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ARTICLE

Apple Proanthocyanidins Do Not Reduce the Induction of Preneoplastic Lesions in the Colon of Rats Associated with Human Microbiota

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Since the gut microbiota metabolizes various dietary constituents unabsorbed by the small intestine and modulates colon function, it plays an essential role in colon carcinogenesis. First, we have developed a model of human microbiota-associated rats (HMA), fed a human-type diet and injected with 1-2, dimethylhydrazine (DMH). We observed that the number and size of DMH-induced aberrant crypt foci (ACF) were significantly higher in HMA rats than in germ-free or conventional rats. Second, we used this model to assess the protective effect of an apple proanthocyanidin-rich extract (APE) on colon carcinogenesis. In this model, ACF number and multiplicity were not reduced by APE at 0.001% and 0.01% in drinking water. They were higher with APE 0.1% than with APE 0.01%. Therefore, the cross-talk between human microbiota and the colon epithelium should be taken into account in carcinogenesis models. Moreover, attention should be paid prior to using proanthocyanidin extracts as dietary supplements for humans.

KEYWORDS: Rat colon carcinogenesis; aberrant crypt foci; human microbiota; human-type diet; apple proanthocyanidins

INTRODUCTION

Diet plays an important role in colon carcinogenesis. As reviewed recently, whereas a significant reduction of colon cancer risk is associated with fruit and vegetable consumption in numerous case-control studies, no significant reduction has been found in cohort studies, except for fruit consumption in women (*1*). Fruits and vegetables provide numerous phytochemicals which, in part, may explain their beneficial effect. These effects on colon carcinogenesis are studied in rodent models. Corpet and co-workers have reviewed hundreds of prevention studies made with classical colon cancer models such as azoxymethane-treated rats (*2*) and Min mice (*3*). As compared to these classical models, models of rats harboring human fecal microbiota and fed a human-type diet would allow a better understanding of the preventive effect of phytochemicals on colon carcinogenesis since ingested food, host, and gut microbiotia interact with each other. Indeed, gut microbiota can modulate intestinal function (*4*) and appear to be an essential factor in pathological disorders including colon cancer (*5*). It metabolizes various dietary plant constituents, in particular undigested fibers and polyphenols, and it modifies their bioavailability (*6*) and effects (*7*) on the host. In addition, composition and functions of human gut microbiota are influenced by the host. For example, human and rat gut microbiota profoundly differ. In addition, they are influenced by dietary habits (*8*). Modulation of the genotoxicity and carcinogenicity of several substances by human gut microbiota has been demonstrated by several groups including ours. In mice, the effects of human gut microbiota on DNA adduct formation induced by various environmental mutagens are different from those of mouse microbiota (*9*). In germ-free (GF) rats colonized with human fecal microbiota (human microbiota-associated rats, HMA), the genotoxicity of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is lower than in conventional (CV) ones (*10*). Likewise, a carragenan-rich diet increased the multiplicity of aberrant crypt foci (ACF) after azoxymethane treatment in CV rats but not in HMA rats (*11*). Finally, HMA rats have been used to study the effect of different dietary risk factors on 1,2-dimethylhydrazine (DMH)-induced carcinogenesis by Rowland and co-workers (*12*). More recently, we demonstrated the dietary modulation of IQ genotoxicity (*10*).

The interest in proanthocyanidin extracts is now increasing due to their potential phytopharmaceutical applications in various pathologies, including cardiovascular and cancer diseases (*13*). Indeed, among polyphenols, proanthocyanidins (also called condensed tannins or oligomeric proanthocyanidin complexes) represent the major part of flavonoids ingested. They include dimers, oligomers, and polymers of catechins, which are poorly absorbed by the small intestine and can be metabolized in the

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Table 1. Phenolic Composition of APE[a]

phenolic compounds		amount (mg/g of dry matter)	%
flavan-3ols	monomers	37.3	5.0
	proanthocyanidins	663.9	89.8
	average degree of polymerization	2.95	
dihydrochalcones		17.0	2.3
hydroxycinnamic acid		21.6	2.9
flavonols		Not detected	
total		739.8	100

[a] The phenolic composition was determined by HPLC.

colon by microbiota (*14*). They are abundant in various foods and apples are one of the major dietary sources. As compared with dessert apples, unripe apples or cider varieties contain as much as 10 times more polyphenols, largely identified as polymerized proanthocyanidins (*15*). Apple polymerized proanthocyanidins have been shown to inhibit in vitro the metabolism of polyphenols by human fecal microbiota (*16*).

The first aim of the present study was to settle the model of HMA rats receiving a human-type diet after DMH-treatment. We characterized animal growth, human microbiota stability, cecal content as well as the number and size of DMH-induced ACF, in comparison with GF and CV rats. The second aim was to use this animal model for assessing the effects of an apple proanthocyanidin-rich extract (APE) on colonic ACF development.

MATERIALS AND METHODS

Composition of the Apple Proanthocyanidin-Rich Extract (APE). APE was kindly provided by the Pernod Ricard Company (Créteil, France). On the basis of dry matter composition, it contained as high as 75% of total polyphenols. Its phenolic composition was determined by HPLC according to the procedure described by Guyot et al. (*17*). Proanthocyanidins were predominant and accounted for about 90% of total polyphenols, with an average degree of polymerization of 2.95 (**Table 1**).

Animals and Diet. GF male F344 rats were provided by our germ-free rodent breeding facilities. They were reared as described elsewhere (*7*). The sterility of isolators containing GF rats was tested every week. Rats were given free access to sterilized water and to the experimental diet. As shown in **Table 2**, the experimental diet (SAFE, Augy, France) has been formulated to match a human-type diet with protein and fat from both animal and plant origins, and carbohydrates as saccharose and starch (potato and corn) (*7*). Before its introduction into isolators, the diet was sterilized by γ irradiation at 45 kGy (IBA Mediris, Fleurus, Belgium). Once a week, stock solutions of APE were prepared and stored at room temperature in isolators: APE was dissolved in 100 mL of tap water at different concentrations (0.01%. to 1%) and then sterilized by filtration through a 0.22 μm Millipore filter. Three times a week, the stock solution was diluted 1:10 (vol/vol) in sterile water and immediately distributed into drinking bottles. Any remaining beverage was discarded. To check a possible degradation of APE during its preparation and storage in isolators, a 1% stock solution was prepared and left at room temperature for 7 days. Every day, a 100 μL sample was aliquoted and stored at -70 °C until the assay with HPLC. All samples were analyzed on an RP18 column (5 μm $\times$ 150 mm) using a methanol/0.01 M KH_2PO_4 (pH 2.6) gradient at 1 mL/min. (+)-Catechin (Sigma-Aldrich, L'Isle d'Abeau, France) was used as a standard. The (+)-catechin peak area was equal in all samples, suggesting that the polyphenol solution was stable during the preparation and storage periods.

Inoculation of Microbiota and DMH-Treatment. Two-month old GF rats (300 g) were transferred into separate isolators and divided into different groups. They were fed the human-type diet. To generate CV and HMA groups, the rats were inoculated orally with whole fecal microbiota (either from a home-bred rat or a healthy human donor) as described previously. Nine days and 9 weeks after microbiota inoculation, fecal samples were collected from HMA rats and immediately frozen to monitor microbiota composition by PCR-TTGE. Two weeks after microbiota inoculation, the rats received two intraperitoneal injections of DMH (a total of 50 mg/kg). The consumption of drinking water was recorded every other day, and the rats were weighed once a week. Ten weeks after microbiota inoculation, they were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg·kg^{-1}) prior to decapitation. The colons were collected for scoring of ACF. All aspects of the protocol conformed to the international guiding principles for biomedical research involving animals. All procedures were carried out in accordance with European Guidelines for the Care and Use of Laboratory Animals.

Table 2. Composition of the Human-Type Diet[a]

component	amount (g/kg)
corn starch	280
mashed potato	280
casein	50
soya isolate[b]	120
corn oil	30
saccharose	40
lard	50
cellulose	70
mineral additive[c]	70
vitamin additive[d]	10

[a] Analytical composition was proteins (N $\times$ 6.25), 16.63%; crude fat, 7.56%; ash, 5.12%; energy, 17.85 MJ/kg (Eurofins Scientific Analytics, Nantes, France). [b] Nurish 1500 (DuPont Protein Technologies). [c] The mineral additive provided (g/kg diet) calcium, 2.11; phosphorus, 5.46; sodium, 2.74; potassium, 3.67; magnesium, 1.02; iron, 0.10; copper, 0.09; manganese, 0.55; zinc, 0.31; iodine, 4.3×10^{-3}; cobalt, 0.7×10^{-3}. [d] The vitamin additive provided (units/kg diet) retinol, 20000 IU; cholecalciferol, 2500 IU; tocopherol, 175 IU; menadione, 17.6 mg; thiamin, 20 mg; riboflavin, 15 mg; nicotinic acid, 100 mg; pantothenic acid, 6.9 mg; pyridoxine, 10 mg; folic acid, 5 mg; biotin, 0.3 mg; cyanocobalamine, 0.05 mg; L-ascorbic acid, 0.8 mg; choline, 1.36 g; myoinositol, 150 mg.

Experimental Design. In a first experiment, we tested the effect of the origin of gut microbiota on the induction of ACF by DMH. Fifteen GF rats were divided into three groups: GF, CV, and HMA. In a second experiment (APE experiment), we tested the effects of different doses of APE (0.001%, 0.01%, and 0.1%) on the induction of ACF by DMH in HMA rats. APE treatment started from the day of inoculation of the human microbiota to GF rats and was prolonged until euthanasia. The APE experiment was performed in two separate sets. In the first set, 45 rats were divided into three groups receiving APE at final concentrations of 0, 0.001, and 0.01%. These doses correspond approximately to 0 to 10 mg/kg body weight. In the second set, doses of APE were 0, 0.01, and 0.1%, and 36 rats were used (12 rats per group). Cecal contents were collected for the SCFA assay.

Analysis of Fecal Samples by PCR-TTGE. PCR-TTGE profiles were obtained as previously described (*18*). Briefly, total DNA was extracted from 0.2 g of feces as previously described. Concentration and integrity of DNA were determined visually by electrophoresis. Universal primers U968-GC-forward (5′ GC clamp-GAA CGC GAA GAA CCT TAC 3′) and L1401-reverse (5′ GCG TGT GTA CAA GAC CC 3′) were used to amplify V6 to V8 regions of bacterial 16S rRNA. We used the Dcode Universal Mutation Detection System (Bio-Rad, Paris, France) for sequence-specific separation of PCR products. The molecular marker was obtained by mixing the PCR products of 7 cloned rDNA from intestinal bacteria. Gels were stained in a solution of SYBR Green I (Roche Diagnostic, Meylan, France) and read on a Storm system (Molecular Dynamics).

SCFA Assay. After water extraction of acidified samples, SCFAs were analyzed using a gas−liquid chromatograph (Nelson 1020, Perkin-Elmer, Saint-Quentin-en-Yvelines, France) equipped with a flame ionization detector and a wide-bore column (15 m $\times$ 0.53 mm) (FSCAP Nukol, Supelco, Saint-Quentin-Fallavier, France) impregnated with SP 1000. Carrier gas (He) flow rate was 10 mL/min, inlet temperature was 100 °C, and detector temperature was 280 °C. 2-Ethylbutyrate was used as an internal standard.

ACF Determination. The colons were flushed with saline and opened from the anus to cecum. They were then fixed flat on filter paper in 10% buffered formalin for 2 to 10 days. After coding (for blind scoring), fixed

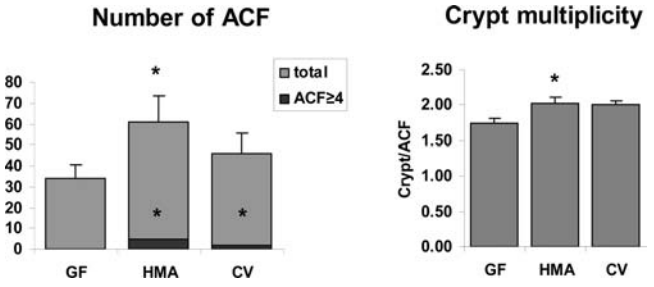

Figure 1. Effect of the bacterial status on the number of aberrant crypt foci (ACF) and on the mean crypt multiplicity after DMH treatment. GF, germ-free rats; HMA, human microbiota-associated rats; CV, conventional rats. *$P < 0.05$ when compared to GF rats.

colons were stained with 0.5% methylene blue according to Bird's method (*19*). Within 24 h, the number of ACF/colon and the number of aberrant crypts in each focus were determined microscopically at 40× magnification. Colons were independently scored by two observers. ACF were categorized as 1, 2, 3, and ≥ 4 crypts per focus, to analyze their size and mean multiplicity.

Statistical Analysis. Numerical data were expressed as means ± SEM. Statview software (version 5.0, SAS Institute, Cary, NC, USA) was used. The statistical significance was accepted when $p < 0.05$. ACF data were obtained by duplicate scoring by two different observers. Therefore, we performed a two-way analysis of variance per set of experiment to take into account the effect of the observer. ANOVA was followed by PLSD Fisher regression. For all of the other data, we performed one-way ANOVA followed by PLSD Fisher regression. In the APE experiment, data from the two sets of experiment cannot be merged since we cannot exclude a set effect which could not be discriminated by statistical analysis. Therefore, they were analyzed separately. TTGE profiles were compared using Gelcompar II software (version 2.0, Applied Maths, Kortrijk, Belgium). Analysis included a calculation of the number, position, and intensity of bands for each lane, and between-pattern comparisons using the Pearson correlation coefficient calculated as a measure of the degree of similarity; a value of 100 indicates that samples are identical. To estimate the relationship between TTGE profiles and APE, a PLS predictive model was established using SIMCA-P software, version 9.0 (UMETRI, Umea, Sweden).

RESULTS

Human Microbiota Increased the Number and Size of DMH-Induced ACF. By injecting 50 mg/kg of DMH into rats, we succeeded in obtaining ACF within 2 months in all colons observed (**Figure 1**). The human microbiota remained stable throughout the first experiment since the PCR-TTGE profiles were maintained (data not shown). Whatever the gut bacterial status, the growth of rats fed the human-type diet and submitted to DMH treatment was normal (data not shown). The total number of ACF per rat was higher in HMA rats (61 ± 12) than in CV rats (46 ± 9) or GF rats (37 ± 7). The increase was significant when compared to GF rats ($P < 0.05$). The mean crypt multiplicity per colon, defined as the mean of number of aberrant crypts in foci ($n = 1, 2, 3$, or $n \geq 4$), was significantly higher in HMA rats than in GF rats (2.0 ± 0.1 vs 1.7 ± 0.1, $P < 0.05$), while the difference was not statistically different between CV and GF rats. The number of ACF ≥ 4 was significantly higher in HMA and CV rats ($P < 0.05$ when compared to GF rats).

In HMA Rats, APE Did Not Reduce the Number and Size of DMH-Induced ACF. This protocol was performed in two sets of experiments which have not been merged in statistical analysis (see above). Therefore, results are reported separately. In addition, since the statistical analysis demonstrated an observer effect, we report in this section means and SEM calculated for both observers.

Low doses of APE (0.001% and 0.01%) were tested in the first set of experiments. In the control group, depending on the observer, the ACF number per colon reached an average of 76 ± 9 and 63 ± 9, respectively, and there were 6 ± 1 and 7 ± 2 ACF ≥ 4. The mean crypt multiplicity was 2 ± 0. Regardless of the observer, consumption of APE at doses of 0.001 and 0.01% did not alter these parameters (data not shown).

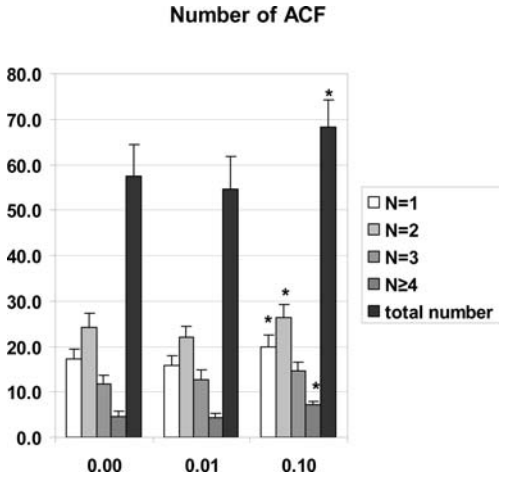

Figure 2. Effect of different doses of APE (0, 0.01%, and 0.1%) on the number and size of aberrant crypt foci (ACF) after DMH treatment in HMA rats (observer 1).*$P < 0.05$ when compared to the 0.01% APE group.

Higher doses of APE (0.01% and 0.1%) were tested in the second set of experiments. In the control group, there was an average of 57 ± 7 and 66 ± 12 ACF per colon depending on the observer and 5 ± 1 ACF ≥ 4 regardless of the observer. The mean crypt multiplicity was 2 ± 0. Regardless of the observer, 0.1% APE significantly increased the number of ACF containing 1, 2, and ≥4 crypts, as well as the total number of ACF per colon as compared to 0.01% APE ($P < 0.05$) but not to the control. **Figure 2** shows the data obtained for observer number 1. Those from observer 2 were comparable.

In HMA Rats, APE Did Not Alter Physiological Parameters. The consumption of APE did not alter rat growth. Whatever the amount of APE in their drinking water, rats drank the same amount throughout the experiment. Body weight gains and liver weights at sacrifice were identical between groups.

Bacterial populations as assessed by PCR-TTGE were not altered by APE treatment (**Table 3**). At nine days after inoculation of human fecal microbiota and the beginning of the APE treatment, the percentage of similarity between PCR-TTGE profiles of rats within each group was high: 86.7 ± 6.3, 82.3 ± 8.1, and 89.5 ± 7.8, respectively, for the control, 0.01% APE, and 0.1% APE groups. Nine weeks after introduction of APE in drinking water, the population similarity between the three experimental groups became superior to 90%.

Cecal pH and cecal weights were not altered by the consumption of APE. In ceca, the main SCFA were acetate, propionate, and butyrate, and their concentrations were similar in all groups (**Figure 3**).

DISCUSSION

For the first time, our model of rats both harboring human microbiota and fed a human-type diet has been successfully combined with DMH-induced preneoplastic lesions. Interestingly, we have demonstrated that the number and size of DMH-induced ACF were higher in HMA rats than in GF rats and that the number of ACF ≥ 4 was significantly higher in HMA rats than in GF and CV rats. In order to apply this model to the study of dietary modulation of ACF formation, it was important to check several parameters. This has been performed successfully.

Table 3. Percentage of Similarity between PCR-TTGE Profiles in the HMA Rats of the Different Dietary Groups[a]

experiment stage	within control group	within 0.01% APE group	within 0.1% APE group	control vs 0.01% APE	control vs 0.1% APE
day 9	86.7 ± 6.3	82.3 ± 8.1	89.5 ± 7.8	69.0 ± 6.8	80.7 ± 9.8
week 9	97.2 ± 1.0	96.7 ± 1.2	96.1 ± 1.9	93.6 ± 2.3	94.0 ± 2.5

[a] Fecal samples from 15 rats per group were collected and analysed in pool per cage by PCR-TTGE as described in Material and Methods. Results are expressed as means ± SEM.

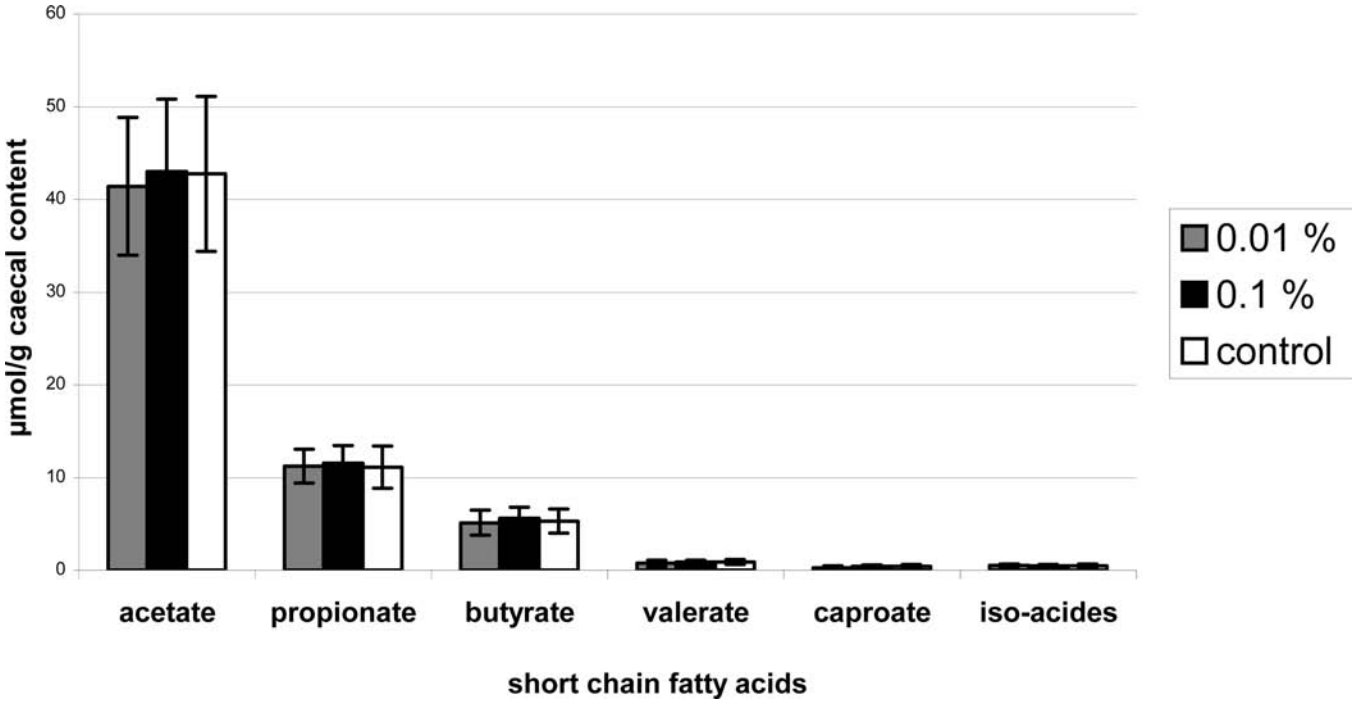

Figure 3. Concentrations of short chain fatty acids (SCFA) in cecal content of HMA rats exposed to APE in drinking water.

Inoculated human microbiota was stable, and fermentation activity measured by SCFA production showed typical values throughout the experiments. Then, this model could be helpfully used to study the effects of food constituents such as proanthocyanidins.

We have then shown that APE (0.001, 0.01%, and 0.1% in drinking water) had no protective effect on the number and size of DMH-induced ACF in HMA-rats. We cannot exclude an antagonistic effect of gut microbiota on a potentially protective effect of proanthocyanidin extracts on colon carcinogenesis. Our results must be compared to the few and discordant data available in the literature regarding these effects in rats, although the disparity of study design makes any comparison risky. Indeed, all previous studies vary in different aspects: source of the proanthocyanidin extract, doses, and vehicles, rat strains and gender, and carcinogens. This is without taking into account gut microbiota and diet composition, which we did in the present study. For example, grape seed proanthocyanidins (0.1% or 1%) appeared to decrease the number and multiplicity of AOM-induced ACF in female Sprague−Dawley rats (*20*), while cacao liquor proanthocyanidins (at 0.025% and 0.25% in the diet) failed to inhibit colon tumor formation in the male F344 CV rat multiorgan initiation model (*21*). Second, composition of the extract may be important as suggested by two studies. In wine, low or high molecular weight polyphenols had no effect, while the total polyphenolic extract reduced colorectal adenomas induced by AOM in male F344 rats (*22*). Cloudy, but not clear, apple juice reduced the number of large ACF in DMH-treated rats (*23*). Last, Gossé et al. reported that 0.01% apple proanthocyanidins in drinking water reduced the number of ACF in CV Wistar rats exposed to AOM (*24*). Essential differences between this latter study and ours are the presence of human microbiota and feeding with a human-type diet. Although data describing the metabolism of proanthocyanidins by gut microbiota are scarce, they suggest that proanthocyanidins are differently metabolized by rat and human gut microbiota. Indeed, it has been shown in vitro in anaerobic conditions, that human gut microbiota catabolizes purified proanthocyanidin oligomers in low-molecular-weight phenolic acids (*14*), whereas in CV rats fed proanthocyanidins from willow tree catkins, oligomers are poorly metabolized (*25*). In addition to strain specificities of human gut microbiota, the human-type diet may also have influenced the metabolic functions of microbiota, as suggested elsewhere (*11*, *16*).

We also found that 0.1% APE significantly increased the number and size of ACF as compared to 0.01%APE, but there was no significant difference with the control group. If confirmed, this potential adverse effect of APE would complete data from toxicology and safety evaluation studies: until now, the no-observed-adverse-effect level (NOAEL) of proanthocyanidins was reported to be 2000 mg/kg body weight/day using an apple extract in CV Sprague−Dawley rats (*26*) and 1410 mg/kg body weight/day using a grape seed extract in male CV F344 rats (*27*). If NOAEL was lower than admitted presently, the significance of the adverse effects of high doses of phytochemicals in human diets should be discussed. Indeed, increases of ACF incidence by high doses of other phytochemicals, such as β-carotene and lutein, have also been demonstrated (*28*). Therefore, it is important to evaluate doses of proanthocyanidins that are currently administered to animals and the quantity consumed by humans to determine exposure levels. The lack of reliable concentration data for proanthocyanidins in foods, in apples, and derivatives (compote, cider, juice, etc.) makes it difficult to evaluate dietary

intake. In the American diet, the daily average intake of proanthocyanidins from different sources has been estimated to be about 50 mg, i.e., around 1 mg/day/kg body weight (*13*). Besides, for several decades, the use of various proanthocyanidin extracts as additives or nutritional supplements has been increasing regularly in USA, Japan, and European countries, leading supplement consumers to ingest several hundred milligrams of proanthocyanidins per day (*29*). A recent meta-analysis illustrates the potential adverse effect of supplements since it shows that regular consumption of antioxidant substances taken as supplements does not reduce the risk of digestive cancers but significantly augments mortality (*30*). Even in extreme situations, human exposure would not exceed 10 mg/day/kg body weight (*30*). In animal studies, such an exposure level is reached with about 0.01% proanthocyanidin extract in the drinking water or diet. At this dose, we have seen that colon carcinogenesis studies gave discordant results.

In conclusion, our data raise relevant questions regarding human health. It is important to take into account the cross-talk between gut microbiota and the colon epithelium. Although the use of HMA rats cannot be systematic because of the burden linked to gnotobiotic animal experimentation, such a model is particularly useful to shed light on the potential adverse effects on colon carcinogenesis of dietary components usually considered as protective. Furthermore, our results confirm that particular attention should be paid prior to using food components with potential cancer-inhibiting effect in chemopreventive diets or dietary supplements in humans.

ABBREVIATIONS USED

ACF, aberrant crypt foci; APE, apple proanthocyanidin-rich extract; CV, conventional; DMH, 1-2, dimethylhydrazine; GF, germ-free; HMA, human microbiota-associated; TTGE, temporal temperature gradient gel electrophoresis; SCFA, short chain fatty acids.

SAFETY

The carcinogenesis studies were performed in a separate unit equipped with three plexiglass isolators which could be maintained under negative pressure. When possible, disposable material was preferred, and all wastes were eliminated in sealable plastic tubing directly connected to the isolator. By using these specific procedures, we could guarantee maximal security both to the operators working in this unit and to the environment.

Isolators were maintained under negative pressure. During DMH treatment and 3 days thereafter, the air was directly eliminated outdoors. By switching off the air pump of each isolator, we eliminated all risk of contamination of the indoor air by DMH gaseous metabolites. Three days after the second injection, the air pumps were re-established for each isolator.

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