Colonic Bacterial Activity and Serum Lipid Risk Factors for Cardiovascular Disease

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Antibiotics are being proposed for the treatment of cardiovascular disease. In the past, antibiotics were advocated for the control of hypercholesterolemia. We have therefore investigated the relation between colonic bacterial activity and serum lipids. In a four-phase randomized crossover study, we fed a different starch supplement during each 2-week phase to 24 healthy subjects. In two phases, supplements containing resistant starches were fed that reach the colon and are largely fermented by colonic bacteria. Fecal starch recovery therefore reflects the metabolic activity of colonic microflora. The control treatments were conventional starches. Blood lipid levels were obtained at the start and 4-day fecal collections at the end of each phase. Resistant starch supplements increased fecal starch excretion by 3.8 ± 1.2 g/d more than conventional starches ($P = .006$). Mean starch excretion was related positively to pretreatment serum high-density lipoprotein (HDL) cholesterol ($r = -.57$, $P = .003$) and negatively to low-density lipoprotein (LDL) cholesterol ($r = -.57$, $P = .004$), apolipoprotein B:AI ($r = -.56$, $P = .005$), and fecal output of fusobacteria ($r = -.73$, $P = .003$) and bacteroides ($r = -.72$, $P = .003$). The ratio of fusobacteria to total anaerobes was also related to pretreatment LDL cholesterol ($r = .56$, $P = .037$). Differences in starch excretion between healthy subjects, as a measure of bacterial activity, accounted for 32% of the variation in pretreatment LDL cholesterol. The activity of colonic microflora therefore appears to influence serum lipid levels. Alterations of bacterial number and activity may provide an additional strategy to control serum lipid risk factors for cardiovascular disease.

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THERE IS GROWING EVIDENCE for bacterial involvement in diseases not previously thought to be of bacterial origin. The link is well established for *Helicobacter pylori* in peptic ulcer disease, and there is now considerable interest in the possible association of *Chlamydia pneumoniae* and *H pylori* with cardiovascular disease.[1-3] Furthermore, there are reports of the successful use of antibiotics in reducing cardiovascular events.[2,3] However, colonic bacteria have been largely neglected in this respect despite the fact that over 25 years ago, antibiotic treatment with neomycin, which influences colonic microflora, was also recommended in hyperlipidemia as part of the strategy to reduce the risk of cardiovascular disease.[4]

We have therefore determined whether the blood lipid concentrations of healthy subjects are related to colonic microbial activity, as assessed by the completeness of colonic starch fermentation, when challenged with dietary supplements containing either available or resistant starches.

SUBJECTS AND METHODS

Subjects

Twenty-four healthy subjects (12 men and 12 premenopausal women) were recruited from university and hospital staff and students. The mean age was 33 ± 2 years (mean ± SE; range, 22 to 53y) and the body mass index (BMI) was 23.7 ± 0.6 kg/m^2 (range, 19.4 to 34.2). None of the subjects had a history of diabetes or renal or hepatic disease, and none were taking medications. The study was approved by the University of Toronto Ethics Committee and the ethics committee of St. Michael's Hospital. Informed consent was obtained from all participants.

Protocol

On four different occasions, fasting blood samples were obtained and body weight was measured, and subjects were assigned in random order to consume one of four wheat or cornstarch supplements as muffins and breakfast cereal for a 2-week period in a crossover design separated by a 2-week washout period. The supplements were low-fiber wheat starch (wheat flour) control, high-fiber wheat starch (wheat bran) control, high-amylose cornstarch, and retrograded cornstarch. Every effort was made to conduct a double-blind study, although the high-fiber wheat bran control cereal and muffin were a darker color than the other supplements.

Supplements contributed, on average, 32% of the subjects' recorded energy intake and approximately 100 g/d starch (range, 98 to 104). These supplements all shared a similar macronutrient profile: 11% fat, 14% protein, and 75% available carbohydrate (as a percent of energy) and 4 g/d fiber, apart from the high-fiber wheat bran supplement, which provided 23 g/d dietary fiber. In addition, the supplements provided differing amounts of small-intestinally unavailable or "resistant" starch (RS): low-fiber control, 2.3 g/d; high-fiber control, 1.5 g/d; high-amylose cornstarch (RS2), 21.5 g/d; and retrograded cornstarch (RS3), 27.9 g/d.[5] Seven-day diet histories obtained during the last week of each phase demonstrated a similar caloric intake (2,124 to 2,260 kcal/d), macronutrient intake (58% to 61% available carbohydrate, 22% to 24% fat, and 15% to 16% protein), and fatty acid profile for all four phases. The mean dietary fiber consumption on all four study phases calculated without the supplement was also similar (9 to 11 g/1,000 kcal).

Subjects were provided with underseat lavatory frames and plastic bags for fecal collection on days 11 to 14 of each study. Labeled samples were stored on frozen CO_2 in polystyrene containers. Immediately after passing feces, a 1-mL core suction biopsy was obtained from the fecal mass by the subject for microbiological analysis, discharged into a preweighed plastic storage tube containing peptone water and 0.03% cysteine hydrochloride to maintain anaerobic conditions, and placed on frozen CO_2.[6,7] Fourteen subjects provided samples for

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Table 1. Fecal Starch Output (n = 24) and Fecal Microflora (n = 14)

Parameter	Low-Fiber Control	High-Fiber Control	RS2	RS3
Fecal starch (g/d)	1.4 ± 0.4	2.4 ± 0.5	6.1 ± 1.6	5.2 ± 1.6
Total aerobes*	8.3 ± 0.4	8.1 ± 0.4	8.3 ± 0.4	8.5 ± 0.4
Total anaerobes*	10.5 ± 0.2	10.6 ± 0.3	10.7 ± 0.3	11.1 ± 0.3
Anaerobes/aerobes	1.3 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.0
Bifidobacteria*	9.0 ± 0.3	9.0 ± 0.4	8.3 ± 0.4	8.6 ± 0.6
Bacteroides*	8.5 ± 0.8	8.5 ± 0.5	7.3 ± 1.1	8.6 ± 1.0
Fusobacteria*	9.7 ± 0.4	9.3 ± 0.5	10.1 ± 0.4	10.0 ± 0.4

NOTE. Results are the mean ± SE.
*Log_{10} CFU/g wet weight.

microbiological analysis on all four phases All samples were delivered to the laboratory upon completion of the collections.

Details of the study relating to short-chain fatty acids, breath gases, and blood lipids after consumption of starch supplements are reported elsewhere.[8]

Analyses

Frozen serum was stored at −70°C. All samples from each individual were analyzed in the same batch for total cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol, after dextran sulfate and magnesium chloride precipitation, using the techniques of the Lipid Research Clinics.[9] The level of low-density lipoprotein (LDL) cholesterol was calculated.[10] Serum levels of apolipoproteins Al and B were measured by an enzyme-linked immunosorbent assay technique.[11]

Total fecal starch was analyzed on freeze-dried fecal homogenates of 4-day collections.[12] Fecal biopsies were analyzed for microbiology using selective media.[6,7] Bacterial populations were expressed as the log_{10} of colony forming units per gram of fresh sample (log CFU/g).

Supplements were analyzed for macronutrients[13] and resistant starch,[5] and dietary fiber was assessed from food composition tables.[14] Diet histories were assessed using a database derived from US Department of Agriculture data.[15]

Statistical Analysis

The results are expressed as the mean ± SE. Differences between the treatment mean values were assessed by a Student-Newman-Keuls multiple-range test using the SAS program after prior determination of a significant difference using ANOVA.[16] For paired data, Student's *t* test (two-tailed) was also used to assess the difference. The strength of linear association between various factors was determined by Pearson product-moment correlations. Partial correlations were used to adjust for age and BMI (PROC CORR/SAS).[16]

RESULTS

Subject records indicated that 98.7% ± 0.5% of cereal supplements and 96.0% ± 1.2% of muffin supplements were consumed, with no significant differences in compliance between treatments. There were also no differences in body weight change.

Fecal Starch Output

Both resistant starches tended to result in higher fecal starch loss compared with the low- and high-fiber wheat starch controls (Table 1). The difference in mean fecal starch output between the two resistant starch challenges and two control challenges was 3.8 ± 1.2 g/d ($P = .006$). No difference was observed between the resistant starch supplements. The ranking for fecal starch output in a given individual tended to be preserved across all four treatments (Figs 1 and 2). Thus, fecal starch output on one treatment correlated significantly with fecal starch output on each of the other three treatments ($P \leq .019$).

Fecal Microbiology

Consumption of the starch supplements did not result in differences in the output of total bacteria, bifidobacteria,

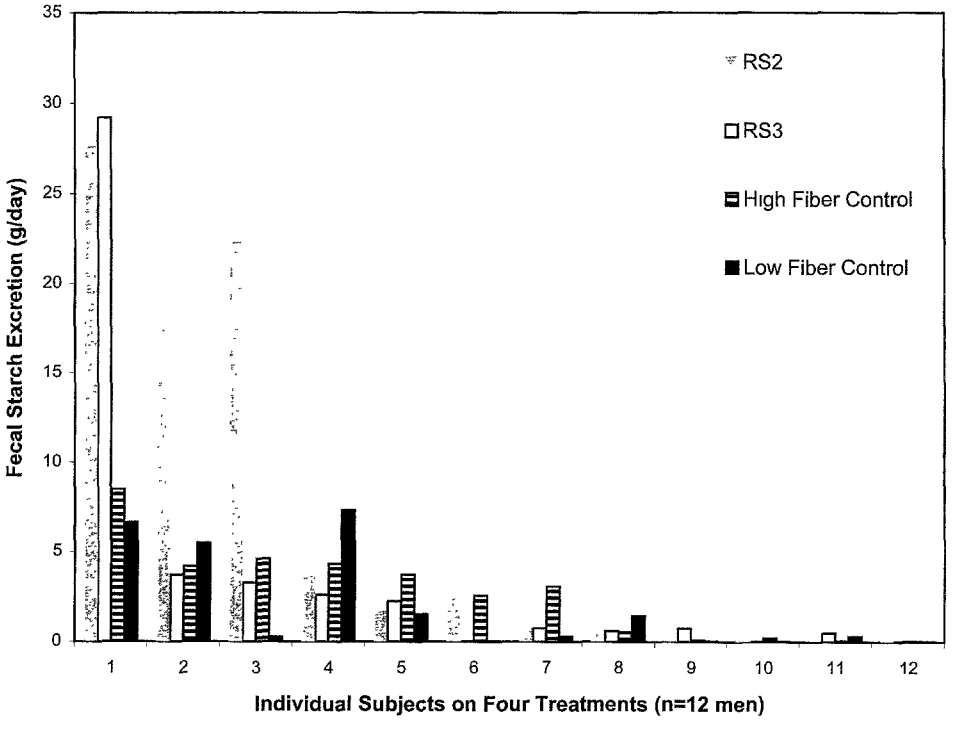

Fig 1. Fecal starch excretion for each man (n = 12) on all 4 treatments. Subjects are ranked in descending order (left to right) on the basis of mean starch output for the 4 treatments.

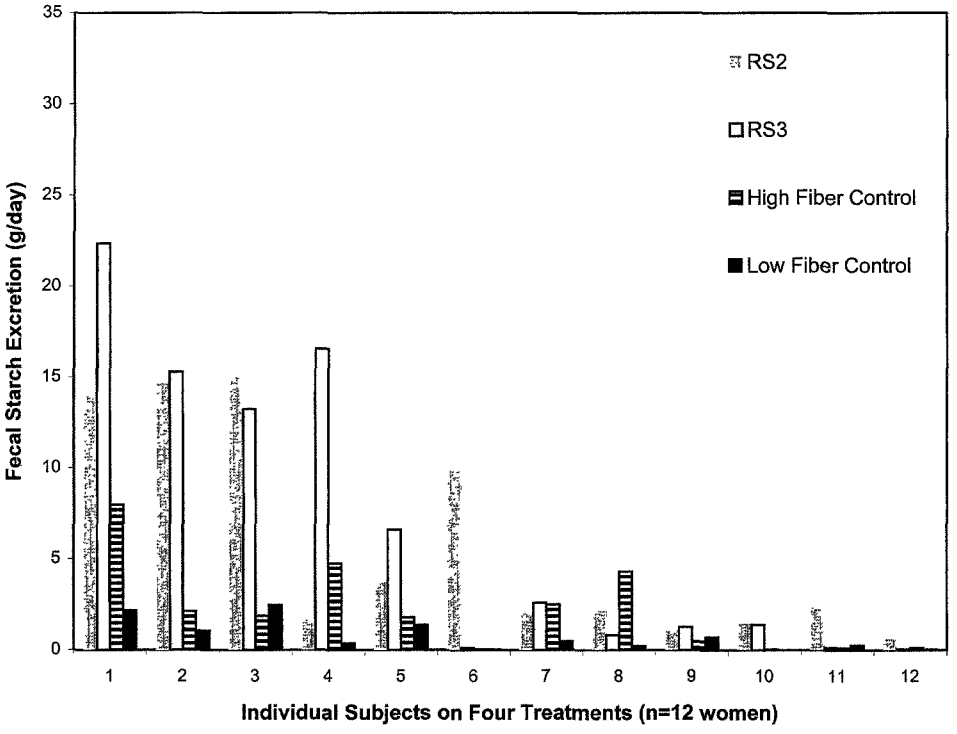

Fig 2. Fecal starch excretion for each woman (n = 12) on all 4 treatments. Subjects are ranked in descending order (left to right) on the basis of mean starch output for the 4 treatments.

fusobacteria, or bacteroides assessed by the Student-Newman-Keuls test (Table 1). However, as with the fecal starch output, subjects tended to maintain their individual pattern of colonic microflora on the low-fiber control and resistant starch supplements, with the ranking on one diet being similar to the other two for fecal output of bacteroides ($P \leq .003$) and fusobacteria ($P \leq .011$) (Table 2).

Serum Lipids and Lipoproteins

Mean baseline blood lipid levels for all subjects were within National Cholesterol Education Program (NCEP) guidelines (LDL cholesterol < 4.1 mmol/L)[17] and did not differ between treatments.

Table 2. Between-Treatment Correlations for Fecal Bacteria (n = 14)

Bacteria	High-Fiber Control	RS2	RS3
Bacteroides			
Low-fiber control			
r	.17	.72	.80
P	.564	.003	.001
High-fiber control			
r	1.00	.03	.25
P		.909	.383
RS2			
r		1.00	.70
P			.005
Fusobacteria			
Low-fiber control			
r	.14	.67	.65
P	.622	.009	.011
High-fiber control			
r	1.00	.32	.21
P		.270	.463
RS2			
r		1.00	0.52
P			.059

Relation of Fecal Starch, Microbiology, and Serum Lipids

Age was related positively to the mean baseline total cholesterol ($r = .52$, $P = .010$) and LDL cholesterol ($r = .52$, $P = .010$). The BMI was related negatively to HDL cholesterol ($r = -.47$, $P = .021$). All associations between blood lipids and other factors were therefore assessed using partial correlations controlling for age and BMI. No associations were found between the BMI, age, or dietary carbohydrate intake and the mean fecal starch output.

When the mean starch excretion data for all four treatments were compared with the mean pretreatment lipid and lipoprotein data, significant negative associations were observed between fecal starch output and total cholesterol ($r = -.41$, $P = .047$), LDL cholesterol ($r = -.57$, $P = .004$), apolipoprotein B ($r = -.55$, $P = .006$), and the ratios total: HDL cholesterol ($r = -.52$, $P = .009$), LDL:HDL cholesterol ($r = -.57$, $P = .003$), and apolipoprotein B:AI ($r = -.56$, $P = .005$) and there was a positive relation with HDL cholesterol ($r = .44$, $P = .031$) (Fig 3).

Mean fecal starch excretion was also related to fusobacteria ($r = -.73$, $P = .003$), bacteroides ($r = -.72$, $P = .003$), and the ratio of fusobacteria to anaerobes ($r = -.62$, $P = .019$) and bacteroides to anaerobes ($r = -.73$, $P = .003$). In addition, the ratio of fusobacteria to anaerobes was related positively to LDL cholesterol ($r = .56$, $P = .037$). No other association between fecal bacteria and blood lipids reached significance.

DISCUSSION

The association between blood lipids and the aspect of bacterial activity measured in this study appeared to account for

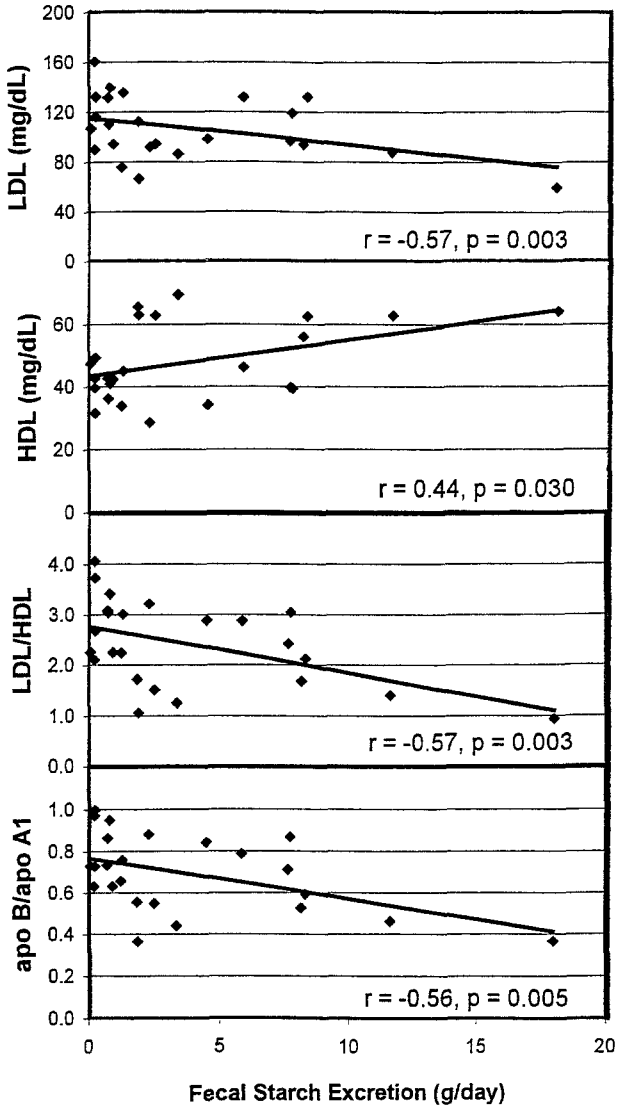

Fig 3. Correlation of fecal starch excretion with blood lipids and lipoprotein ratios after adjustment for age and BMI. Values are the mean fecal starch excretion and baseline blood lipid and lipoprotein concentrations for all 4 treatments in 24 subjects.

17% to 32% of the variation in blood lipids, depending on the lipid and lipoprotein fraction (r = .41 to .57). By comparison to genetic and dietary influences, these associations of serum lipids with colonic events were substantial. Of the common genetic determinants of serum lipids, the apolipoprotein E polymorphism possibly explains 8% of the total variance in LDL cholesterol.[18] A reduction of saturated fat (<7% of dietary calories) and dietary cholesterol (<200 mg/d) (NCEP Step 2 diet)[17] may achieve a 19% reduction in serum cholesterol.[19] Other dietary manipulations including viscous soluble fibers, soy protein, and plant sterols may also reduce serum cholesterol by approximately 5% to 10% each,[20-23] while hypolipidemic drug therapy with 20 to 40 mg/d of the commonly used hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitors reduces LDL cholesterol by 21% to 44%.[24]

To challenge the ability of the colonic microflora to ferment carbohydrate, we used supplements containing resistant starches, which by definition, in a fashion analogous to dietary fiber, are not digested in the small intestine.[25,26] Fecal recovery of these starches is therefore dependent on the activity of colonic microflora.[26] The mean fecal starch output and wide range of values between individuals were similar to previously reported studies.[26]

It is unlikely that fecal starch output is causally related to blood lipids, since feeding significant amounts of resistant starch did not change the blood lipid profile at the end of the supplementation period in this study[8] or others.[27] It is more likely that individual differences in colonic bacterial numbers and metabolic activity that determine fecal starch loss independently influence the blood lipid profile.

In this study, starch excretion was related negatively to the fecal output of fusobacteria and bacteroides. One of the metabolic activities of colonic microflora that may influence serum lipids is the production of acetate during carbohydrate fermentation. Acetate infused rectally[28] or generated within the colon[29] appears to increase serum cholesterol. On the other hand, propionate generation by colonic bacteria may decrease serum cholesterol.[30] Another marker of colonic bacterial activity shown to relate to serum cholesterol is the level of fecal bacterial 7α-dehydroxylase. Serum cholesterol levels in both normolipidemic children[31] and adults[32] have been shown to relate to the activity of this enzyme, which converts primary bile acids to secondary bile acids, although the exact reason for this association is not evident. It is therefore of interest that over 25 years ago the antibiotic neomycin was advocated to decrease cholesterol, although as a result of concerns over toxicity and opportunistic infections, it was never widely used.[4] At that time, Samuel[4] found that a number of antibiotics in addition to neomycin reduced serum cholesterol levels in hyperlipidemic subjects, dependent on their ability to reduce fecal 7α-dehydroxylase levels.[4] Whether the cholesterol decrease with neomycin was due to a general depression of bacterial metabolic activity or selective growth of fusobacteria, which is known to occur in the presence of neomycin, is not clear.[33] However, the successful use of antibiotics in cardiovascular disease[2,3] may relate not only to their effects on bacterial targets such as *C pneumoniae* and *H pylori* but also to their effect on the colonic microflora.

There is also evidence that dietary fiber, as a nonabsorbable carbohydrate, may influence colonic bacteria, as shown by an increase in the ratio of anaerobes to aerobes.[34] Studies have clearly shown that inhibition of small-intestinal carbohydrate absorption using acarbose also resulted in altered bacterial activity and short-chain fatty acid synthesis, with possible benefits for colonic mucosal health.[35] There is now considerable interest in the prospect that the colonic microflora numbers and metabolic activity can be manipulated, for example, using fructooligosaccharides, which are not absorbed in the small intestine. With this "prebiotic" approach, it has been shown that bifidobacterial numbers can be selectively increased together with alterations in blood lipids in laboratory animals.[36] Therefore, there appear to be a number of options available, including the use of prebiotic substrates or antibiotics that may alter the colonic microflora to the benefit of the mucosa with possible systemic effects on the blood lipid profile.

Our study suggests that the colonic microbial activity of healthy subjects is related to lipid risk factors for cardiovascular

disease. The negative associations for HDL cholesterol and apolipoprotein AI with colonic bacterial activity support previous findings with *H pylori*.[1,37] We are not implying that the colonic microflora responsible for these effects are pathogens. However, certain profiles of colonic microflora may share some of the effects on serum lipids and other risk factors for heart disease reported for *H pylori* and possibly *C pneumoniae*.[1,38] The strength of the relation may be due to the bacterial density and sheer volume of the colonic biomass. Any future trials of antibiotics in cardiovascular disease should consider the antibiotic effect on the colonic bacteria and their metabolic activity. Ultimately, the aim of possible treatment strategies may involve not only the elimination of defined pathogens in the upper gastrointestinal tract and elsewhere, but also modification of the profile of an otherwise healthy colonic microflora.

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