

Diet Induced Changes in the Colonic Environment and Colorectal Cancer

Effects of a Controlled Diet and Black Tea Drinking on the Fecal Microflora Composition and the Fecal Bile Acid Profile of Human Volunteers in a Double-Blinded Randomized Feeding Study¹

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ABSTRACT Although diet has been clearly associated with human health many potential mechanisms remain undefined. For instance, although the intestinal bacterial microflora has long been postulated to contribute to human health, little is known about the effects of diet on the bacterial microflora composition and the specific contributions of the microflora to human health. Thus, we analyzed 1) changes in the fecal microflora composition by fluorescent in-situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) and 2) changes in the fecal bile acid profile, in a crossover feeding study that investigated the effects of black tea drinking on blood lipids in hypercholesterolemic volunteers. DGGE analysis shows that each study subject harbors a specific bacterial profile that exhibits little change over time. Change from a “free” living diet to the controlled study diet or to black tea drinking did not significantly change these bacterial profiles. FISH analysis revealed that even though black tea did not affect the specific bacterial groups that were analyzed, it did decrease the amounts of bacteria that were detected by the universal bacterial probe, but not by any of the specific probes. We did not detect any consistent effects of either diet or black tea drinking on the levels and proportions of fecal bile acids. Our results indicate that tea drinking affects some microflora components. Larger studies with well defined end points that control for the observed variation are needed to improve our understanding of the effects of diet on intestinal microflora and fecal bile acid profile. *J. Nutr.* 134: 473–478, 2004.

KEY WORDS: • fecal microflora • black tea • human • FISH • DGGE • diet

Diet has long been known to contribute to human health and various associations between diet and human disease including cancer are well established (1–3). However, the complexity of dietary interactions has hampered the investigation of some of the potentially important mechanisms through

which diet might affect health. For instance, our knowledge of the role of diet in regulating the composition of the bacterial gut microflora and the potential contributions of the microflora to human health is scarce.

The colon is heavily inhabited by a variety of microorganisms, mainly bacteria, fungi and protozoa that contribute to health by metabolizing pro-carcinogens and carcinogens that include bile acids and facilitating their excretion through binding, activating beneficial compounds such as phytoestrogens for uptake by the colonic epithelium; by producing fermentation end products such as short chain fatty acids, excluding pathogenic microorganisms; and by stimulating the immune system (4,5). The gut microflora likely influences the effects of diet on human health, especially through its participation in the metabolism of nutrients that reach the colon (6). Thus, effects of some dietary components that have long been proposed to contribute to human health, such as dietary fiber, might depend on the ability of an individual's particular gut microflora to ferment them into beneficial end product such as butyrate. However, little is known about interactions

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between dietary substances and the composition of the microflora and how changes in microflora composition affect the colon physiology. Directed changes in the intestinal physiology through modification of the gut microflora by dietary interventions (pre- and probiotics) offer the potential for disease prevention (7,8). Although various products claiming to promote intestinal health are already commercially available, the scientific data supporting such claims are weak.

Molecular tools based on 16S rDNA sequence similarities such as fluorescent in-situ hybridization (FISH)³ and denaturing gradient gel electrophoresis (DGGE) have helped to overcome limitations of conventional microbiological plating methods in studying the fecal microflora composition (4,9). These tools have been successfully applied to study the development of the infant microflora, changes in the human microflora during aging, the effects of pre- and probiotics on the human microflora composition, and the effects of dietary interventions on the intestinal microflora in various animal models (7,10–13). These studies have advanced our understanding of the microflora but they have also shown a large degree of individual variation. Some but not all of this observed variation is likely due to differences in dietary intake. Except for prebiotics, probiotics, and synbiotics, the effects of diet on the human microflora have not been extensively studied with molecular tools and very few of the existing studies were performed with a controlled dietary regimen.

The black tea pilot study, from which we now report on fecal microflora and bile acids, was a double-blinded randomized crossover feeding study that investigated the effects of black tea drinking on blood lipids in hypercholesterolemic volunteers. The study reported that black tea drinking vs. placebo significantly reduced total cholesterol and LDL cholesterol while HDL cholesterol and triglycerides were not affected (14a).

One possible mechanism by which tea could reduce LDL cholesterol is through changes in the composition and metabolic activity of the microflora. Polyphenols, which are abundant in tea, have antimicrobial properties (14b) that can affect microflora composition. While some bacteria express the enzyme 7 α -dehydroxylase and can metabolize primary bile acids into potentially harmful secondary bile acids, other bacteria can facilitate bile acid excretion through binding (15). Because bile acid levels are replenished by synthesis from serum cholesterol in the liver, increased bile acid excretion could contribute to lower serum cholesterol levels.

We describe here changes that we observed during the tea pilot study in the fecal microflora composition and the effects of the intervention on fecal bile acid profiles.

MATERIALS AND METHODS

Study design

Volunteers were recruited to participate in a randomized, double-blind, crossover study of black tea (T) compared to a placebo (P). Beverages for T and P treatments were prepared from dry powders, similar to instant tea. Prior to the first treatment period volunteers were placed in a 2-wk run-in period. Treatments were separated by a 4-wk washout period. During this break, alcohol and tea consumption were not allowed. During all phases of the study (run-in, wash-out, and treatment periods) volunteers ingested either one cup of caffeinated coffee or two caffeinated diet sodas daily thus establishing a

consistent baseline level of caffeine intake and preventing possible caffeine withdrawal symptoms. Subjects eliminated other caffeine-containing foods and medications throughout the study.

Controlled diet

During the two treatment periods, volunteers consumed the same background controlled diet. All foods and beverages were prepared and supplied by the Human Studies Facility at the Beltsville Human Nutrition Research Center (Beltsville, MD). Food items were weighed, served in proportion to caloric requirements, and color-coded according to the treatment beverage. Dietitians monitored food and treatment beverage selections at each meal. Composites of foods in the 7-d menu cycle were prepared and analyzed for macronutrients and fatty acids (Covance Laboratories). Seven-day menus were prepared in 200-kcal increments and designed to follow a National Cholesterol Education Program Step I type diet. The diets provided 58% of calories from carbohydrates, 26% from fat, and 16% from protein. The fat had a ratio of polyunsaturated to monounsaturated to saturated fatty acids of 1:1:0.8. The diet provided 71 mg cholesterol, 13.6 g dietary fiber, and 8.5 mg iron per 1000 kcal. At the average energy intake for the study of 2760 kcal, this translates to a daily intake of 196 mg cholesterol, 33.6 g dietary fiber, and 23.5 g iron. Except for calcium and iron when prescribed by the volunteer's personal physician, vitamin and mineral supplementation was not permitted. Each weekday volunteers were weighed and energy intake was adjusted as needed to keep body weight constant. Dinner and breakfast were consumed at the Center during the week; carryout lunches and snacks were provided. Weekend foods and treatment beverages were packaged with instructions for home consumption.

Fecal collections

Fecal samples were collected on days 1, 14, and 21 of each intervention for a total of six fecal samples per subject. Subjects obtained a cooler filled with ice for storage of the sample until delivery to the lab. All samples were delivered on ice within 12 h of defecation. Samples were processed by kneading in a strong plastic bag immediately upon arrival in the laboratory. A small portion of the sample was fixed for FISH analysis as described below and the remainder was stored at -70°C.

FISH analysis

Fecal sample (~0.5 g) was added to 4.5 mL of PBS and the samples were prepared for FISH analysis as described previously (16). In short, samples were homogenized by vortexing with a dozen glass beads for 5 min, the fecal debris was removed by centrifuging at low speed, and the bacteria containing supernatant was fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) over night. Aliquots of the samples were stored at -70°C until time of hybridization. For hybridization 10 μ L of appropriate dilutions of the samples was applied to gelatin coated microscopic slides and fixed to the slides with 95% ethanol as described previously (17) except that the dilutions were made in PBS and not in 5% Tween solution. The slides were hybridized with the 5 ng/ μ L of the respective probes using the conditions described previously (16,18,19). The following probes were used: the EU338 probe detecting almost all bacteria (20); probe Bac303 for the genera *Bacteroides* and *Prevotella* (21); the Elgc01 probe detecting *Faecalibacterium*-like species (22) probe Erec482 for eubacteria, clostridia and ruminococci belonging to *Clostridium* cluster XIVa (16); Ato291 for the Atopobium group, with *Collinsella aerofaciens* as the predominant fecal species (18); Rfla730/Rbro729 for ruminococci and clostridia of *Clostridium* cluster IV; the Bif164 probe for the all bifidobacteria (23), the EC1532 for *Escherichia coli* (24). Fluorescent cells were enumerated by automated counting as described (17) with a computer controlled Leica DMRXA epifluorescence microscope (Leica, Wetzlar, Germany), except when the number of cells was lower than 4×10^8 cells/g wetweight; then the cells were counted visually with a Olympus BH2 epifluorescence microscope.

³ Abbreviations used: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent in-situ hybridization; P, compared to a placebo; PBS, phosphate buffered saline; T, black tea.

DGGE analysis

Bacterial genomic DNA was isolated from the fecal sample by the bead beating method. This method allows for the efficient lysis of most bacterial cells and appears to have little bias (25). A 457 bp fragment from the V6 to V8 region of the bacterial 16S rDNA was amplified with primers U968-GC (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401 (5' GCG TGT GTA CAA GAC CC) as described by Zoetendal et al. (26). The GC clamp facilitates separation by DGGE. DGGE was performed on an 8%[wt:vol] acrylamide gel with a gradient from 40% at the top to 50% at the bottom at a temperature of 60°C. One hundred percent denaturing conditions were defined as 7 M urea and 40% formamide. Gels were run for 16 h at 65 V and stained with Cyber Green. Images of the stained gels were scanned in and analyzed with Quantity One software (Biorad).

Bile acid analysis

Bile acids were extracted and partially purified following the method of Lockett and Gallaher (27) and reverse-phase HPLC was

used to separate individual bile acids (28). Detection was achieved by use of a second column containing immobilized 3- α -hydroxysteroid dehydrogenase. A buffer containing NAD (0.1 mol/L Tris-HCl, pH 8.5, 2.7 mmol/L EDTA, 0.82 mmol/L dithiothreitol, and 0.5 mmol/L NAD) was introduced by means of a tee between the first and second columns at a constant rate of 1 mL/min. NADH produced by the reaction of bile acids and NAD⁺ with the immobilized enzyme was detected fluorometrically. Peak areas were calculated and bile acids quantified using detector response factors established with known standards. We determined levels of: diol: 3 α , 7 α -dihydroxy-12-keto-5 β -cholanoic acid; UDCA: ursodeoxycholic acid; HDCA: hyodeoxycholic acid; CA: cholic acid; 7-keto: 3 α -hydroxy-7-keto-5 β -cholanoic acid; 12-keto: 3 α -hydroxy-12-keto-5 β -cholanoic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid.

Statistical analysis

The amounts of bacteria were analyzed as total counts and log₁₀ transformed counts. The effect of the intervention diet on bacterial

TABLE 1

Enumeration of bacteria with DAPI and seven FISH probes in the tea pilot study^{1,2}

	Total cells DAPI	Bacteria Eub338	<i>Bacteroides/</i> <i>Prevotella</i> Bac303	<i>Clostridium</i> cluster XIVA Erec482	<i>Faecalibacterium</i> -like spp. Elgc01	<i>Atopobium</i> - group Ato291	Ruminococci Rbro729/ Rfla730	Bifido- bacteria Bif164
A1 ^{T,P}	10.72	10.76	9.98	9.97	9.83	9.15	9.76	9.54
A2	10.91	10.89	10.05	10.09	9.75	9.09	10.03	9.40
A3	11.26	11.12	10.35	10.37	10.00	9.21	10.26	9.66
A4	11.07	10.98	10.19	10.43	10.14	9.22	10.29	9.76
A5	11.26	11.14	10.35	10.42	10.24	9.55	10.30	9.78
A6	11.17	11.08	10.25	10.39	10.11	9.48	10.26	9.59
B1 ^{T,P}	11.25	11.13	10.54	10.35	10.18	9.74	10.05	9.22
B2	11.18	11.04	10.53	10.31	10.08	9.51	9.98	9.48
B3	10.97	10.83	10.34	10.08	9.83	9.33	9.78	9.15
B4	11.25	11.12	10.61	10.31	10.16	9.94	10.04	9.29
B5	10.84	10.75	10.19	9.93	9.81	9.26	9.51	9.07
B6	11.17	10.94	10.39	10.25	9.99	9.83	9.76	9.41
C1 ^{T,P}	10.70	10.67	10.16	9.91	9.48	9.16	9.50	8.91
C2	10.79	10.72	10.32	10.00	9.63	8.48	9.89	8.52
C3	11.05	10.86	10.34	10.09	9.88	8.78	9.99	8.60
C4	10.65	10.54	10.28	10.04	9.71	9.33	9.43	9.43
C5	11.14	10.99	10.55	10.36	10.13	9.10	9.87	9.12
C6	11.10	10.99	10.39	10.25	10.00	8.91	10.17	9.12
D1 ^{P,T}	10.54	10.31	9.97	9.77	9.30	9.08	9.51	7.90
D2	10.43	10.36	10.10	9.64	9.34	8.70	9.40	7.60
D3	10.63	10.53	10.15	9.75	9.73	8.92	9.60	7.60
D4	11.07	10.86	10.25	10.09	9.41	9.32	9.97	8.81
D5	10.76	10.63	10.38	9.96	9.08	8.93	9.99	8.45
D6	10.77	10.74	10.32	9.94	9.29	8.90	9.91	8.72
E1 ^{P,T}	10.11	10.12	9.51	9.48	7.92	8.24	8.88	7.62
E2	10.42	10.26	9.54	9.83	8.82	8.32	9.34	7.81
E3	9.86	9.87	9.49	8.91	7.28	7.74	9.03	7.60
E4	10.85	10.64	9.51	9.80	9.14	9.14	9.67	8.25
E5	10.96	10.60	9.60	9.74	9.34	8.87	10.09	8.21
E6	11.01	10.92	10.25	10.20	9.95	9.26	10.16	8.34
F1 ^{P,T}	11.00	10.52	9.34	9.94	9.63	8.59	10.01	8.60
F2	10.96	10.55	9.68	9.68	9.35	8.95	10.09	8.75
F3	10.89	10.50	9.43	9.64	9.24	8.99	9.92	8.98
F4	11.14	10.65	9.82	10.00	9.77	9.29	10.11	8.96
F5	11.12	10.86	10.20	10.43	9.93	9.17	10.31	9.02
F6	11.06	10.65	9.92	10.04	9.49	9.00	10.03	8.81

¹ Bacterial counts (log₁₀) for subjects A–F at sampling points 1–6.

² T,P period 1 tea, period 2 placebo. P,T period 1 placebo, period 2 tea.

TABLE 2

Difference in the numbers of bacteria detected by FISH analysis during black tea drinking period

Bacterial group	Probe	Mean difference (cells/g of wet weight feces)	P-value
<i>Bacteroides/Prevotella</i>	Bac303	4 × 10 ⁹	(0.14)
<i>Clostridium</i> cluster XIVa	Erec482	3 × 10 ⁹	(0.34)
<i>Faecalibacterium</i> -like spp.	Elgc01	8 × 10 ⁶	(0.99)
Atopobium-group	Ato291	-3 × 10 ⁸	(0.50)
Ruminococci	Rbro729/Rfla730	3 × 10 ⁹	(0.15)
Bifidobacteria	Bif164	3 × 10 ⁸	(0.26)
Not specifically detected bacteria	Eub338—other probes ¹	-1.4 × 10 ¹⁰	0.02

¹ Eub338—other probes: bacteria hybridizing to universal bacterial probe Eub338 - Ω bacteria hybridizing to specific probes.

numbers is defined as the mean during the intervention periods (sample points 2,3 and 5,6) minus the mean on the free diet (sample points 1 and 4). The effect of tea drinking on bacterial numbers is defined as the mean during the black tea intervention minus the mean during the placebo period subtracted by the mean numbers during the free diet. Subtraction of the amounts during the free diet was done to correct for time trend. The statistical significance of the effects is based on two-sided unpaired *t*-statistics. Due to the exploratory nature of this pilot study, we did not adjust the *P*-values for the multiple comparisons that were conducted.

The bile acid concentration was analyzed by $\log_e(1 + \text{the raw bile acid concentration in } \mu\text{g/mg of dry feces})$. Raw bile acid concentrations below the detection limit were assumed to be zero. The effect of the intervention diet is defined as the mean concentration while on the intervention diet minus the concentration while on the free diet. The effect of black tea drinking is defined as the mean concentration during the black tea period minus the concentration during the placebo period. Statistical significance of the effects was judged with two-sided unpaired *t*-statistics. We also evaluated intervention effects on the following: the sum of DCA and LCA; the ratio of DCA and LCA; the ratio of CDCA to the sum of DCA and LCA; and the ratio of the sum of DCA and LCA to the total bile acid concentration. In the rare cases where the denominator was zero, we set the value for that observation to be the maximum value observed.

For the bacterial profiles generated by DGGE we used imaging software (Quantity One, Biorad) to scan in the gel images, calculate a similarity matrix based on Pearson correlation coefficients and generate phylogenetic trees based on various algorithms (Ward, UPGMA).

RESULTS AND DISCUSSION

Our FISH analysis of changes in the fecal bacterial microflora in this study shows a large degree of inter- and intraindividual variability in the amounts of bacteria that were measured at six time points by specific probes directed against regions of the 16S rDNA. This observation confirms earlier reports that described a high degree of variability in the microflora composition (16,29). The numbers of bifidobacteria in the fecal flora were lower than those observed in other populations and we only detected *E. coli* in one study subject. These differences in the flora might reflect differences in diet between study populations. Neither the change from a "free"

living diet to the controlled intervention diet nor the black tea intervention compared to the placebo resulted in a statistically significant difference in the numbers of bacteria to which specific probes hybridized (Table 1). However, during the black tea consumption we detected a decrease in the amount of "other bacteria," bacteria that were detected with the universal eubacterial probe but not with any of the more specific probes (Table 2). This observation indicates that black tea consumption does indeed suppress the growth of some bacteria in the fecal microflora, but the inhibited bacteria do not belong to any of the bacterial groups that we could detect with the specific probes.

DGGE allows for the separation of DNA fragments of the same length that differ slightly in their nucleotide sequence, due to differences in duplex denaturing characteristics. Fragment separation is improved by the addition of a GC clamp to one end of the duplex DNA. In this study DGGE analysis of PCR amplified fragments of the V6-V8 16S rDNA region with total bacterial DNA extracted from fecal samples shows that each study individual harbors a characteristic bacterial profile that remains stable during the study period (Fig. 1). We obtained phylogenetic trees that correctly designated separate branches for each of the five subjects from a correlation coefficient based matrix that compared lanes on the DGGE gel. Although the phylogenetic trees depended on the algorithm used we obtained similar patterns with different algorithms (data not shown). We did not detect any differences in the DGGE profile caused by either the change from a "free" living diet to the controlled intervention diet or the black tea intervention compared to placebo. Although DGGE does not allow for the enumeration of the bacteria present in the sample, we concluded that neither the change in diet during the feeding study nor the drinking of black tea significantly changed the profile of bacteria present in the fecal samples. This conclusion from the DGGE analysis is not in contrast to our above observation by FISH analysis that black tea drinking reduced the amounts of "other" bacteria. Although bacterial numbers

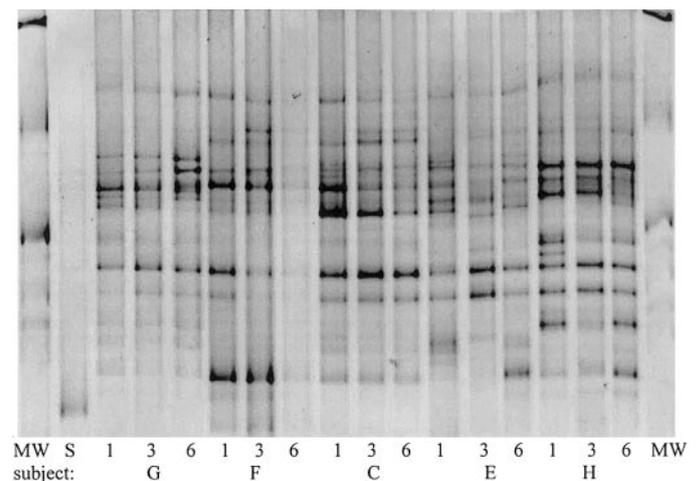


FIGURE 1 DGGE analysis of fecal bacterial profiles at three sampling points (1, 3 and 6) from subjects in the tea pilot study. DGGE profiles of total fecal bacterial DNA at sampling points 1, 3 and 6 for subjects G, F, C, E, and H. Separated bands represent bacterial species with a sequence difference in the amplified V6-V8 region of 16S rDNA. Fecal microflora from the same samples of subjects E, C and F were also analyzed by FISH (see Table 1).

TABLE 3

Mean difference (log conc.($\mu\text{g}/\text{mg}$) + 1) in bile acids and their respective P-values comparing free vs. intervention diet (DIET) and black tea vs. placebo (TEA) for 13 volunteers

	DCA ¹	LCA	CDCA	HDCA + CA	12keto	DCA + LCA	CDCA/DCA + LCA	DCA + LCA/total
Diet	-0.9	-0.2	0.1	0.89	-0.1	-0.8	-0.1	-0.1
P-value	(0.07)	(0.76)	(0.95)	(0.1)	(0.76)	(0.11)	(0.46)	(0.09)
Tea	1.1	0.4	-1.1	-0.7	0.76	1.1	-0.1	0.1
P-value	(0.11)	(0.38)	(0.25)	(0.17)	(0.08)	(0.14)	(0.11)	(0.20)

¹ DCA: deoxycholic acid; LCA: lithocholic acid; CDCA: chenodeoxycholic acid; HDCA + CA: hydoxycholic acid + cholic acid; 12keto: 3 α -hydroxy-12-keto-5 β -cholanoic acid.

can be quantified by FISH analysis, low numbers of specific bacterial groups that are below the detection limit for the FISH analysis ($\sim 10^7$ bacteria/g of fecal sample) can still be amplified by PCR and detected by DGGE.

We further analyzed fecal samples from study subjects for changes in their fecal bile acid profile. We observed a large degree of variation in the fecal bile acid profile, as reported by others (30), and did not detect any effect of black tea drinking on the concentrations or ratios of any of the bile acids (Table 3). Our analysis of bile acid excretion was limited to changes in the bile acid concentrations because we collected individual stools rather than 24-h stool and thus cannot determine total daily excretion levels. Thus, we cannot exclude the possibility that increased excretion of total bile acids contributed to the observed decrease in serum LDL cholesterol levels by black tea drinking. However, our data do not support the hypothesis that changes in the metabolism of primary bile acids into secondary bile acids by the bacterial enzyme 7 α -dehydroxylase are associated with the regulation of serum LDL cholesterol levels.

Our results confirm earlier observations that large inter- and intraindividual variations exist in the human fecal microflora composition and the fecal bile acid profile. Larger studies with the appropriate power to detect the effects of dietary interventions on the fecal microflora composition and bile acid profile are clearly needed to advance this field. Molecular microflora analysis methods can facilitate the determination of dietary effects on the microflora composition as shown by our observation that black tea drinking seems to inhibit at least some groups of "other" fecal bacteria. However, a more comprehensive microflora analysis approach that includes conventional microbiological plating methods and metabolic activity/mutagenicity assays will increase the likelihood of detecting potentially important associations between diet and either composition or activity of the gut microflora. Establishing any such associations will be instrumental for the future design of specific dietary interventions aimed at improving human health through changes in the microflora. Although commercial products that claim such health benefits are already widely available, rigorous scientific data supporting the effectiveness of these products and the development of new products based on such data will facilitate a wider acceptance of this promising approach in the foreseeable future.

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LITERATURE CITED

- McGinnis, J. M. & Foege, W. H. (1993) Actual causes of death in the United States. *J. Am. Med. Assoc.* 270: 2207-2212.
- Doll, R. & Peto, R. (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J. Natl. Cancer Inst.* 66: 1191.
- Willett, W. C. (2000) Diet and cancer. *Oncologist* 5: 393-404.
- Tannock, G. W. (1999) Analysis of the intestinal microflora: a renaissance. *Antonie Leeuwenhoek* 76: 265-278.
- McCracken, V. J. & Lorenz, R. G. (2001) The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. *Cell Microbiol.* 3: 1-11.
- Guarner, F. & Malagelada, J. R. (2003) Gut flora in health and disease. *Lancet* 361: 512-519.
- Steer, T., Carpenter, H., Tuohy, K. & Gibson, G. R. (2000) Perspectives on the role of the human gut microbiota and its modulation by pro- and prebiotics. *Nutr. Res. Rev.* 13: 229-254.
- Hirayama, K. & Rafter, J. (2000) The role of probiotic bacteria in cancer prevention. *Microbes. Infect.* 2: 681-686.
- Vaughan, E. E., Schut, F., Heilig, H. G., Zoetendal, E. G., De Vos, W. M. & Akkermans, A. D. (2000) A molecular view of the intestinal ecosystem. *Curr. Issues Intest. Microbiol.* 1: 1-12.
- Mountzouris, K. C., McCartney, A. L. & Gibson, G. R. (2002) Intestinal microflora of human infants and current trends for its nutritional modulation. *Br. J. Nutr.* 87: 405-420.
- Harmsen, H. J., Wildeboer-Veloo, A. C. & Raangs, G. C., Wagendorp, A. A., Klijn, N., Bindels, J. G. & Welling, G. W. (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J. Pediatr. Gastroenterol. Nutr.* 30: 61-67.
- McCracken, V. J., Simpson, J. M., Mackie, R. I. & Gaskins, H. R. (2001) Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. *J. Nutr.* 131: 1862-1870.
- Tajima, K., Aminov, R. I., Nagamine, T., Matsui, H., Nakamura, M. & Benno, Y. (2001) Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* 67: 2766-2774.
- Davies, M. J., Judd, J. T., Baer, D. J., Clevidence, B. A., Paul, D. R., Edwards, A. J., Wiseman, S. A., Muesing, R. A. & Chen, S. C. (2003) Black tea consumption reduces total and LDL cholesterol in mildly hypercholesterolemic adults. *J. Nutr.* 133: 3298S-3302S.
- Puupponen-Pimia, R., Nohynek, L., Meier, C., Kahkonen, M., Heinonen, M., Hoppa, A. & Oksman-Caldentey, K. M. (2001) Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* 90: 494-507.
- Debruyne, P. R., Bruyneel, E. A., Li, X., Zimber, A., Gespach, C. & Mareel, M. M. (2001) The role of bile acids in carcinogenesis. *Mutat. Res.* 48: 359-369.
- Franks, A. H., Harmsen, H. J., Raangs, G. C., Jansen, G. J., Schut, F. & Welling, G. W. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 64: 3336-3345.
- Jansen, G. J., Wildeboer-Veloo, A. C., Tonk, R. H., Franks, A. H. & Welling, G. W. (1999) Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. *J. Microbiol. Methods* 37: 215-221.
- Harmsen, H. J., Wildeboer-Veloo, A. C., Grijpstra, J., Knol, J., Degener, J. E. & Welling, G. W. (2000) Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. *Appl. Environ. Microbiol.* 66: 4523-4527.
- Harmsen, H. J., Raangs, G. C., He, T., Degener, J. E. & Welling, G. W. (2002) Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Appl. Environ. Microbiol.* 68: 2982-2990.
- Amann, R. I., Krumholz, L. & Stahl, D. A. (1990) Fluorescent-oligonu-

cleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172: 762-770.

21. Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* 142: 1097-1106.

22. Wilson, K. H. & Blitchington, R. B. (1996) Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* 62: 2273-2278.

23. Langendijk, P. S., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. & Welling, G. W. (1995) Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* 61: 3069-3075.

24. Poulsen, L. K., Licht, T. R., Rang, C., Krogfelt, K. A. & Molin, S. (1995) Physiological state of *Escherichia coli* BJ4 growing in the large intestines of streptomycin-treated mice. *J. Bacteriol.* 177: 5840-5845.

25. Miller, D. N., Bryant, J. E., Madsen, E. L. & Ghiorse, W. C. (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* 65: 4715-4724.

26. Zoetendal, E. G., Akkermans, A. D. & De Vos, W. M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* 64: 3854-3859.

27. Locket, P. L. & Gallaher, D. D. (1989) An improved procedure for bile acid extraction and purification and tissue distribution in the rat. *Lipids* 24: 221-223.

28. Gallaher, D. D., Locket, P. L. & Gallaher, C. M. (1992) Bile acid metabolism in rats fed two levels of corn oil and brans of oat, rye and barley and sugar beet fiber. *J. Nutr.* 122: 473-481.

29. Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P. & Dore, J. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 66: 2263-2266.

30. Setchell, K. D., Ives, J. A., Cashmore, G. C. & Lawson, A. M. (1987) 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* 162: 257-275.