 1977a; Reddy and Maruyama, 1986). Monitoring the production of secondary products from primary sterols also appears to be a sensitive indicator of changes in the bacterial ecology, since the bacteria that perform these reactions occur at only about 10^5/g of contents and represent only 0.0001% of the total microflora.

MATERIALS AND METHODS

This study was conducted at TechLab (Blacksburg, VA) using cecal contents collected during part of a rat 13-week subchronic toxicity study at Hazleton Wisconsin, Inc. (Madison, WI). Detailed information on the experimental design and methods for the subchronic rat study can be found in the toxicology study (Hayes et al., 1994).

Test and Control Materials. SALATRIM 23CA lot A014 and SALATRIM 32CA lot A015 were provided by Nabisco Foods Group (NFG), East Hanover, NJ. Analytical determinations show that SALATRIM 23CA lot A014 is a mixture of triacylglycerols composed predominantly of diacetylstearoylglycerol and that SALATRIM 32CA lot A015 is a triacylglycerol mixture composed predominantly of dipropionylstearoylglycerol (Softly et al., 1994).

Total fatty acid profile analyses for these two fats were conducted at EPL Bio-Analytical Services, Inc. (EPL-BAS), Decatur, IL, and the results are presented in Tables 1 and 2. A selected fatty acid profile of the basal diet was analyzed by NFG and is presented in Table 3. Total fatty acid profile data for the higher molecular weight groups (≥C12:0) were obtained by saponification of the triacylglycerol mixture with methanolic sodium hydroxide followed by esterification with methanolic boron trifluoride. Methyl esters of the fatty acids were quantified by gas chromatography. For lower molecular weight groups (<C12:0), profile data were obtained by saponification of the sample with sodium hydroxide followed by acidification with concentrated hydrochloric acid. The fatty acids were quantified by gas chromatography. Standard curves were constructed bracketing the concentration level of the analyte.

Table 3. Selected Fatty Acid Profile for NIH-07 Rodent Chow[a,b]

fatty acid name	designation	wt %
stearic (octadecanoic)	C18:0	2.69 ± 0.02
acetic	C2:0	<0.10
propionic	C3:0	<0.10
palmitic (hexadecanoic)	C16:0	12.61 ± 0.12
arachidic (eicosanoic)	C20:0	0.21 ± 0.00
behenic (docosanoic)	C22:0	0.14 ± 0.01
oleic (9-octadecenoic)	C18:1	9.70 ± 0.04
lignoceric (tetracosanoic)	C24:0	2.08 ± 0.04
linoleic (9,12-octadecadienoic)	C18:2	30.81 ± 0.25
lauric (dodecanoic)	C12:0	<0.10

[a] Data represent the mean ± standard deviation for triplicate determinations. Weight percent is based upon crude fat extracted from diet. Crude fat was 4.96% of diet. [b] Data developed by Nabisco Foods Group, East Hanover, NJ.

Table 4. Free Fatty Acid and Peroxide Value Analyses for Two SALATRIM Fats[a,b]

	SALATRIM	
	23CA lot A014	32CA lot A015
free fatty acid (wt %)	0.86 ± 0.01	0.42 ± 0.03
peroxide value (mequiv/kg)	0.3 ± 0.1	0.699 ± 0.001

[a] Data represent the mean ± standard deviation for triplicate determinations. [b] Data developed by EPL Bio-Analytical Services, Inc.

Free fatty acid concentration (by titration) and peroxide value analyses were conducted by EPL-BAS. Titratable acid values were obtained according to the AOCS. Official Method Ca 5a–40 (AOCS, 1990a). The peroxide values were obtained using the AOCS Official Method Cd 8–53 (AOCS, 1990b). The free fatty acid concentrations and peroxide values for the three triacylglycerol mixtures were low. These results are presented in Table 4.

Dosing and Diets. Twenty male and 20 female rats were exposed to either SALATRIM 23CA lot A014 or SALATRIM 32CA lot A015 at 10% (w/w) of the diet for at least 13 weeks. An equal number of rats was exposed to corn oil at 10% (w/w) of the diet to control for the high lipid content of the diets. An additional 20 rats per sex were fed the control chow diet. Test diets were prepared weekly during weeks 1 and 14 and biweekly for all other weeks of the study. When diets were prepared biweekly, they were divided into two portions. The first portion was fed during the first of the two weeks and the second portion frozen (−20 °C) until fed during the second week. Both SALATRIM fats and corn oil were mixed with NIH-07 Rat and Mouse Ration 5018 (Purina Mills, Inc.) and fed *ad libitum*. Water was provided *ad libitum*. Homogeneity of the fats in the diets was demonstrated, and the actual dosing concentrations of the SALATRIM fats and corn oil were confirmed by analysis of each batch of prepared diets.

Animals. Crl:CD BR VAF rats were from Charles River Laboratories, Inc. (Portage, MI). Animal husbandry complied with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86-23, 1985). Rats were identified by ear tags and housed singly in stainless steel wire-bottom cages in an animal room set to maintain 22 ± 3 °C and 50 ± 20% relative humidity, with a 12-h light/12-h dark cycle. Randomization into treatment groups was unrestricted except the body weight of each rat could not vary by more than 2 standard deviations from the mean body

weight of all rats of the same sex. Group mean body weights could not differ statistically ($p \leq 0.05$) before study initiation.

Sample Collection. At necropsy, following a 24-h fasting period, the cecum was exposed and ligated with cotton thread in three places: at the distal tip, proximal to the ileocecal junction, and distal to the exit into the colon. The cecal tip was removed for histology, and the remainder of the cecum was removed and frozen at −20 °C until use. Upon thawing of each cecum, its contents were thoroughly mixed by kneading within the cecum and then removed. Samples were analyzed without knowledge of their group designation.

Scanning Electron Microscopy (SEM). A portion of the cecal contents from five male rats from each dietary group was examined for changes in the dominant bacterial morphotypes. The methods for the preparation and examination of samples for SEM were modifications of those described by Borriello et al. (1986). A portion of the cecal contents (about 200 μg) was suspended in phosphate-buffered saline (1 mL; pH 7.2–7.4) and the larger particulate matter allowed to settle. Aliquots (150 μL) of the suspension were added to cacodylate buffer (1 mL, 0.1 M; pH 7.2–7.4) and centrifuged (15000*g* for 2 min). Bacterial pellets were suspended in cacodylate buffer (1 mL) supplemented with glutaraldehyde (3% by volume). The suspensions were mixed with glycerol (4:1) and stored at −10 °C. On thawing, bacteria were washed in cacodylate buffer (1 mL), and 200 μL of the suspension was placed on a polycarbonate Nucleopore filter (Costar Corp., Pleasanton, CA). The filter was submerged in fresh cacodylate buffer. The filter was postfixed for 1 h in cacodylate buffer containing aqueous osmium tetraoxide (1% by volume). Prepared filters were dehydrated successively through 15, 30, 50, 70, 95, and 100% ethanol and critical point dried in a Ladd 2800 (Ladd Research Industries, Burlington, VT). Samples were examined in a JEOL-JEM 35C scanning electron microscope (JEOL USA, Inc., Peabody, MA). Five fields from each animal were photographed and visually compared.

Cecal pH. A portion of the thawed cecal contents (100–200 mg) from each rat was placed into individual sterile tubes. Following the general methods of Treon et al. (1989), the cecal contents were diluted in sterile deionized water (9 volumes, adjusted to pH 7.00 with 0.05 M sodium hydroxide) and vortexed for 30 s. After being allowed to settle at room temperature for 5 min, its hydrogen ion activity was measured using an Accumet 610A pH meter (Fisher Scientific, Atlanta, GA).

Bile Acids. Analytical methods employed were developed from those described by Setchell et al. (1983, 1987). Thawed cecal contents from each rat were placed into individual sterile containers and lyophilized. Known weights (200–300 mg) of the lyophilized cecal contents were ground to a fine powder and extracted four times with 80% ethanol (10 mL) at 70 °C for 20 min. Insoluble matter was pelleted and discarded. Approximately 2 mL of each aqueous ethanolic extract was acidified with hydrochloric acid (1 M) to pH 2–4. The acidified extracts were passed through an SP-Sephadex column (SP-Sephadex C-25, exclusion limit 3×10^4 Da, Sigma, St. Louis, MO) to remove pigments (Setchell et al., 1983). The eluent was collected and dried at 60 °C under argon. The sample was resuspended in sodium hydroxide (1 mL, 0.1 M) and extracted three times with 1 mL of methanol/diethyl ether (30/70 v/v) and twice with 1.6 mL of diethyl ether. Following low-speed centrifugation for 1 min, the two layers were separated. The lower methanolic phase contained the bile acids and the upper (ether) phase the neutral sterols.

The methanolic phase was acidified to below pH 2 using hydrochloric acid (6 M). The solution was extracted three times with ethyl acetate (1 mL), dried over anhydrous sodium sulfate (0.5 g) and magnesium sulfate (0.05 g), and evaporated to dryness under argon prior to derivatization. The dried residue was redissolved in methanol (0.1 mL) and diazomethane (300 μL) added. The mixture was incubated at room temperature for 10 min and then evaporated to dryness under argon. Trimethylsilyl (TMS) derivatization reagent (300 μL), prepared according to the procedure of Setchell et al. (1983), was added and the mixture heated at 60 °C for 30 min. Excess reagents were removed by evaporation under argon and the residue dissolved in chloroform (250 μL). Cholesterol methyl ester (25 μg) was added as an internal standard. The solution was transferred and sealed in airtight vials.

Samples were analyzed with an HP 5890 gas chromatograph fitted with an HP-1 capillary column (25 m × 0.2 mm × 0.33 μm) (Hewlett-Packard Co., Palo Alto, CA). Nitrogen (2 mL/min) was the carrier gas; air (380 mL/min) and hydrogen (32 mL/min) were used for flame ionization detection. The oven was programmed as follows: 100 °C for 1 min, 40 °C/min to 220 °C, 220 °C for 0.3 min, 10 °C/min to 280 °C, 280 °C for 0.3 min, and 2 °C/min to 300 °C, 300 °C for 7 min. The injector and detector temperatures were 300 °C. Calibration was done with 13 steroid nuclei standards obtained from Steroids, Inc., Wilton, NH; Sigma Chemical Co., St. Louis, MO; and Aldrich Chemical Co., Milwaukee, WI.

To confirm the identity of the bile acids, two samples were further characterized using GC/mass spectrometry (Setchell et al., 1983, 1987). The following primary compounds were identified: cholic and α-, β-, and ω-muricholic bile acids. The secondary metabolites deoxycholic, lithocholic, hyodeoxycholic, and unsaturated ω-muricholic acids were identified. Other unidentified acids were sometimes present at such low concentrations compared to the other analytes that their contribution to the total bile acid level was ignored.

Neutral Sterols. Following methanol/diethyl ether extraction (see above), the upper (ether) layer was removed and dehydrated by passage through an anhydrous sodium sulfate column (2-mL maximum bed volume). The ether phase was dried under a stream of argon prior to saponification.

One milliliter of sodium hydroxide (5% NaOH in 80% aqueous methanol) was added to each dried extract and heated to 60 °C for 3 h. After saponification, methanol was removed by evaporation under argon. Water (1 mL) was added and the sample acidified with hydrochloric acid (6 M) to below pH 2. The sample was extracted three times with ethyl acetate (3 mL). The upper (organic) phase was dehydrated over anhydrous sodium and magnesium sulfate, dried under argon, derivatized as described above, and analyzed by gas chromatography.

To confirm the identity of the neutral sterols, two samples were further characterized using GC/mass spectrometry. Compounds of animal origin were cholesterol and its secondary metabolite coprostanol. Primary phytosterols detected were 24β-ethylcholesterol (sitosterol) and 24β-methylcholesterol. Secondary phytosterol metabolites detected were 24α-methylcoprostanol (campestanol), 24β-methylcoprostanol, 24α-ethylcoprostanol (stigmastanol), and 24β-ethylcoprostanol (sitostanol).

Statistical Determinations. Statistical differences were determined by analysis of variance (SAS PROC GLM) (SAS Institute, 1990) and Dunnett's (SAS Institute, 1990) two-tailed t-test to compare the SALATRIM and corn oil groups to the chow-fed control group and the SALATRIM groups to the corn oil group. Data that differed by $p \leq 0.05$ were considered to be statistically significant.

RESULTS

Chemical Characterization of SALATRIM Fats. The total fatty acid profile of SALATRIM 23CA lot A014 is presented in Table 1 and that of SALATRIM 32CA lot A015 in Table 2. As predicted, SALATRIM 23CA lot A014 contains acetic and stearic acids as the predominant fatty acids, while SALATRIM 32CA lot A015 contains propionic and stearic acids as the predominant fatty acids. Table 3 illustrates the corresponding fatty acid profile of the chow control diet fed the rats. Peroxide values were low for SALATRIM 23CA lot A014 and SALATRIM 32CA lot A015, 0.3 ± 0.1 and 0.699 ± 0.001, respectively (Table 4).

Dominant Morphotypes of Cecal Bacteria. Variability in the microflora between animals within the same dietary group (data not shown) restricted the sensitivity of this method to the detection of only major changes. No dramatic differences were detected in the populations of bacterial morphotypes in the five fields from each of the five males from each dietary group. The predominant morphotype was long, thin, tapered (fusiform) rods. A

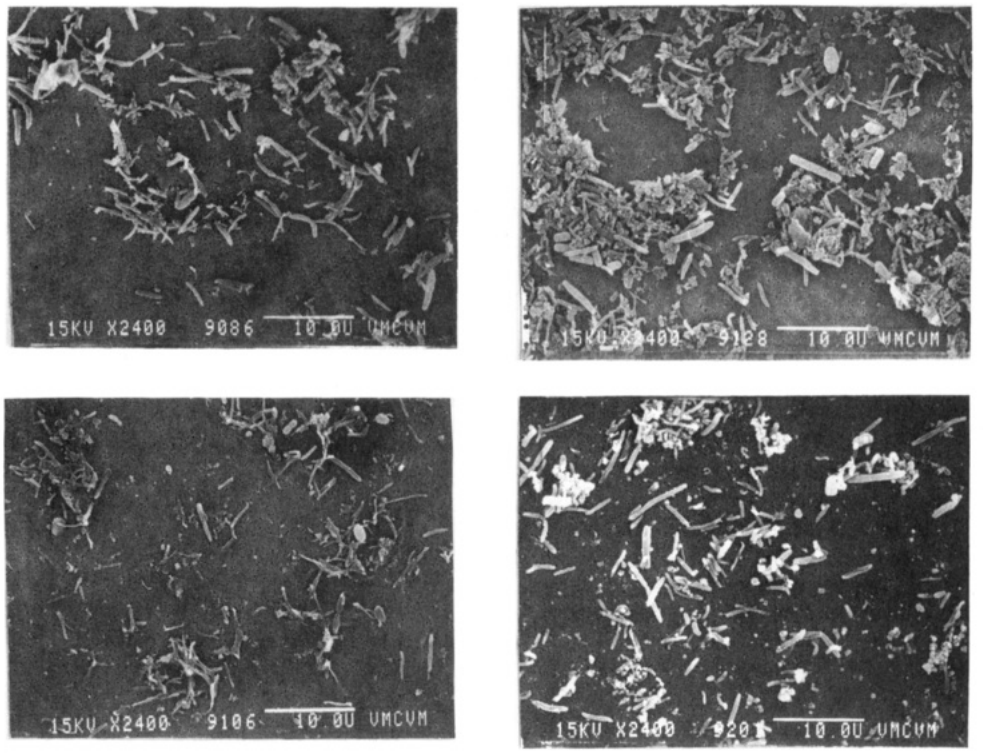

Figure 1. Scanning electron micrographs of the typical bacterial flora of rats fed for 13 weeks: (a, top left) chow; (b, bottom left) chow with 10% SALATRIM 23CA lot A014; (c, top right) chow with 10% SALATRIM 32CA lot A015; (d, bottom right) chow with 10% corn oil. Magnification was 2400×. (Figure is reproduced here at 50% of original.)

Table 5. pH of Cecal Contents of Rats Fed SALATRIM Triacylglycerols and Corn Oil

	control		SALATRIM 23CA lot A014		SALATRIM 32CA lot A015		corn oil	
	males	females	males	females	males	females	males	females
pH[a]	7.80	7.74	7.90	7.85	7.80	7.91**	7.68	7.69
SD of pH[b]	0.35	0.34	0.35	0.29	0.28	0.24	0.29	0.23

[a] Data represent mean pH and range of 20 cecal samples per treatment group. Values are statistically significant ($p \leq 0.05$) as compared to chow control diet (*) or corn oil diet (**). [b] Standard deviation of pH.

single field, typical of the flora associated with each diet, is presented in Figure 1.

Cecal pH. Neither SALATRIM dietary regime resulted in any significant changes in the pH of the cecal contents when compared to rats in the control chow-fed group (Table 5). The value for female rats fed SALATRIM 32CA lot A015 was statistically significantly higher than the female rats fed corn oil, while no difference was noted in either sex when SALATRIM 32CA lot A015 was compared to chow controls. Because it occurred in only one instance, this does not appear to be a physiologically significant change and may be spurious.

Bile Acids. Table 6 presents the primary and secondary bile acids that occurred in the cecal contents of rats fed either 10% SALATRIM 23CA lot A014, 10% SALATRIM 32CA lot A015, 10% corn oil, or chow diets. The only statistically significant changes in the primary bile acids was a decrease in ω-muricholic acid in males fed SALATRIM 23CA lot A014 compared to males fed the chow diet and a decrease in α-muricholic acid in females fed either SALATRIM compared to corn oil-fed females. It is difficult to assess the biological significance of these changes due to the large interanimal variation. With respect to the secondary bile acids, unsaturated ω-muricholic was significantly decreased in male rats fed SALATRIM 23CA lot A014 compared to male rats fed chow diets and unchanged in female rats fed the same fat. This decrease may be associated with the lower ω-muricholic acid noted in these males. Lithocholic acid was significantly lower in both male and female rats fed either SALATRIM compared to the 10% corn oil group but unchanged when compared with the chow group. In contrast, both male and female rats fed corn oil exhibited significantly higher concentrations of lithocholic acid compared with the chow-fed group. No other changes were noted in the secondary bile acids. This indicates that the SALATRIM fats did not produce a biologically significant alteration in intestinal bacterial conversion of primary bile acids to secondary bile acids.

The metabolism of primary to secondary bile acids is further illustrated in Figures 2 and 3. Total cecal bile acids are shown in Figure 2. There were no significant differences between the three dietary fats and the control diet with respect to total bile acids. Secondary bile acids as a percent of total bile acids are shown in Figure 3. No significant differences were noted in the secondary bile acids, indicating that the dietary fats did not alter bile acid metabolism by the intestinal microflora in either sex.

Conversion of Cholesterol to Coprostanol. Table 7 presents the concentrations of cholesterol and coprostanol in the cecal contents of rats fed either 10% SALATRIM 23CA lot A014, 10% SALATRIM 32CA lot A015, 10% corn oil, or chow diets. Cholesterol increased to 160% of the control in the corn oil-fed male rats. No statistically significant changes were noted in any of the other dietary groups. Coprostanol produced by the intestinal microflora was statistically increased in male rats fed SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, and corn oil as

Table 6. Primary and Secondary Bile Acids in the Cecal Contents of Rats Fed SALATRIM Triacylglycerols and Corn Oil[a]

	control (mg/g)[b]		SALATRIM 23CA lot A014 (mg/g)		SALATRIM 32CA lot A015 (mg/g)		corn oil (mg/g)	
bile acid	males	females	males	females	males	females	males	females
primary								
cholic acid	5.15 ± 5.29	10.60 ± 17.20	8.48 ± 9.16	10.50 ± 22.50	15.70 ± 32.80	12.10 ± 18.90	15.40 ± 19.90	13.20 ± 14.50
α-muricholic acid	6.80 ± 3.02	12.70 ± 8.85	6.31 ± 3.20	8.67 ± 6.53**	8.42 ± 5.59	9.97 ± 7.92**	9.32 ± 4.97	18.00 ± 10.90
β-muricholic acid	13.80 ± 8.16	6.04 ± 4.18	11.18 ± 8.11	4.32 ± 3.48	13.75 ± 8.36	7.27 ± 8.32	14.40 ± 8.51	8.13 ± 4.37
ω-muricholic acid	28.70 ± 14.90	12.80 ± 11.50	16.20 ± 7.24*	9.59 ± 6.86	23.10 ± 13.10	10.30 ± 5.81	23.60 ± 15.30	14.00 ± 6.60
secondary								
lithocholic acid	5.47 ± 1.80	7.99 ± 3.70	4.08 ± 1.94**	7.70 ± 3.53**	4.73 ± 1.66**	7.20 ± 3.14**	7.07 ± 2.36*	11.00 ± 3.65*
deoxycholic acid	45.90 ± 13.30	33.90 ± 17.20	44.20 ± 18.20	30.00 ± 12.80	48.60 ± 17.90	36.20 ± 19.60	47.60 ± 16.60	42.00 ± 14.70
hyodeoxycholic acid	22.35 ± 10.20	14.60 ± 6.80	16.60 ± 9.43	13.76 ± 7.15	20.23 ± 8.17	12.85 ± 6.01	20.61 ± 11.40	13.17 ± 6.17
unsaturated ω-muricholic acid	5.82 ± 3.00	2.08 ± 1.71	2.96 ± 1.51*	1.51 ± 1.14	4.16 ± 2.21	1.69 ± 1.12	4.64 ± 2.77	1.97 ± 1.32

[a] Data represent the mean ± standard deviation of 20 rats per sex per group. Values are statistically significant ($p \leq 0.05$) as compared to chow control diet (*) or corn oil diet (**). [b] Units are mg/g dry weight of cecal contents.

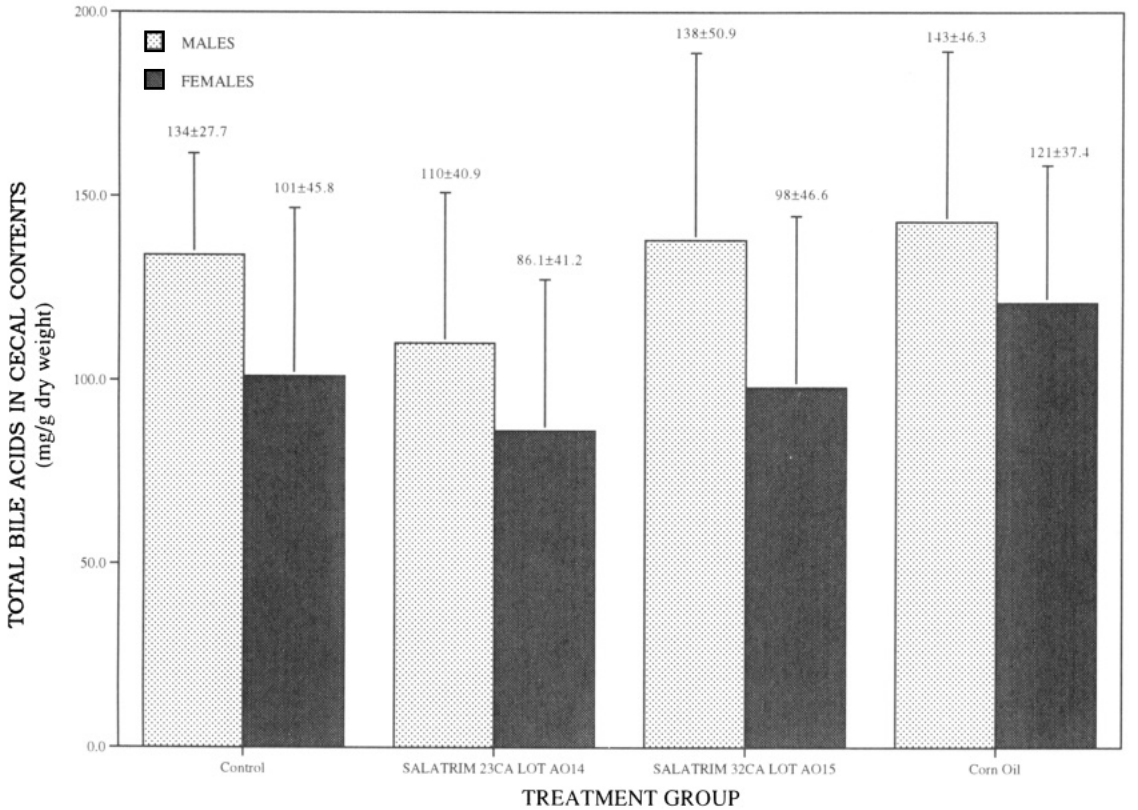

Figure 2. Effect of 13-week dietary exposure to SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, and corn oil on cecal total bile acid concentrations in male and female rats. Data represent the mean ± standard deviation for 20 rats per sex per group. Statistically significant differences from the chow control diet ($p \leq 0.05$) are noted by an asterisk. The mean ± standard deviation for each sex is presented above the specific bar.

compared to the control group. Since all of these groups had increased (although not statistically significant in the SALATRIM-fed rats) concentrations of cholesterol in the cecal contents, the increases in coprostanol could be due to the higher substrate concentration available for microflora metabolism. The data do not differentiate between increased metabolism associated with higher substrate concentrations and changes in the microflora. A truer picture of the effect of SALATRIM on microflora activity with respect to cholesterol conversion may be obtained by comparing the ratio of coprostanol to cholesterol in the SALATRIM-fed rats with that of the controls. No significant changes were observed, as shown by the values presented in Table 7.

Phytosterols. Primary and secondary phytosterols in the cecal contents of the rats fed the different dietary fats and the chow-fed controls are presented in Table 8. In cases where differences were significant, primary phytosterols tended to be higher in the corn oil-fed rats and lower in the SALATRIM-fed rats as compared to the chow-fed rats. The SALATRIM-fed rats exhibited no significant differences in 24β-ethylcholesterol concentrations. In contrast, concentrations were significantly higher in the corn oil-fed rats. SALATRIM-fed females exhibited significant decreases in 24β-methylcholesterol, while males tended to have higher concentrations than the chow-fed group. However, the level of this primary phytosterol was even higher for corn oil-fed males. Compared to the corn oil-fed rats, all of the SALATRIM-fed rats tended to have lower primary phytosterol levels. These results probably arise from the higher primary phytosterol concentration found in corn oil (Softly et al., 1994). A similar pattern is evident when the concentrations of secondary phytosterols are compared among dietary groups. All four secondary phytosterol levels rose in rats fed corn oil; only one (24α-methylcoprostanol) increased in rats fed SALATRIM 32CA lot A015. In general, SALATRIM-fed rats of both sexes produced slightly less of the three remaining

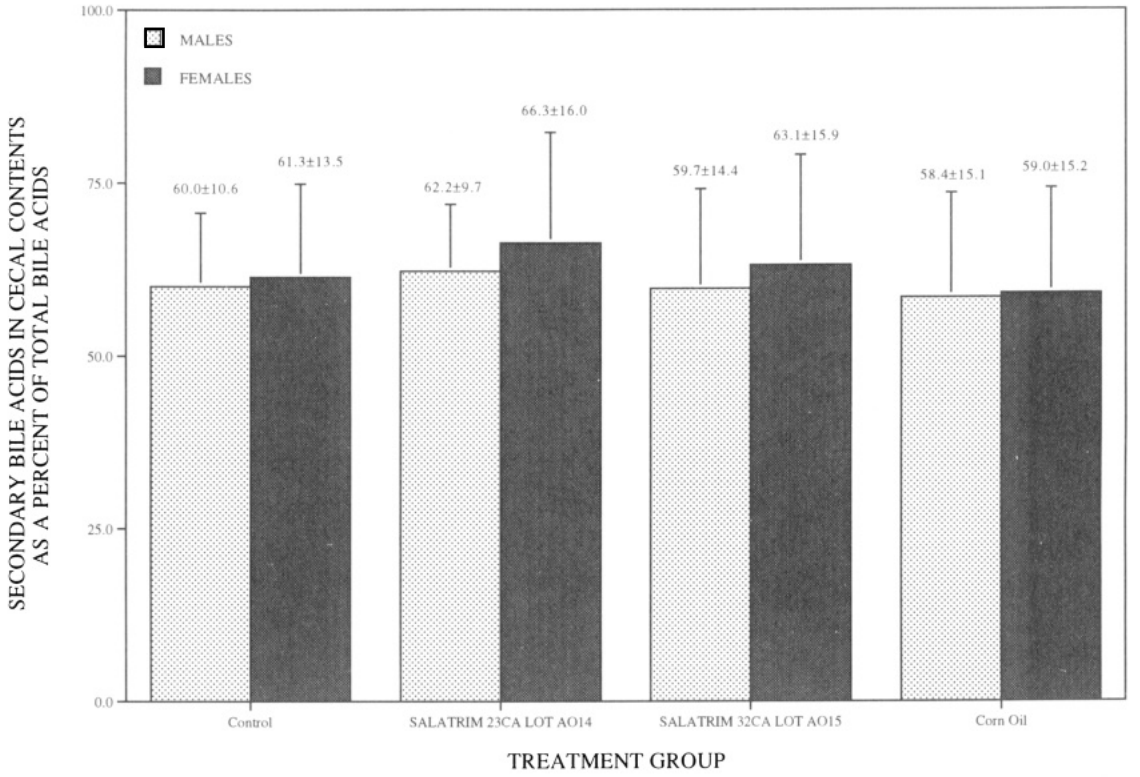

Figure 3. Effect of 13-week dietary exposure to dietary SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, and corn oil on total cecal secondary bile acid concentration in male and female rats. Data are expressed as the percentage of the total bile acids metabolized by bacteria to secondary bile acids. Data represent the mean ± standard deviation for 20 rats per sex per group. Statistically significant differences from the chow control diet ($p \leq 0.05$) are noted by an asterisk. The mean ± standard deviation for each sex is presented above the specific bar.

Table 7. Cholesterol and Coprostanol in the Cecal Contents of Rats Fed SALATRIM Triacylglycerols and Corn Oil[a]

	control (mg/g)[b]		SALATRIM 23CA lot A014 (mg/g)		SALATRIM 32CA lot A015 (mg/g)		corn oil (mg/g)	
sterol	males	females	males	females	males	females	males	females
cholesterol	13.5 ± 3.3	15.2 ± 4.6	15.8 ± 3.7	16.5 ± 5.1	16.3 ± 5.6	15.0 ± 5.4	21.6 ± 13.0*	17.1 ± 5.1
coprostanol	28.7 ± 8.9	31.8 ± 11.5	39.0 ± 16.3*	38.2 ± 9.7	41.3 ± 10.8*	37.0 ± 10.3	38.6 ± 10.3*	33.2 ± 11.1
coprostanol/cholesterol	2.25 ± 0.9	2.16 ± 0.67	2.62 ± 1.24	2.58 ± 1.18	2.77 ± 1.01	2.65 ± 0.91	2.29 ± 1.17	2.19 ± 0.92

[a] Data represent the mean ± standard deviation of 20 rats per sex per group. Values are statistically significant ($p \leq 0.05$) as compared to chow control diet (*) or corn oil diet (**). [b] Units are mg/g dry weight of cecal contents.

secondary phytosterols than chow-fed rats, while rats in the corn oil-fed group produced more.

Figure 4 illustrates the effects of the dietary fats on the total phytosterols in the cecal contents. The total phytosterols detected in rats fed SALATRIM 23CA lot A014 and SALATRIM 32CA lot A015 did not differ from the chow-fed rats with the exception of a decrease in females fed SALATRIM 23CA lot A014. However, male and female rats fed corn oil had significantly higher concentrations compared to the controls. Figure 5 represents the secondary metabolites of the phytosterols produced by the intestinal microflora as a percent of total phytosterols. These data indicate that the microflora in each dietary group retained an equivalent capability to produce secondary phytosterol metabolites compared to the control.

DISCUSSION

When compared to chow-fed rats, SALATRIM 23CA lot A014- and SALATRIM 32CA lot A015-fed rats did not exhibit changes considered to be biologically significant in the MACs measured in this study. Also, the SALATRIM fats did not produce biologically significant differences compared to the corn oil group.

The SALATRIM fats caused no discernible loss of fusiform rods in the cecum as determined by electron microscopic scans of the cecal contents. The normal rat cecal flora is dominated by long, thin bacterial rods tapered at both ends (fusiform rods), mainly clostridia (Wilkins, 1981). Dietary manipulation can cause dramatic alterations to this component of the flora. When fed a defined powdered diet supplemented with cornstarch, casein, ground corn, kaolin, or cellulose, only mice fed the cellulose retained their fusiform flora (Wilkins, 1981). To validate the morphotype methods used in this study, the microflora of rats fed either chow or ground beef were compared. Only the chow-fed rats retained their original flora (S. Scheinbach, J. R. Hayes, R. J. Carman, R. L. Van Tassell, and T. D. Wilkins, unpublished observations, 1991). Changes of this type were not seen in the studies of dietary fats reported here. We conclude that SALATRIM triacylglycerols did not affect the fusiform flora. In support of this conclusion, a similar study with another representative of the SALATRIM family (SALATRIM 4CA lot A006) did not alter the fecal bacterial morphotypes (Scheinbach et al., unpublished observations, 1992).

No biologically significant differences were noted between the pH of cecal contents from rats fed either SALATRIM when compared to control rats fed chow. When compared to the corn oil-fed rats, females fed SALATRIM 32CA lot A015 exhibited a statistically significantly higher cecal pH. Because the increase is

Table 8. Primary and Secondary Phytosterols in the Cecal Contents of Rats Fed SALATRIM Triacylglycerols and Corn Oil[a]

	control (mg/g)[b]		SALATRIM 23CA lot A014 (mg/g)		SALATRIM 32CA lot A015 (mg/g)		corn oil (mg/g)	
phytosterol	males	females	males	females	males	females	males	females
primary								
24β-ethyl-cholesterol	3.54 ± 0.95	3.26 ± 0.61	2.49 ± 0.49**	2.32 ± 0.45**	2.95 ± 0.60**	2.87 ± 0.55**	6.99 ± 2.58*	7.86 ± 5.09*
24β-methyl-cholesterol	3.03 ± 1.00	4.01 ± 4.05	5.55 ± 3.39*	1.82 ± 0.79*,**	4.02 ± 1.24**	2.12 ± 1.04*,**	6.03 ± 1.93*	3.21 ± 1.24
secondary								
24β-methyl-coprostanol	3.66 ± 0.64	3.34 ± 0.56	3.26 ± 0.63**	3.08 ± 0.45**	3.26 ± 0.45**	2.82 ± 0.45*,**	4.52 ± 0.92*	4.53 ± 0.81*
24α-methyl-coprostanol	5.77 ± 0.96	6.24 ± 1.16	5.99 ± 1.01**	6.22 ± 0.78**	7.23 ± 1.01*,**	7.83 ± 1.42*,**	12.33 ± 2.64*	12.46 ± 3.55*
24β-ethyl-coprostanol	14.56 ± 1.83	15.70 ± 2.40	13.71 ± 1.94*	14.10 ± 1.59**	15.54 ± 1.47**	16.58 ± 2.56**	32.00 ± 7.04*	33.58 ± 6.07*
24α-ethyl-coprostanol	3.31 ± 0.70	3.53 ± 0.66	3.25 ± 0.75**	3.43 ± 0.53**	3.13 ± 0.58**	3.30 ± 0.75**	4.79 ± 1.30*	4.79 ± 1.08*

[a] Data represent the mean ± standard deviation of 20 rats per sex per group. Values are statistically significant ($p \leq 0.05$) compared to chow control diet (*) or corn oil diet (**). [b] Units are mg/g dry weight of cecal contents.

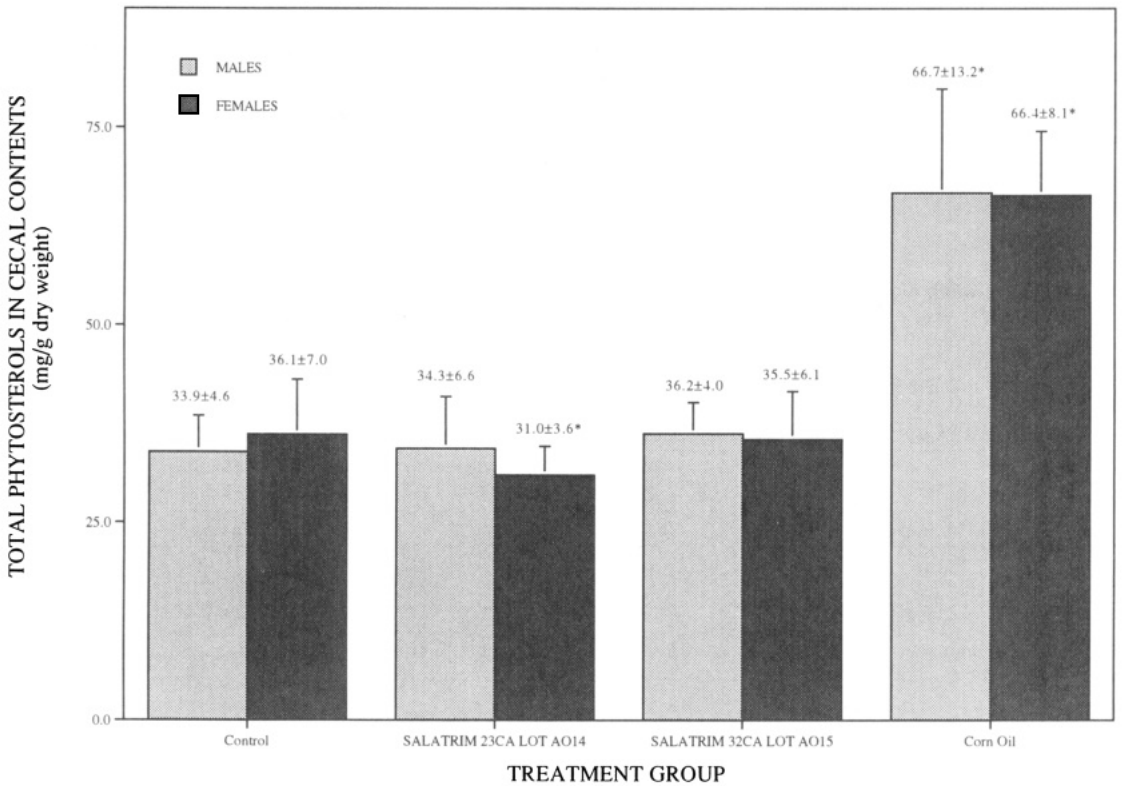

Figure 4. Effect of 13-week dietary exposure to SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, and corn oil on cecal concentrations of total phytosterols in rats. Data represent the mean ± standard deviation for 20 rats per sex per group. Statistically significant differences from the chow control diet ($p \leq 0.05$) are noted by an asterisk. The mean ± standard deviation for each sex is presented above the specific bar.

limited to the females of one group and because the cecal pH of the corn-oil fed rats appears to drop slightly, the biological meaning of this result is unclear. Previous papers describe either rat feeding trials for dietary compounds other than fats or trials monitoring the effects of fats using other animal species. Treon et al. (1989) reported menhaden oil fed to mice significantly reduced fecal pH compared to mice fed either coconut or safflower oil at the same level. However, they presented no data for the fecal pH of chow-fed mice. The degree to which any of these values changed from its "normal" level was not established. How closely the data reported here compare with the study of Treon et al. (1989) is not clear; differences exist. They were able to show a statistical difference because the 95% confidence limits of their measurements were not greater than about 0.2 pH units. The data reported here had a larger variation within each group, and in the one case where a difference was observed, it was an increase. In their studies, groups were comprised of only six mice and they reported fecal pH, not cecal content pH. Also, Treon et al. (1989) fed their mice during the fecal collection period. In the study reported here, the rats were fasted for 24 h before sacrifice. Fasting of rats may cause an elevation in cecal pH (Ward and Coates, 1987).

The two SALATRIM fats and corn oil neither increased the amount of bile acids reaching the cecum nor affected bacterial conversion of primary to secondary bile acids. Although early studies reported increases in bile acid levels in rats fed corn oil (Reddy et al., 1977a), the results reported here are consistent with those of several more recent studies that reported a lack of effect of corn oil on bile acid concentrations in rats (Glauert and Bennink, 1983; Sato et al., 1987; Schuette and Rose, 1986; Gallaher et al., 1992). Regardless of the methods used by these workers, no

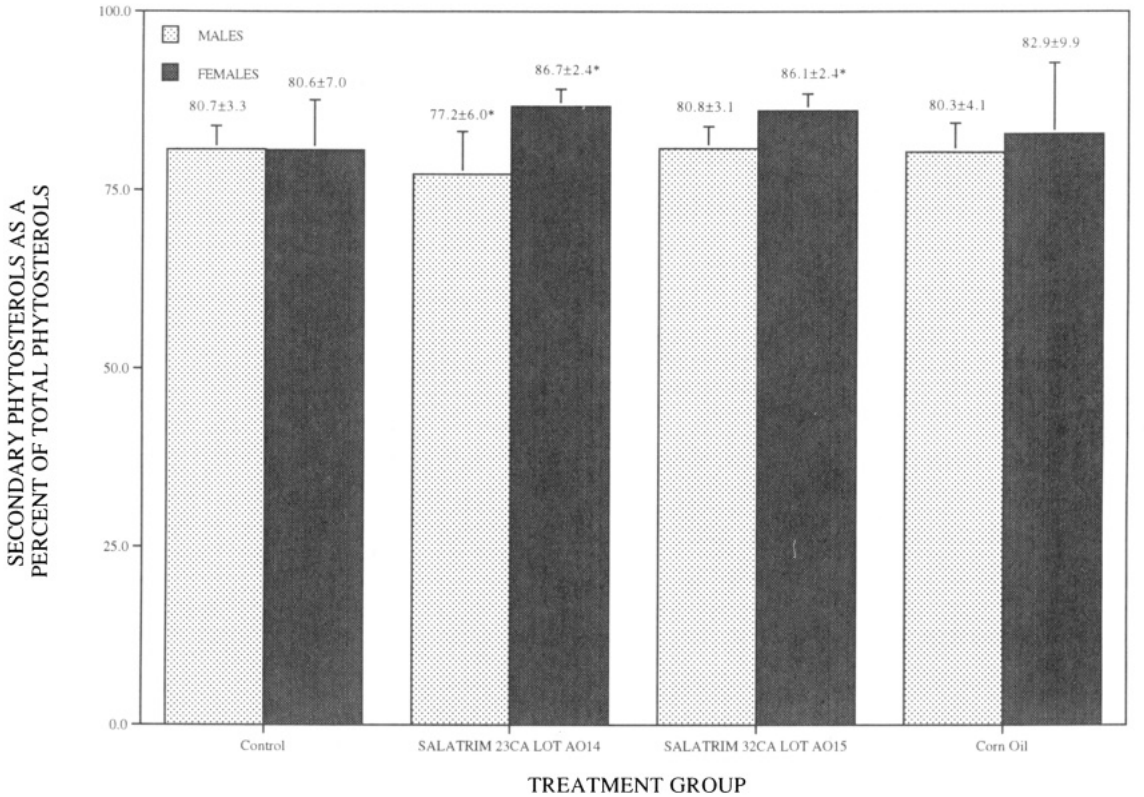

Figure 5. Effect of 13-week dietary exposure to SALATRIM 23CA lot A014, SALATRIM 32CA lot A015, and corn oil on cecal secondary phytosterols concentrations in male and female rats. Data are expressed as the percentage of the total phytosterols and represent the mean ± standard deviation for 20 rats per sex per group. Statistically significant differences from the chow control diet ($p \leq 0.05$) are noted by an asterisk. The mean ± standard deviation for each sex is presented above the specific bar.

significant changes in intestinal bile acid concentrations and metabolism were observed.

Corn oil significantly increased the concentration of cecal cholesterol in male rats, whereas no significant changes were produced by either SALATRIM fat. Increased fecal and serum concentrations of cholesterol and increased biliary excretion of cholesterol have been observed by others studying the effects of high corn oil diets in rats (Reddy et al., 1977a; Bansal et al., 1978; Smit et al., 1991). The results reported here using cecal material are consistent with the effects observed in these studies. There were no large differences among the controls, the SALATRIM fats, and corn oil, in either males or females, in coprostanol as a percent of the control (135–144%). Moreover, no significant differences in the ratio of coprostanol to cholesterol were observed among the groups, indicating the ability of the cecal microflora to metabolize animal sterols is unchanged in any of the treatment groups. The total amount of phytosterols was highest in the cecal contents of rats fed corn oil. This is not surprising since corn oil normally contains about 1.1 wt% phytosterols (Sonntag, 1979) compared to lower values for SALATRIM fats (Softly et al., 1994). There were some very small but statistically significant differences in the amounts of secondary phytosterols in SALATRIM-fed rats compared to the chow-fed control and corn oil groups. However, these changes do not appear to be biologically significant. Interpretation of the data concerning the conversion of primary to secondary phytosterols is confounded by the occurrence of secondary phytosterols in the diet (McNamara et al., 1981). In fact, the secondary phytosterols 24α-methylcoprostanol and 24α-ethylcoprostanol are found in higher concentrations in SALATRIM than in corn oil (Softly et al., 1994). These higher levels of secondary phytosterols may account for the higher levels of these phytosterols in male rats fed either SALATRIM. Grundy et al. (1968) have shown that phytosterols and animal sterols are metabolized by the intestinal microflora to a similar extent. In our study, we found that secondary phytosterols represented approximately 80% of the total, whereas secondary bile acids represented approximately 65% of the total and approximately 68% of the cholesterol was converted to coprostanol. The difference in our study between phytosterols and animal sterol metabolism is probably associated with the occurrence of secondary phytosterols in the diet, which increases the percent of secondary phytosterols as a percentage of total phytosterols.

While each of the variables measured here has been examined in other animal studies, most of the other studies have used neither feeding periods as long as those reported here nor such a large number of animals per experimental group. The 13-week duration of this study was longer than the vast majority of similar studies and was probably sufficient to observe any potential perturbation in the variables measured. Aside from being an indicator of the health and stability of the colonic ecosystem, one variable, bile acid concentration, has additional significance because of studies linking secondary bile acid levels to the incidence of colon cancer (Reddy et al., 1977b). Our finding that SALATRIM fats do not increase the levels of either primary or secondary bile acids when fed at 10% in the diet for 13 weeks is therefore significant in its own right. The combined results from this study indicate that SALATRIM had little or no effect on the MACs measured here and, in general, less effect on the intestinal microecology than did corn oil.

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LITERATURE CITED

AOCS. Free Fatty Acids. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed.; Firestone, D., Ed.; AOCS: Champaign, IL, 1990a; Method Ca 5a-40.

AOCS. Peroxide Value. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th ed.; Firestone, D., Ed.; AOCS: Champaign, IL, 1990b; Method Cd 8-53.

Bansal, B. R.; Rhoads, J. E., Jr.; Bansal, S. C. Effect of Diet on Colon Carcinogenesis and the Immune System in Rats Treated with 1,2-Dimethylhydrazine. *Cancer Res.* **1978**, *38*, 3293–3303.

Borriello, S. P.; Davies, H. A.; Carman, R. J. Cellular Morphology of *Clostridium spiroforme*. *Vet. Microbiol.* **1986**, *11*, 191–195.

Chung, K. T.; Fulk, G. E.; Slein, M. W. Tryptophanase of Fecal Flora as a Possible Factor in the Etiology of Colon Cancer. *J. Natl. Cancer Inst.* **1975**, *54*, 1073–1078.

Felix, Y. F.; Hudson, M. J.; Owen, R. W.; Ratcliffe, B.; van Es, A. J. H.; van Velthuisjen, J. A.; Hill, M. J. Effect of Dietary Lactitol on the Composition and Metabolic Activity of the Intestinal Microflora in the Pig and in Humans. *Microb. Ecol. Health Dis.* **1990**, *3*, 259–268.

Gallaher, D. D.; Locket, P. L.; Gallaher, C. M. Bile Acid Metabolism in Rats Fed Two Levels of Corn Oil and Brans of Oat, Rye and Barley and Sugar Beet Fiber. *J. Nutr.* **1992**, *122*, 473–481.

Glauert, H. P.; Bennink, M. R. Influence of Diet or Intracecal Bile Acid Injections on Colonic Epithelial Cell Proliferation in Rats Previously Injected with 1,2-Dimethylhydrazine. *J. Nutr.* **1983**, *113*, 475–482.

Grundy, S. M.; Ahrens, E. H., Jr.; Salen, G. Dietary β-sitosterol as an Internal Standard to Correct for Cholesterol Loss in Sterol Balance Studies. *J. Lipid Res.* **1968**, *9*, 374–387.

Hayes, J. R.; Wilson, N. H.; Pence, D. H.; Williams, K. D. Subchronic Safety Evaluation of SALATRIM Structured Triacylglycerols in Rats: 2. Triacylglycerols Composed of Stearate, Acetate and Propionate. *J. Agric. Food Chem.* **1994**, one of several papers in this issue.

Hentges, D. J., Ed. *Human Intestinal Microflora in Health and Disease*; Academic Press: New York, 1983; 568 pp.

Hill, M. J., Ed. *Microbial Metabolism in the Digestive Tract*; CRC Press: Boca Raton, FL, 1986; 253 pp.

Macfarlane, G. T.; Cummings, J. H. The Colonic Flora, Fermentation, and Large Bowel Digestive Function. In *The Large Intestine: Physiology, Pathophysiology, and Disease*; Phillips, S. F., Pemberton, J. H., Shorter, R. G., Eds.; Raven Press: New York, 1991; pp 51–92.

Mallet, A. K.; Rowland, I. R.; Bearne, C. A.; Flynn, J. C.; Fehilly, B. J.; Udeen, S.; Farthing, M. J. G. Effect of Dietary Supplements of Apple Pectin, Wheat Bran or Fat on the Enzyme Activity of the Human Fecal Flora. *Microb. Ecol. Health Dis.* **1988**, *1*, 23–30.

McNamara, D. J.; Proia, A.; Miattinen, T. A. Thin-layer and Gas-liquid Chromatographic Identification of Neutral Steroids in Human and Rat Feces. *J. Lipid Res.* **1981**, 474–484.

NIH Publication 86-23. Institute of Laboratory Animal Resources Commission of Life Sciences, *Guide for the Care and Use of Laboratory Animals*, U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health: Bethesda, MD, 1985.

Reddy, B. S.; Maruyama, H. Effect of Different Levels of Dietary Corn Oil and Lard During the Initiation Phase of Colon Carcinogenesis in F344 Rats. *J. Natl. Cancer Inst.* **1986**, *77*, 815–822.

Reddy, B. S.; Mangat, S.; Sheinfil, A.; Weisburger, J. H.; Wynder, E. L. Effect of Type and Amount of Dietary Fat and 1,2-Dimethylhydrazine on Biliary Bile Acids, Fecal Bile Acids and Neutral Sterols in Rats. *Cancer Res.* **1977a**, *37*, 2132–2137.

Reddy, B. S.; Watanabe, K.; Weisburger, J. H.; Wynder, E. L. Promoting Effect of Bile Acids in Colon Carcinogenesis in Germ-Free and Conventional F344 Rats. *Cancer Res.* **1977b**, *37*, 3238–3242.

Rumney, C. J.; Rowland, J. R. *In Vivo* and *In Vitro* Models of the Human Colonic Flora. *Crit. Rev. Food Sci. Nutr.* **1992**, *31*, 299–331.

SAS Institute. *SAS/STAT User's Guide*, version 6, 4th ed.; SAS Institute: Cary, NC, 1990; Vol. 2.

Sato, Y.; Furihata, C.; Matsushima, T. Effects of High Fat Diet on Fecal Contents of Bile Acids in Rats. *J. Cancer Res. (Gann)* **1987**, *78*, 1198–1202.

Schuette, S. A.; Rose, R. C. The Effects of Diets High in Fat and/or Fiber on Colonic Absorption of DMH in the Rat. *Nutr. Cancer* **1986**, *8*, 257–265.

Setchell, K. D. R.; Lawson, A. M.; Tanida, N.; Sjovall, J. General Methods for the Analysis of Metabolic Profiles of Bile Acids and Related Compounds in Feces. *J. Lipid Res.* **1983**, *24*, 1085–1100.

Setchell, K. D. R.; Ives, J. A.; Cashmore, G. C.; Lawson, A. M. On the Homogeneity of Stools with Respect to Bile Acid Composition and Normal Day-to-Day Variations: A Detailed Qualitative and Quantitative Study Using Capillary Column Gas Chromatography-Mass Spectrometry. *Clin. Chim. Acta* **1987**, *162*, 257–275.

Smit, M. J.; Temmerman, A. M.; Wolters, H.; Kuipers, F.; Beynen, A. C.; Ronk, R. J. Dietary Fish Oil-Induced Changes in Intrahepatic Cholesterol Transport and Bile Acid Synthesis in Rats. *J. Clin. Invest.* **1991**, *88*, 943–951.

Softly, B. J.; Huang, A. S.; Finley, J. W.; Petersheim, M.; Yarger, R. G.; Chrysam, M. M.; Wieczorek, R. L.; Otterburn, M. S.; Manz, A.; Templeman, G. J. Composition of Representative SALATRIM Fat Preparations. *J. Agric. Food Chem.* **1994**, one of several papers in this issue.

Sonntag, N. O. V. Structure and Composition of Fats and Oils. In *Bailey's Industrial Oil and Fat Products*, 4th ed.; Swern, D., Ed.; Wiley: New York, 1979; Vol. 1, p 55.

Treon, S. P.; Fox, E. S.; Broitman, S. A. Marine Oils in the Modulation of Colonic Flora and pH: Considerations for Colon Cancer. *Microb. Ecol. Health Dis.* **1989**, *2*, 115–122.

Ward, F. W.; Coates, M. E. Gastrointestinal pH Measurement in Rats: Influence of the Microbial Flora, Diet and Fasting. *Lab. Anim.* **1987**, *21*, 216–222.

Weinstein, W. M.; Onderdonk, A. B.; Bartlett, J. G.; Gorbach, S. L. Experimental Intra-Abdominal Abscesses in Rats: Development of an Experimental Model. *Infect. Immun.* **1974**, *10*, 1250–1255.

Wilkins, T. D. Microbiological Considerations in Interpretation of Data Obtained with Experimental Animals. In *Gastrointestinal Cancer: Endogenous Factors*; Bruce, W. R., Correa, P., Lipkin, M., Tannebaum, S. R., Wilkins, T. D., Eds.; Cold Spring Harbor: Plainview, NY, 1981; 468 pp.

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