Gut microbiota facilitates dietary heme-induced epithelial hyperproliferation by opening the mucus barrier in colon

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Colorectal cancer risk is associated with diets high in red meat. Heme, the pigment of red meat, induces cytotoxicity of colonic contents and elicits epithelial damage and compensatory hyperproliferation leading to hyperplasia. Here we explore the possible causal role of the gut microbiota in heme-induced hyperproliferation. To this end, mice were fed a purified control or heme diet (0.5 μmol/g heme) with or without broad-spectrum antibiotics for 14 days. Heme-induced hyperproliferation was shown to depend on the presence of the gut microbiota, since hyperproliferation was completely eliminated by antibiotics, although heme-induced luminal cytotoxicity was sustained in these mice. Colon mucosa transcriptomics revealed that antibiotics block heme-induced differential expression of oncogenes, tumor-suppressors and cell-turnover genes, implying that antibiotic treatment prevented the heme-dependent cytotoxic micelles to reach the epithelium. Our results indicate that this occurs because antibiotics reinforce the mucus barrier by eliminating sulfide-producing bacteria and mucin-degrading bacteria (e.g. Akkermansia). Sulfide potently reduces disulfide bonds and can drive mucin denaturation and microbial access to the mucus layer. This reduction results in formation of trisulfides that can be detected in vitro and in vivo. Therefore, trisulfides can serve as a novel marker of colon mucolysis and thus as a proxy for mucus barrier reduction. In feces, antibiotics drastically decreased trisulfides, but increased mucin polymers that can be lysed by sulfide. We conclude that the gut microbiota is required for heme-induced epithelial hyperproliferation and hyperplasia by their capacity to reduce mucus barrier function.

colorectal cancer | red meat | mucus barrier | mucolysis | (tri)sulfides

Introduction

Colorectal cancer, the second leading cause of cancer death in Western countries, is associated with diets high in red meat (1), whereas consumption of white meat does not have this association (2). Heme, the iron-porphyrin pigment, is present at much higher levels in red- compared to white meat. Epidemiological studies show that heme intake is related to colon cancer risk (3,4). Our previous studies show that when rodents consume heme, their colonic contents become more cytotoxic (5, 6). This increased cytotoxicity injures the colonic epithelial surface cells. To replace the injured surface cells, hyperproliferation from the stem cells in the crypts is initiated. Together with inhibition of apoptosis, this compensatory hyperproliferation leads to hyperplasia (6), which eventually can develop into colorectal cancer.

Dietary heme is poorly absorbed in the small intestine; approximately 90% of dietary heme enters the colon (7). Besides the toxic effect of heme on the colonic mucosa, dietary heme affects the microbiota. The relationship between intestinal microbiota and colon cancer has long been suspected (8). In humans, a red-meat diet increases Bacteroides spp. in feces (9). We recently showed that in mice, a heme diet changed the microbiota drastically, majorly increasing the Gram-negative bacteria (mainly Bacteroidetes, Proteobacteria and Verrucomicrobia) (10). The gut microbiota can induce hyperproliferation via mechanisms occurring in the colon lumen, such as modulation of oxidative and cytotoxic stress or by influencing the mucus barrier. Oxidative stress induces the formation of peroxidized lipids, which react with heme to form the cytotoxic heme factor (CHF), thereby increasing cytotoxic stress (5, 11). In a time-course study we showed that there is a lag time in the formation of CHF and in the induction of hyperproliferation when mice are transferred from a control to heme diet (11). This could be due to a time-dependent adaptation of the microbiota to the heme diet. Notably, heme does not increase cytotoxicity and epithelial hyperproliferation in the small intestine (12), indicating that formation of CHF only occurs in the colon where bacterial density is high. Moreover, these experiments suggested that CHF-induced hyperproliferation coincided with a reduced mucus barrier function (11), leading to enhanced contact of colonocytes with microbiota and toxic substances. In the present study we investigate whether bacteria play a causal role in heme-induced cytotoxicity and hyperproliferation by using broad-spectrum antibiotics (Abx). Our results

Significance

Consumption of red meat is associated with increased colorectal cancer risk. We show that the gut microbiota is pivotal in this increased risk. Mice receiving a diet with heme, a proxy for red meat, show a damaged gut epithelium and a compensatory hyperproliferation that can lead to colon cancer. Mice receiving heme together with antibiotics do not show this damage and hyperproliferation. Our data indicate that microbial hydrogen sulfide production and thereby maintain the mucus barrier that prevents heme-induced hyperproliferation. Our study indicate fecal trisulfide as a novel biomarker of mucus barrier integrity, which could be of relevance in human colon disease diagnostics.

Reserved for Publication Footnotes
Table 1. Effects of heme and Abx on body weight and fecal parameters

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<thead>
<tr>
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<th>Control Heme</th>
<th>Control + Abx</th>
<th>Heme + Abx</th>
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<tr>
<td>Body weight (g)</td>
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<td>24.9 ± 0.5 b</td>
<td>27.2 ± 0.3 a</td>
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<td>Fecal wet weight (g/day)</td>
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<td>0.60 ± 0.05 a</td>
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<td>Fecal dry weight (g/day)</td>
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<td>0.11 ± 0.01 a</td>
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<td>TBARS (MDA equivalents, μmol/L)</td>
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<td>59.84 ± 2.46 b</td>
<td>11.06 ± 1.63 a</td>
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<td>Cytotoxicity (% lysis)</td>
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<td>66.90 ± 10.45 b</td>
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Mean ± SEM (n=9 per group), differences were tested by ANOVA and Bonferroni post-hoc test. Superscripts indicate significant differences (p<0.05).

Fig. 1. A. Counts of total bacteria, Bacteroidetes and Firmicutes measured by qPCR. Control bars are set at 100%, other bars are relative to controls, mean ± SEM (n=9/group). B. Bile acids (BA) profiles determined in HPLC, mean ± SEM (n=3/group). Letters indicate significant different groups (p<0.05).

Results

Heme- and Abx-induced changes in the colonic lumen. Mice were divided into 4 groups receiving either a control diet (further referred to as C-group), a heme diet (H-group), a control diet with Abx treatment (CA-group), or a heme diet with Abx treatment (HA-group). After 2 weeks of intervention the H- and HA-group had a lower body weight as compared to their controls (Table 1). Fecal dry and wet weight was significantly increased in the HA-group (Table 1).

To confirm that Abx decreased the abundance of bacteria, qPCR analyses with specific primers targeting total bacteria, Bacteroidetes or Firmicutes were performed (Fig. 1A). Abx treatment significantly reduced the abundance of total bacteria and Firmicutes ~100-fold and Bacteroidetes ~1,000-fold. Moreover, the H-group had a significantly increased abundance of Bacteroidetes compared to the C-group, which corroborates previous observations (10). Abx thus drastically decreased the bacterial density, affecting both the Gram-positive Firmicutes and Gram-negative Bacteroidetes. To study how this impacts the normal microbial modification of host compounds, we determined the fecal bile acid composition. No conjugated bile acids were detected in fecal water in the C- and H-group, where unconjugated and secondary bile acids were predominant (Fig. 1B). However, with Abx almost all bile acids were primary and conjugated with glycine or taurine (ratio about 3), showing that Abx blocked microbial bile deconjugation and dehydroxylation almost completely.

Heme increases oxidative and cytotoxic stress in the colon (10, 11). Reactive oxygen species (ROS) induce the formation of lipid peroxides which react with heme to form CHF, thereby increasing the cytotoxicity of luminal contents (5, 11). We determined lipid peroxidation product levels by measuring TBARS in fecal water. TBARS were low in the C- and CA-group (Table 1) and increased significantly and to a similar extent in the H- and HA-group, implying that heme, both in presence and absence of Abx, induced ROS-stress. Analogously, fecal water cytotoxicity (Table 1) was significantly increased in the H- and HA-group compared to their controls. Since Abx drastically reduced microbiota density (100-1000 fold), but only slightly reduced cytotoxicity (2-fold) and TBARS (1.3-fold), it is unlikely that bacteria play a major role in the formation of TBARS and cytotoxicity.

Heme- and Abx-induced changes in the colonic mucosa. Morphology analyses of H&E stained colon tissue (Fig. 2A) confirmed the previously reported heme-induced increased crypt depth (H- vs C-group). Abx treatment did not affect the colon morphology in the CA- vs C-group, but completely restored tissue morphology in the HA- vs H-group. The crypt depth increase in the H-group did not result from inflammation since neutrophil and macrophage infiltration in the lamina propria was comparable to the C-group. Analogous to earlier reports (6), cell-proliferation quantification using Ki67-staining (Fig. 2B and 2C) shows that the heme diet strongly induced cell proliferation (H- vs C-group), leading to expansion of the proliferative compartment and increased crypt depth. Abx treatment led to slightly reduced numbers of cells per crypt in the CA- vs C-group, but did not significantly affect their labeling index or amount of proliferative cells. However, Abx treatment in the heme diet (HA- vs H-group) completely suppressed heme-induced hyperproliferation and hyperplasia to levels observed in the C- and CA-groups (Fig. 2C). In conclusion, heme-induced hyperproliferation and hyperplasia in mouse colon only occurs in the presence of the gut microbiota.

Abx block the heme-induced expression of cell cycle genes. Using whole genome transcriptomics we investigated whether the physiological changes were reflected in gene expression profiles. The differentially expressed genes in the H- vs C- and HA- vs CA-groups are visualized in Fig. 3A. The heme diet (H- vs C-group) led to 5,507 differentially expressed genes (q-value<0.01), of which almost 90% (4,859) were not significantly affected in the HA vs CA comparison. The 4,859 genes specific for the H-group were analyzed by Gene Set Enrichment Analysis (GSEA), indicating that mainly cell cycle related processes were affected by heme (Fig. S1B). Moreover, mining of these genes for the involved transcription factors (Fig. S1C), revealed that Cdkn2a, Smarcb1, and the tumor suppressors Tp53 and Rb1 were inhibited, while oncogenes such as Myc and Foxm1 as well as cell cycle regulators E2f1 and Tbx2, were activated by heme. Importantly, these processes and transcription factors were not modulated in the HA-group compared to the CA-group. There were only 369 differentially expressed genes unique for the HA-group (Fig. S1A). Notably, none of the modulated processes identified in the HA-group related to the endpoints of our study. Because of the specific heme-Abx interaction, the Abx-mediated differential gene expression profiles and processes were substantially different in the heme diet background (Table S1 and S2) as compared to the control diet background (Table S3 and S4). These observations indicate that heme-induced mucosal gene expression changes of cell cycle related processes require the presence of the microbiota, which is in agreement with the microbiota requirement for the increased labeling index (Fig. 2C).
Fig. 2. A. Histochemical H&E staining and B. immunohistochemical Ki67-staining of colon of control and heme-fed mice. C. Quantification of Ki67 positive cells per crypt, total number of cells per crypt and labeling index (percentage of proliferative cells per crypt); mean ± SEM (n=9/group). Letters indicate significant different groups (p<0.05), ANOVA with Bonferroni post-hoc test.

Fig. 3. A. Gene expression of injury markers Birc5, Ier3, Ripk3 and Slpi. B. immunohistochemical colonic Slpi-staining of control and heme-fed mice. C. Gene expression of mucin genes 1 to 4 and Galnt 3 and 12. Expression levels of control is set at 1. Expression of other bars is relative to controls; mean ± SEM (n=4 for C, H; n=6 for HA). Letters indicate significant differences (p<0.05), ANOVA with Bonferroni post-hoc test.

Abx do not affect the heme-induced antioxidant response. A set of 648 genes were significantly regulated in both the H- and HA-group as compared to their controls (Fig. S1A). Of those shared genes, 599 were similarly regulated in both groups. Notably, this group of genes included the activation of several transcription factors, including the PPARs, involved in fatty acid metabolism, and Nrf2, involved in antioxidant response (Fig. S1B and C). This implies that oxidative stress and lipid peroxidation products induced the antioxidant response and the induction of...
Sulfide reduces S-S bonds leading to mucolysis. Mucus is high in intra- and intermolecular S-S bonds, stabilizing its polymeric, network-like structure. When S-S bonds are broken, mucin molecules dissociate and/or depurate, leading to decreased viscosity and higher mucin accessibility for bacterial degradation (15). SRBs can use mucin-derived sulfate as oxidant in anaerobic respiration, generating sulfide. We hypothesized that sulfide could have a mucolytic effect by reducing the intermolecular S-S bonds, which contributes to an enhanced mucin barrier in the Abx group due to the decreased abundance of SRBs (Fig. 4A) and the corresponding decrease of luminal sulfide production. We tested the reducing potency of several sulfur containing compounds on the model compound DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) containing a central S-S bond. Splitting of this S-S bond in DTNB leads to increased absorbance at 412 nm, and the assay allows the determination of the overall S-S splitting extent as well as the initial S-S splitting rate (Fig. 4B). Cysteine, glutathione and N-acetylcycteine (NAC) were able to split the S-S bond with a similar rate between 24 to 31 µM/min, whereas the negative control Na2SO3 did not affect DTNB integrity. Importantly, sulfide gave a significantly higher rate of S-S bond splitting, 62.4 ± 5.9 µM/min, indicating that sulfide has a 2-fold more potent mucolytic effect as compared to the amino-acid thiols that have been shown to split S-S bonds and make mucus less viscous (16). Moreover, the overall extent of S-S bond splitting by sulfide was also 2-fold higher as compared to the other amino-acid thiols. Based on Ellman’s mechanism (17), this indicates that a reactive persulfide anion originates from the splitting of the first S-S bond, which can subsequently target a second S-S bond, creating a trisulfide bond.

To quantitate the production of trisulfide bonds, we developed a method to determine trisulfide bonds based on the difference between total bound sulfides and acid labile sulfides (18). Indeed, trisulfide bonds were generated when sulfide was used to reduce S-S bonds in DTNB (Fig. 4B), but not upon amino-acid thiol (NAC, GSH or cysteine) treatment of DTNB. The theoretical (Fig. 4C) as well as the measured ratio (Fig. 4B) of sulfide-dependent formation of thiol to trisulfide is 2, indicating that all sulfide reacts with DTNB to form trisulfide bonds.

To test whether similar redox reactions also occurred in vivo, total sulfides and trisulfides were determined in mice fecal water (Fig 4D). Heme increased the levels of total bound sulfides, which is in agreement with literature showing that heme addition to the growth medium stimulates bacterial reduction of sulfate to sulfide (19). Concentrations of trisulfide in fecal water of mice not receiving Abx were much higher (with H>C) than those of mice receiving Abx indicating that sulfide-generating bacteria are not significantly affected by heme in vivo.

An increased mucin barrier could be caused by increased mucin synthesis, or by decreased mucin degradation. Expression levels of secreted Muc2 and cell-associated Muc4 were decreased in the HA-group compared to the H-group (Fig. 3C). Moreover, the KEGG pathway 'Mucin type O-Glycan biosynthesis' was significantly repressed in the HA- compared to the H-group, according to GSEA analysis (q=0.049). For instance, Galnt 3 and 12, involved in the first step of α-glycosylation, were downregulated by Abx (Fig. 3C), indicating that mucin layer production is most probably decreased by Abx. Regarding mucin degradation, it is well established that the microbiota degrades mucins and uses their carbohydrates and amino acids as substrates for growth. In addition, sulfate-reducing bacteria (SRBs) use mucin-derived sulfate as electron acceptor in anaerobic respiration forming sulfides (see also below). As the Abx-treatment drastically reduced the microbiota density, we hypothesized that Abx increased the mucin barrier by preventing microbial degradation of mucin.

Notably, the abundance of the mucin-degrading Akkermansia muciniphila was 8-fold increased by heme, whereas Abx reduced the abundance of this mucin degrader more than 1000-fold (Fig. 4A). This implies that the relative reduction of the Akkermansia population by Abx exceeded the overall effect of Abx on the total microbial load, supporting that Abx treatment could increase mucin barrier function by strongly reducing the levels of mucin degraders such as Akkermansia.
tumor-suppressor and oncogenes, which increases colorectal cancer risk (20, 21). These luminal and epithelial effects of heme are similar to those detailed in our recent studies (6, 10, 11). The crucial finding of the present study is that when the microbial abundance is drastically reduced by Abx, heme does not injure the surface epithelium and does not induce the carcinogenic changes in the crypts, mentioned above. This is not due to the slightly lower levels of cytoxicity and ROS in the HA-group, which can be explained by the higher luminal dilution factor because of the increased fecal wet weight. Recently, we reported that heme diets induce, in the colon lumen, covalent heme modification resulting in the very lipophilic and toxic CHF, which is solubilized in mixed micelles (11). Abx block the mucosal sensing of these cytotoxic micelles, as they prevent the heme-induced changes in epithelial histology, and in up regulation of injury markers such as Slpi. In contrast, Abx do not block mucosal sensing of luminal ROS, as the Nrf2-mediated antioxidant response was initiated and PPARs were activated by oxidized lipids in both heme diet groups. This differential mucosal sensing shows that, with Abx, the mucus layer is still permeable to small molecules, such as oxidized lipids, but no longer to larger micellar aggregates containing CHF. The absence of hyperpolarization in the HA-group also shows that mucosal exposure to ROS does not cause hyperpolarization. This is in line with our previous observation that ROS is instantly formed after consumption of the heme diet, while there is a delay in the appearance of luminal cytotoxicity and the induction of hyperpolarization (11).

Our study implies that the colon microbiota facilitates heme-induced epithelial injury and hyperpolarization by opening the mucus barrier by the concerted action of hydrogen sulfide-producing and mucin-degrading bacteria. The principal steps of this hypothesis (Fig. 5) are (i) mucolysis by hydrogen sulfide to open the compact, protective mucus layer for (ii) further bacterial degradation, thereby (iii) allowing diffusion of luminal, cytotoxic, micelles to the mucosal surface. Consequently, surface epithelial cells are less protected against luminal cytotoxicity, leading to induction of compensatory hyperpolarization. The diffusion barrier function of the mucus layer is illustrated by muc2 KO mice, which display colitis and epithelial hyperproliferation, as well as spontaneous development of colorectal cancer (22). In addition, an in vitro study shows that apically applied mucin creates a diffusion barrier preventing the contact between cytotoxic micelles and colonicocytes (13). That microbiota increase the permeability of mucus barrier is illustrated by a study of recomolized vs Abx-treated rats showing that bacteria colonizing the isolated colon segment increase epithelial injury by luminally added toxic compounds (30). The recent finding that increasing the luminal dilution factor because of the increased fecal wet weight reduces the mucus barrier suggests that our results can only be explained by the absence of hyperpolarization in the mouse colon (24).

Overall, we conclude that the microbiota facilitates the heme-induced hyperpolarization by opening the mucus barrier. Bacterial hydrogen sulfide can reduce the S-S bonds in polymeric mucin, thereby increasing the mucus barrier permeability for mucin-degrading bacteria and for cytotoxic micelles. Consequently, epithelial surface cells are injured by the cytotoxic splitting of S-S is higher with hydrogen sulfide. Moreover, as sulfide donates 2 electrons it splits 2 S-S bonds, whereas thiols only split one. Elaborating on Ellman’s mechanism of S-S splitting (17), we reasoned that the highly nucleophilic persulfide (30), formed in de first reaction, generates a trisulfide bond in the second one. Our in vitro results show that trisulfide formation is indeed specific for S-S reduction by sulfide. Our fecal analysis shows that trisulfide is also formed in vivo and stimulated by dietary heme, probably because bacterial sulfate reduction is heme dependent (19). Abx strongly reduce overall bacterial abundance and suppress this trisulfide formation almost completely, supporting our mechanism that sulfide opens the mucus barrier. In line with this, Abx greatly increased fecal excretion of Muc2 in a high MW polymeric form, as shown by nonreducing SDS-PAGE. Moreover, this polymeric Muc2 dissociates almost completely after reduction by DTT or sulfide, supporting the hypothesis that S-S bond splitting by sulfide opens the mucus barrier. This is supported further by the recent finding that increasing the number of S-S bonds in the Muc2 network increases the mucus barrier in mouse colon (24).

In humans, the colonic mucous layer functions as a barrier. As in mice, it prevents bacterial colonization of the epithelial surface and protects the surface cells from exposure to luminal toxic compounds (26). Three prevalent microbial profiles, so-called ‘enterotypes’ have been proposed to exist in human microbiota (27). Interestingly, for two of those enterotypes mucin-degrading bacteria are identified as microbial drivers. One enterotype is rich in Prevotella and the co-occurring Desulfovibrio. Prevotella degrades mucin and Desulfovibrio may enhance the rate limiting sulfatase step by hydrolyzing glycosyl-sulfate esters. The second mucin-degrading enterotype is rich in Ruminococcus and Akkermania, both able to degrade mucins. We showed previously that dietary heme drastically increases the abundance of Prevotella and Akkermania (10), which may be of relevance for these two enterotypes. The third enterotype is rich in Bacteroides using carbohydrates and proteins as substrates for fermentation (27).

It would be of interest to see whether mucin-barrier differences between different enterotypes exist or that diseases of the gut, such as colorectal cancer and IBD, are associated with mucin-degrading enterotypes. Notably, in some people the colon microbiota do not reduce sulfate to hydrogen sulfide (28). Nevertheless, also in these people our mechanism of mucus barrier breaking may be relevant. In mice, we found recently that dietary heme increases the abundance of Prevotella and Akkermania from cysteine (e.g. Prevotella) or from taurine-conjugated bile acids, see (29) for review. In humans, and in our mice on a Western diet, the glycine/taurine ratio of bile acid conjugation is about 3 (30), implying that about 25% of the bile acids spilled over into the colon contain taurine.

Overall, we conclude that the microbiota facilitates the heme-induced hyperpolarization by opening the mucus barrier. Bacterial hydrogen sulfide can reduce the S-S bonds in polymeric mucin, thereby increasing the mucus barrier permeability for mucin-degrading bacteria and for cytotoxic micelles.
heme and compensatory hyperproliferation is initiated. This hyperproliferation might eventually lead to colorectal cancer (20). Our model, as well as our results imply that fecal trisulfides can serve as a strong inducer of colonic mucositis. Therefore, it would be of interest to measure levels of trisulfide in the human enterotypes, mentioned above, and in gut diseases in which the mucus barrier is compromised, such as IBD (31).

Materials and methods

Animal handling and design of the study. Experiments were approved by the Ethical Committee on Animal Testing of Wageningen University and were in accordance with national law. C57BL6/J mice (Harlan, Horst, The Netherlands) were housed individually in a room with controlled temperature (20-24°C), relative humidity (55±15%) and a 12 h light dark cycle. Mice were fed diets and demineralized water ad libitum. Mice (n=9/group) received either a light diet or a high fat diet plus antibiotics (Abx), containing ampicillin (1 g/L), neomycin (1 g/L), and metronidazole (0.5 g/L), were administered in drinking water during the time of intervention. There were 4 experimental groups; control, heme, control plus Aloxetin baemoglobin induced colitis were quantitatively collected during days 11-14, frozen at -20°C and subsequently freeze-dried. After 14 days, the colon was excised, mesenteric fat was removed and the colon was opened longitudinally, cut into three parts. The middle 1.5 cm colon tissue was formalin-fixed and paraffin embedded for histology. The remaining proximal and distal parts were scraped, pooled per mouse, snap-frozen in liquid nitrogen and grind at -80°C until further analysis. Colonic contents were sampled for microbiota analysis. Chemicals were from Sigma-Aldrich, unless indicated otherwise.

Fecal analyses. Fecal water was prepared by extracting freeze-dried feces with double distilled water to obtain a physiological osmolarity of 300 mOsm, as described previously (9). Cytotoxicity of fecal water was quantified by potassium release from human erythrocytes after incubation, as previously described (5) and validated with human colon carcinoma.

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