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REVIEW

Approaches that ascertain the role of dietary compounds in colonic cancer cells

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Abstract

Preventive approaches against cancer have not been fully developed and applied. For example, the incidence of some types of cancer, including colon cancer, is highly dependent upon lifestyle, and therefore, amenable to prevention. Among the lifestyle factors, diet strongly affects the incidence of colon cancer; however, there are no definitive dietary recommendations that protect against this malignancy. The association between diet-derived bioactives and development of colonic neoplasms will remain ill defined if we do not take into account: (1) the identity of the metabolites present in the colonic lumen; (2) their concentrations in the colon; and (3) the effect of the colonic contents on the function of individual bioactives. We review two approaches that address these questions: the use of fecal water and *in vitro* models of the human colon. Compared to treatment with individual diet-derived compounds, the exposure of colon cancer cells to samples from fecal water or human colon simulators mimics closer the *in vitro* conditions and allows for more reliable studies on the effects of diet on colon cancer development. The rationale and the advantages of these strategies are discussed from the perspective of a specific question on how to analyze the combined effect of two types of bioactives, butyrate and polyphenol metabolites, on colon cancer cells.

Key words: Human colon model; Fecal water; Diet; Colon cancer; Prevention; Butyrate; Polyphenols; WNT signaling

Core tip: Studies on diet and colorectal cancer are in their infancy, and the relevance of many publications on the topic is questionable due to three problems: (1) there is uncertainty about which diet-derived compounds are present in the colon; (2) most studies have focused on individual bioactives; whereas, food intake results in complex metabolite mixtures; and (3) the physiological concentrations of many colonic bioactives are unknown. Here we discuss how the use of fecal water samples and *in vitro* models of human colon address these problems.

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INTRODUCTION

Within the past 100 years, the leading causes of death

have changed dramatically[1]. Approximately a century ago, the three leading causes of death were influenza and/or pneumonia, tuberculosis, and gastrointestinal (GI) infections. However, in 1997 less than 5% of the deaths were attributed to pneumonia, influenza, and human immunodeficiency virus infection; whereas, heart disease and cancers accounted for more than 50% of all deaths[2], In 2008, the American Cancer Society projected that soon cancer will become the leading cause of death worldwide[3], and the 2010 data for United States indicate almost equal number of deaths caused by heart disease and cancer (597689 *vs* 574743, respectively[4]). Recent projections of mortality and causes of death by the World Health Organization also support cancer as emerging leading cause of death in both, economically developed and developing countries[5]. How are these changes explained? The deaths from infectious diseases declined due to the implementation of childhood vaccinations, improvements in sanitation and hygiene, and the discovery of antibiotics. Except for the use of antibiotics, these approaches are classified as preventive measures. The more recent reduction of total cardiovascular death is also attributed to prevention; thus, massive educational efforts have raised the awareness of what constitutes a healthy lifestyle, and novel medications that control high blood pressure and cholesterol levels have been introduced into clinic. Therefore, the decreased deaths from infectious and heart diseases are mainly attributed to the development of preventive measures.

Unfortunately, the full power of prevention has not been applied in the battle against cancer. Presently, the focus is on cancer treatment, and as a result, billions of dollars are invested in drug development. The new arsenal of molecularly targeted anti-cancer drugs has raised hopes; however, it is increasingly clear that although "targeted" therapies prolong patients' lives, their benefit is limited in time by the inevitable acquisition of drug resistance. Combination therapies that incorporate conventional chemoradiation and molecularly targeted drugs might be the next step; however, the lesson from the past is that to obtain a significant victory against any disease, we need to emphasize on primary prevention.

Similar to the trend of personalized cancer treatment, future cancer prevention measures should be stratified by phenotype, genotype, and family history. Cancer prevention strategies could include, but not be limited to, the following: (1) monitoring of the patient's exposome (a set of biomarkers indicative of individual's exposure to cancer promoters[6]); (2) non-invasive imaging techniques that detect the earliest stages of abnormal growth; (3) reliable dietary, physical activity, and other lifestyle recommendations; and (4) vaccines that reduce the risk for specific cancers. In addition to developing future personalized prevention approaches, it is important to expand the existing prevention strategies that address some types of cancer as a public health issue affecting large populations (*e.g.*, educational approaches, influencing legislation, mobilizing communities). The present review focuses on the dietary approach to colorectal cancer (CRC) prevention, and addresses several problems that hinder the progress of this approach in terms of obtaining valid and unambiguous dietary recommendations.

There are over 140000 new cases of CRC and approximately 50000 CRC-related deaths a year in the United States[7]. A distinct characteristic of CRCs is that they develop slowly from benign adenomas: polyps larger than one centimeter in size have a 24% chance of progressing into carcinoma over a 20-year period[8]. The transition of benign adenomas into malignancies and the incidence of colonic neoplasms are modulated by diet-derived compounds[9]. However, studies on diet and CRC are in their infancy, and the relevance of many publications on the topic is questionable due to three problems: (1) there is uncertainty about which diet-derived compounds are present in the colon, and what their half-life; (2) most studies have focused on individual bioactives; whereas, food intake results in a complex mixture of metabolites that could modify each other's effect on neoplastic cells; and (3) the concentrations of many bioactives in the colon are unknown; whereas compounds, for which such information is available, have been frequently analyzed at levels exceeding physiological concentrations.

Here we review two approaches that address these problems, and discuss how these strategies solve a specific question on the interaction between two dietary bioactives: butyrate and polyphenol derivatives. Both bioactives affect the risk for CRC, and although there are other dietary compounds and mechanisms proposed to be protective against the malignancy, this review is limited to one example. Our objective is to highlight the methodologies that unravel the effects of multiple dietary bioactives on colonic cells, and not to comprehensively discuss all classes of dietary bioactives and their plausible physiological effects.

WNT/catenin signaling by butyrate

In 2011, the World Cancer Research Fund and the American Institute for Cancer Research upgraded the protective effect of fiber against colon cancer from "probable" to "convincing"[10] and this effect is attributed in part to the fermentation product of fiber in the colon, butyrate. Butyrate is a short-chain fatty acid (SCFA), the production of which enables the salvage of energy from dietary fiber that would be otherwise lost. It is estimated that SCFAs contribute to about 5%-15% of the total caloric requirements in humans[11]. Various tissues in the body can utilize SCFA for energy generation; however, butyrate is the preferred fuel for the colonic epithelial cells that derive about 70% of their energy from butyrate oxidation[12,13]. Butyrate is regarded as a healthy metabolite due to its positive influence on cell growth and differentiation, as well as its anti-inflammatory properties[12,14]. Butyrate also acts as an inhibitor of histone deacetylases (HDACi). Its colonic concentration is between 2 and 10 mmol[15] and at these levels, butyrate induces apoptosis in most CRC cells *in vitro*. We have provided evidence that this effect is in part due to the ability of butyrate to hyperactivate the WNT/catenin signaling pathway, and several synthetic

HDACis mimic the effect of butyrate on the WNT pathway and apoptosis[16,17]. The hyperactivation of WNT/catenin signaling by HDACis takes place only in colonic neoplastic cells with mutations in the pathway, and such mutations are detected in 80% of the sporadic colon cancers[18-20]. This finding is in agreement with observations that moderate levels of oncogene activities support cancer development; however, hyperactivation of oncogenic functions may result in cell death and senescence[21]. Therefore, WNT/catenin signaling is not "oncogenic" under all conditions, and sometimes its activation correlates with less aggressive cancer phenotypes[22].

Polyphenols as biological food constituents

The intake of fiber (the most important source of butyrate in the colon) is usually associated with that of other bioactive ingredients; for example, many fiber-rich foods are a source of polyphenols (*e.g.,* cereals, fruit, and vegetables). The drinks that accompany our meals further increase the complexity of bioactives: wine, fruit juices, cocoa, tea, and coffee are all rich in polyphenols. The two main classes of dietary polyphenols are the flavonoids and the phenolic acids. In *in vitro* experiments, the flavonoids are powerful antioxidants; however, this activity is exhibited at concentrations exceeding the levels achievable *in vivo*. Thus, after consumption of 10-100 mg of a single compound, the maximum plasma levels of individual flavonoids are approximately 1-3 mol[23,24]. In addition, due to host metabolism the *in vivo* half-life of the precursor polyphenols is short due to their rapid conversion into metabolites, all of which exhibit diminished antioxidant activity[24-26]. More recent studies indicate that at physiological concentrations, polyphenols and their metabolites modulate cell signaling pathways[27], and exhibit anti-inflammatory activity through inhibition of COX-2 protein levels, prostanoid biogenesis, or pro-inflammatory cytokine production[28-31]. Polyphenol metabolites also exhibit anti-proliferative effect on neoplastic cells[32,33], thus, similar to butyrate, some polyphenols and their microbial metabolites exhibit a CRC protective role. For example, quercetin, a flavonol found in citrus fruit, buckwheat, and onions, suppresses the formation of aberrant crypt foci and induces apoptosis in preneoplastic human colonocytes[34,35]. Caffeic acid esters present in propolis are potent inhibitors of human colon adenocarcinoma cell growth, carcinogen-induced biochemical changes, and preneoplastic lesions in the rat colon[36,37]. A CRC-preventive role has also been reported for isoflavons, curcumin, and tea polyphenol in green tea, (-)-epigallocatechin-3-gallate (EGCG)[38].

Synergistic or antagonistic effects of butyrate and polyphenols?

Since the intake of dietary fiber is frequently accompanied by that of polyphenols, it is logical to investigate whether the effect of butyrate on WNT/catenin signaling and apoptosis in CRC cells are modified by polyphenols and their metabolites. Presently, the combined effects of butyrate and polyphenol metabolites on WNT/catenin signaling are unknown; however, there have been reports on the modulation of WNT/catenin signaling by polyphenols. For example, polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine-induced colorectal tumorigenesis in rats, and the researchers proposed that this effect is mediated by suppression of WNT/catenin signaling[39]. EGCG suppresses WNT/catenin transcriptional activity in HCT-116 CRC cells at concentrations of 100-200 mol, which are unachievable *in vivo*[40]. However, at physiologically relevant concentration of 0.5 mol[23,24,32], EGCG inhibits the enzyme glycogen synthase kinase-3 beta (GSK-3beta)[41]. This inactivation of GSK-3beta should result in accumulation of transcriptionally active Ser-37/Thr-41-dephosphorylated beta-catenin, and increased WNT transcriptional activity[42,43]. Polyphenol-rich apple juice extract, as well as the free aglycon phloretin and the flavonol quercetin, also inhibit GSK-3beta in *in vitro* assays[44]. In agreement with this inhibitory effect on the enzyme, quercetin at 10 mol increases WNT/catenin transcriptional activity[41]. The interpretation of these findings is difficult due to the fact that the bioavailability of the compounds has not been taken into account, or is unknown. In addition, polyphenols are biochemically transformed or completely fermented by the gut microbiota to metabolites with a modified biological activity, as discussed below. The inhibition of GSK-3beta by some polyphenols indicates that these compounds may synergize with butyrate in its effect on WNT/beta-catenin signaling. Furthermore, similar to butyrate, some polyphenols and their metabolites inhibit histone deacetylases (HDACs). Thus, fermentation of polyphenol-rich apple juice extracts with human fecal slurry revealed that polyphenol metabolites have a HDAC inhibitory function[45]. Metabolites of polyphenols in the colon, such as *P* Coumaric acid, 3-(4-OH-phenyl)-propionate, and caffeic acid also exhibit HDAC inhibitory function in *in vitro* assays with nuclear extracts from HT-29 human CC cells[46]. Therefore, similar to butyrate[16,17], polyphenol metabolites with HDAC inhibitory function may protect against CC *via* stabilization of beta-catenin and hyperinduction of WNT/beta-catenin signaling. Despite these data, the question of how polyphenols and their metabolites modulate the effects of butyrate on colonic neoplastic cells has remained unanswered. Several problems hinder the progress of the studies: there is little knowledge about the polyphenol derivatives present in the colon, their physiological concentrations, and how the colonic content modulates the functions of the bioactives. The main colonic species might be the polyphenol aglycones and their derivatives: phenolic and non-phenolic aromatic acids. The deglycosylation of polyphenols is catalyzed by microbial beta-glucosidases in the small intestine and primarily the colon, and this process results in aglycone forms that are more absorbable[47]. After absorption in the intestinal cells, the aglycones are metabolized to conjugates of glucuronate and sulfate, which are the major forms in plasma and urine[47]. However, these conjugates have not been detected in the colon, most likely due to the hydrolase activity of the GI microbiota[48-50].

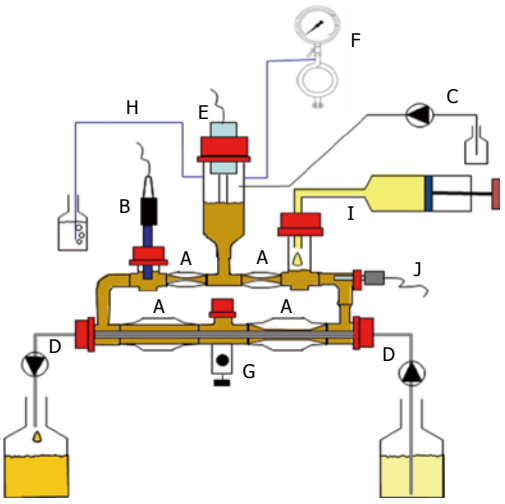

Figure 1 TIM-2 is a validated, computer-controlled system that simulates the human colon. The model consists of glass units with a flexible wall inside (A); Peristaltic movements, achieved by pumping warm water into the space between the glass unit and the flexible walls at regular intervals, simulate peristaltic movements and allow the lumen to be mixed and transported through the loop-shaped system. The system is kept at body temperature (37 ℃). To simulate the pH in the proximal colon, the pH is set at 5.8 and controlled (B) and adjusted by secretion of 2 mol/L NaOH into the system (C). A dialysis membrane consisting of semi-permeable hollow fibres is placed in the lumen (D). Water and fermentation products are removed from the lumen through the dialysis system, thereby maintaining physiological concentrations of microbial metabolites and preventing accumulation of metabolites to toxic levels. Furthermore the model contains an inlet system for the delivery of food (I) and a level sensor to control (E) a constant volume of the luminal content. The system was kept anaerobic by flushing with nitrogen gas (F), which allowed for the growth of a dense, complex microbiota, comparable to that found in the proximal colon of humans. A: Peristaltic compartments containing fecal matter; B: pH electrode; C: Alkali pump; D: Dialysis liquid circuit with hollow fibre membrane; E: Level sensor; F: N2 gas inlet; G: Sampling port; H: Gas outlet; I: "Ileal efflux" container; J: Temperature sensor.

Use of fecal water

The problems listed above are not specific to our example on the combined effect of dietary butyrate and polyphenols on colon cancer cells, as they represent a stumbling block for all studies aimed at characterization of the effects of dietary bioactives. To date, there are two approaches that address these problems: (1) performing analyses with the aqueous phase of feces (fecal water); and (2) utilizing *in vitro* GI models. The first approach is justified by the fact that the colonic epithelium is exposed to the fecal matter *in vivo*[48,51-54] and that fecal water affects the growth of colonocytes more effectively than components of the solid phase of feces[52,53]. Gas chromatography and mass spectrometry analyses of the fecal water of healthy volunteers have identified and quantified the flavonoids and their derivatives in the colon[48]. In these samples, the most prevalent flavonoids were naringenin, quercetin, formononetin, catechin, epicatechin, isorhamnetin, apigenin, and kaempferol, and they were detected at mean concentrations of 1.2, 0.63, 0.17, 0.14, 0.11, 0.10, 0.07 and 0.05 mol, respectively. All polyphenols and derivatives exhibited daily fluctuations in the same individual, and the most prevalent flavonoids naringenin, quercetin, and formononetin reached a maximum concentration of 4.04, 1.30, and 0.84 mol, respectively. Colonic derivatives of the flavonoids in the colon were detected at concentrations up to two orders of magnitude higher than these of their precursors; thus, the total monophenolic acids reached up to 740.7 mol and the total nonphenolic aromatic acids, 1.5 mmol[48]. Recent analyses of fecal water have confirmed the prevalence of the phenolic and non-phenolic aromatic acids in fecal water[28,54]. Therefore, our question of how polyphenols and their metabolites modulate the activity of butyrate may need to be re-stated to how the activity of butyrate is affected by high levels of monophenolic and nonphenolic acids.

In vitro models that mimic the human colon

The combined effect of butyrate and polyphenol metabolites on neoplastic cells, however, is even more complex. The combined effect could be modified by the presence of additional metabolites, as the intake of any diet results in a complex mixture of compounds in the colon. The physiological properties of diet-derived mixtures could be analyzed with *in vitro* models of the human GI tract, and one such model has been developed by TNO in the Netherlands[55]. This system closely mimics the physiological conditions in the GI tract, as established in numerous validation studies[32,56-67]. The GI system is composed of two separate models: TIM-1 that simulates the stomach and the small intestine (not further discussed here), and TIM-2 that simulates the colon[68] and contains compartments with a high density, metabolically active microbiota of human origin. The physiological conditions of the large intestine that are simulated include pH, anaerobiosis and gradual intake of pre-digested meal compounds coming from the small intestine (Figure 1). Physiological amounts of microorganisms in the TIM-2 model are maintained *via* dialysis mechanism. This mechanism takes up electrolytes and microbial metabolites, and ensures that the concentrations of these remain at physiological levels, preventing inhibition of the microbiota by metabolites. The *in vitro* GI system permits the use of an intestinal microbiota from different enterotypes and the comparison between various donors, *e.g.,* healthy *vs* diseased, lean *vs* obese[69]. The technology also allows for controlled analyses on the colonic outputs from various diets. Thus, entire meals representative of different types of diet can be "fed" to the GI model[60] and the resulting real-time fermented samples from the TIM-2 compartments can be tested on neoplastic and normal colonic cells *in vitro*[32]. Although fecal water from human subjects could be used for similar studies, there are several problems associated with this approach: inter-individual differences in metabolic rates and colonic microbiota, noncompliance with diet, preferential absorption of some compounds by the colonocytes, and the impossibility of acquiring samples from different locations of the human GI tract (*e.g.,* pre-colon, proximal colon). The last point is important, since metabolite concentrations change along the colon[11]. Using the *in vitro* GI system has several advantages: it is computer-controlled, allowing standard-

ization of the experiments, it is cheaper than clinical or animal trials, and it does not have the ethical constraints associated with animal and human subject studies. Furthermore, sampling from various locations along the GI tract and at different time points enables kinetic studies of the microbial metabolism of dietary components. The application of the *in vitro* gut approach can facilitate the design of functional foods and dietary supplements that decrease CRC incidence. For example, utilizing the *in vitro* GI system, Gao *et al*[32] discovered that tea, citrus fruit, and soy flavonoids are metabolized in the colon to a few phenolic and aromatic acids, therefore ascertaining the exact compounds that should be screened for effects on CRC cells.

In addition to the studies performed with the computerized human gut TIM-2, there are numerous reports on simpler colon simulators, and the function of some of these has been validated by chemical and microbiological measurements of the intestinal contents of human sudden death victims[70]. These models are fermentation systems that closely reproduce the proximal and distal human colon in terms of physicochemical parameters by utilizing a number of different vessels and continuous or semi-continuous culturing modes[71]. For example, a two-stage compound continuous culture models consisting of a proximal vessel (with lower pH) and a distal vessel (with higher pH) inoculated with human feces have been used to evaluate how various nutrients and supplements affect genotoxicity of the colonic environment and the populations of human gut bacteria[72,73]. Continuous culture models have been applied to analyses of how certain prebiotics affect the fecal metabolite profile, the survival of probiotics, and the interactions between various colonic microbial populations[74-77]. The effect of retention time (colonic transit time) on the catabolism of organic sources of carbon and nitrogen have been analyzed by a three-stage continuous culture model, which revealed that the majority of carbohydrate breakdown and SCFA production takes place in the proximal part of the colon (in the first vessel); whereas, formation of branched-chain fatty acids and phenolic compounds, occurs primarily in the distal part (mimicked by vessels 2 and 3)[70]. Other three-stage continuous culture colonic models inoculated with human fecal material were utilized to quantitate bacteria and evaluate the fermentability of oligosaccharide sources[78,79]. Four-stage semicontinuous model systems of the human colon, in which the four compartments mimic the conditions of the ascending, transverse, descending and sigmoid colon, have been employed to investigate the effects of probiotics, prebiotics, and various synbiotic combinations[80-82].

Applied to our question of whether the apoptotic and WNT signaling-modulating functions of butyrate are affected by diet-derived polyphenol compounds and their metabolites, the strategy utilizing *in vitro* gut models would be a reliable approach.

Thus, digesta samples from *in vitro* fermentation systems or the computerized TIM-2 model, instead of individual compounds, should be used to analyze the effects of various diets on colonic cancer cells.

Screening for dietary components that increase butyrate production by the colonic microbiota

TIM-2 allows determining the potential of dietary fibers to produce butyrate by the microbiota under physiological conditions. In an extensive study comparing 17 fibers Maathuis *et al*[57] showed varying levels of butyrate production for each fiber, with the highest production resulting from pullulan. Interestingly, this fiber also produced high levels of lactate, an intermediate intestinal metabolite that accumulates when there is a fast fermentation of a substrate. Lactate is usually converted into propionate[83] and butyrate[84] and through cross-feeding between different members of the microbiota. Butyrate is also produced through cross-feeding from acetate; thus, using ^{13}C-starch Maathuis *et al*[58] have shown that cross-feeding between *Ruminococcus bromii* and *Eubacterium rectale* results in production of butyrate from acetate[56]. Similarly, using ^{13}C-labeled galacto-oligosaccharides it was shown that lactate, produced by *Bifidobacteria* and *Lactobacilli*, was converted into butyrate. These two cross-feeding reactions in the colon could be quantified[85,86] and an *in silico* model can be used to predict production of the various SCFA by the colonic microbiota.

Analyses of human fecal samples also allow for focused analyses of how dietary changes affect butyrate levels in different individuals. Thus, considerable variations in fecal butyrate concentrations have been detected among individuals consuming resistant starch or nonstarch polysaccharides in a randomized cross-over study[87]. McOrist and colleagues reported that intake of resistant starch overall increases butyrate concentrations in most, but not all, individuals[87].

Analyses with a semi-continuous colonic simulator revealed that *Lactobacillus acidophilus* NCFM™ in combination with lactitol increases the numbers of *Bifidobacteria*, and stimulates synergistically the production of butyrate[82]. Similar colonic simulation system consisting of three vessels and inoculated with fecal slurry from healthy nonmethane producing donors established the parameters of SCFA production, including this of butyrate[70].

Use of in vitro models to study the microbial metabolism of polyphenols in the colon

Approximately 90%-95% of dietary polyphenols are not absorbed in the small intestine and reach the colon intact[88]. In the case of monomeric units, studies performed with ileostomy patients have shown that almost 70% of the ingested monomeric flavanols are accumulated in the colon, with 33% corresponding to the intact parent compounds[89]. As mentioned above, the major colonic metabolites of the polyphenols are phenolic acids. Thus, (epi) catechin and the monomeric units of procyanidins are degraded into several phenolic acids, namely various substituted phenylvaleric, phenylpropionic, phenylacetic, benzoic, and hippuric acid[90-92]. Additional metabolites from catechin and epicatechin such as 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone

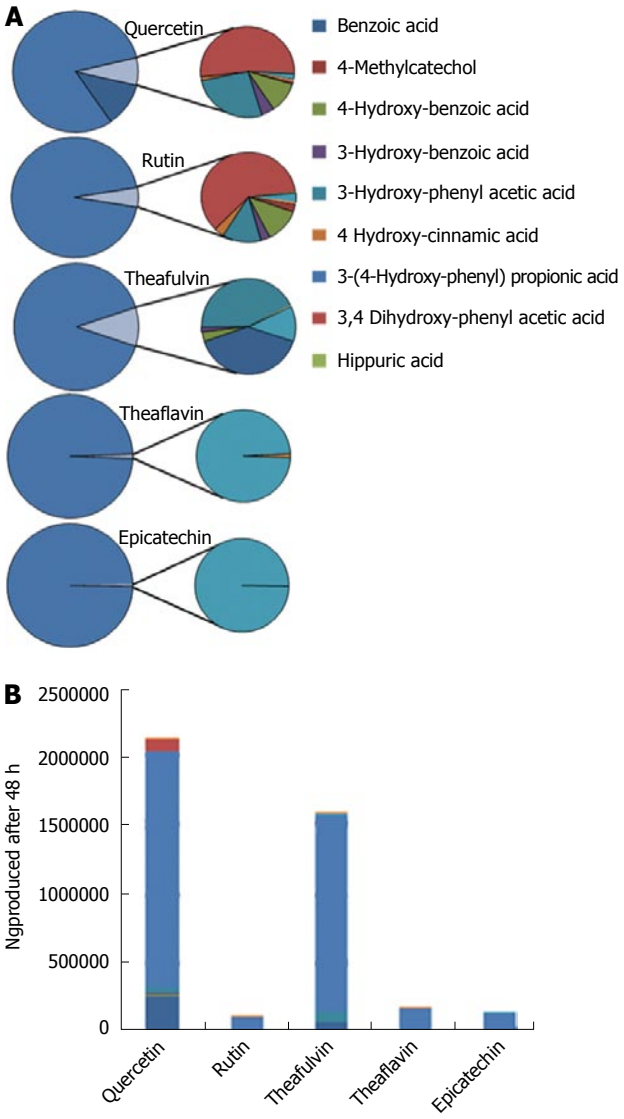

Figure 2 Cumulative production of 'simple' phenolic metabolites after 48 h fermentation of different polyphenols in TIM-2. At time zero a single shot of individuals polyphenols (1 microgram in dimethyl sulfoxide) was introduced into TIM-2 throigh the sampling port (Figure 1G). At regular intervals for the next 48 h samples were taken from the lumen and dialysate and analyzed using LC-MS for the microbial metabolites generated by the gut microbiota. The ratio (bar graph, in percentage) and absolute cumulative production (B; in ng) at t = 48 h of microbial metabolites after fermentation in TIM-2 were subsequently calculated and compared amongst the different polyphenols.

have been identified in man[90,91,93].

Under physiological conditions, the monomeric polyphenols are fermented rapidly; therefore, it is unknown whether these compounds have sufficient half-life to affect colonic (neoplastic) tissue from the luminal side, or whether the resulting microbial metabolites exert a stronger biological effect. Studies with human gut models can facilitate the answer to this question. In unpublished studies with TIM-2, we have observed that the same microbiota metabolizes different polyphenols to different low-molecular weight aromatic acids with variable hydroxylation profile and length of the aliphatic side chain (Figure 2). The number of produced microbial metabolites ranged from two (for epicatechin) to 12 (for quercetin). Even glycosylation of the polyphenols (*e.g.,* quercetin versus rutin) affected the production of microbial metabolites, likely because different groups of colonic microorganisms ferment quercetin and rutin. Thus, compared to other polyphenols, fermentation of rutin resulted in decreased proportion of benzoic acid and other metabolites (Figure 2), as well as an about 20-fold lower absolute amount of metabolites.

Analyses with colonic simulators allow for the detection of new colonic metabolites. In urine, the most frequent metabolite found after polyphenol ingestion is hippurate. This metabolite, a conjugate of benzoic acid and glycine, is considered to be produced by co-metabolism of the host and the microbiota. Benzoic acid is produced from the phenolic acids produced by the microbiota, and the glycine is thought to be coupled to benzoic acid in the liver. However, in the *in vitro* human gut TIM-2, which lacks the host metabolism component, we have shown that hippurate is also produced, indicating that the colonic microbiota by itself produces the metabolite (Figure 2).

Studies with colonic models could also address the question on the half-life of monomeric flavanols. For example, in studies on the dimeric forms of chocolate procyanidins Appeldoorn *et al*[90] have shown that the human microbiota produce several metabolites: 2-(3,4-dihydroxyphenyl)acetic acid, 2-(3-hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid and 3-(3-hydroxyphenyl)propionic acid, as well as various hydroxylated phenylvaleric acids, phenylvalerolactones,and 1-(3',4'-dihydroxyphenyl)-3-(2',4',6'-trihydroxyphenyl)propan-2-ol. The researchers also indicated that the formation of smaller metabolites was due to the direct degradation of dimers instead of cleavage of the monomeric form as previously assumed[90]. It is still possible that some procyanidin dimers are converted into monomeric flavanols before being fermented into phenolic acids; however, monomeric flavanols are rapidly metabolized, and therefore their presence is difficult to analyze[94].

Finally, phenolic acids produced from flavanols by the colonic microbiota significantly inhibit pathogenic human intestinal bacteria, such as *Clostridium perfringens*, *Staphylococcus aureus*, *E. coli*, and *Salmonella,* while exhibiting a much lower inhibition of commensal bacteria and probiotics, *Clostridium*, *Bifidobacterium* and *Lactobacillus*[95,96]. One mechanism mediating this activity is the destabilization of the outer membrane of *Salmonella* species[97]. Since changes in microbiota composition influence the production of butyrate from dietary fiber, the combined effects of polyphenols and fibers need to be thoroughly investigated in colonic simulator systems that include the naturally occurring colonic microorganisms.

CONCLUSION

Analyses on individual bioactives pinpoint their molecular targets in cells; however, such studies (1) should utilize physiological concentrations of the compounds; and (2)

should be accompanied by analyses with colonic digesta from different diets, since the activity of individual metabolites is likely modified by the complex colonic milieu. Such studies can be facilitated by the use of artificial GI systems and fecal water samples. This type of analyses will assist the design of functional foods and/or dietary supplements with CRC-preventive role.

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