Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model

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Cruciferous vegetables, such as Brassica, which contain substantial quantities of glucosinolates, have been suggested to possess anticarcinogenic activity. Cutting and chewing of cruciferous vegetables releases the thioglucosidase enzyme myrosinase, which degrades glucosinolates to isothiocyanates and other minor metabolites. Cooking of cruciferous vegetables inactivates the myrosinase enzyme, allowing intact glucosinolates to reach the large intestine, where they can be degraded by the indigenous microflora into isothiocyanates. This local release of isothiocyanates where they can be degraded by the indigenous microflora may explain the protective effect of cruciferous vegetables on the colon epithelium. However, little is known about the amounts and identities of glucosinolate metabolites produced by the human microflora. The production of allyl isothiocyanate from sinigrin was investigated in a dynamic in vitro large-intestinal model, after inoculation with a complex microflora of human origin. Sinigrin and allyl isothiocyanate concentrations were analysed in the lumen and dialysis fluid of the model. Peak levels of allyl isothiocyanate were observed between 9 and 12 h after the addition of sinigrin. The model was first set up with a pooled and cultured human microflora, in which 1 and 4% of, respectively, 1 and 15 mM sinigrin, was converted into AITC. However, the conversion rate was remarkably higher if different individual human microflora were used. Between 10% and 30% (mean 19%) of the sinigrin was converted into allyl isothiocyanate. The results of this study suggest that allyl isothiocyanate is converted further into other, yet unknown, metabolites.

Introduction

Based on epidemiological studies, frequent consumption of relatively large amounts of a wide variety of fruits and vegetables has been associated with a lower incidence of colorectal cancer (as reviewed by Block et al. and Steinmetz and Potter (1,2)). Especially cruciferous vegetables, such as Brassica, which contain substantial quantities of glucosinolates, have been suggested to possess anticarcinogenic activity (3–5). However, most epidemiological studies have not clearly resolved the question whether the protective effects should be attributed to cruciferous vegetables per se or to vegetables in general (6). On the other hand, Voorrips et al. recently observed an inverse association between Brassica vegetables consumption and the incidence of colon cancer for both men and women (7).

More than one hundred glucosinolates have been identified, 15–20 of which have been found in cruciferous vegetables of the genus Brassica, such as Brussels sprouts, broccoli, cabbage and kohlrabi (8–10). Their concentration varies among the different species and cultivars of Brassica vegetables, the cultivation conditions and the seasons, as well as the different parts of the plant (11).

The consumption of Brassica vegetables varies among different populations (12). For example, per capita consumption in the UK amounts to 45 g/day (13). Milford and Evans reported that the glucosinolate content varied between 9 to 35 µmol/g in crops (14). However the average daily consumption of all glucosinolates in the UK is estimated to be ~50 mg (15). The consumption of Brassica vegetables is quite high in the UK compared with Canada and the United States, in which the consumption was estimated at 15.6 and 18 g/day, respectively (16,17). In contrast, in Japan the consumption of Brassica vegetables was 113 g/day in 1975 (18). In the Netherlands, the consumption of Brassica vegetables is estimated to be 32–36 g/person/day, which equals an estimated average intake of 22 mg of glucosinolates per person per day (6,19,20).

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Depending on target tissue and the type of compound, different mechanisms of action have been suggested to explain the anticarcinogenic actions of glucosinolates and their breakdown products, among which are the isothiocyanates (ITCs). The most frequently proposed cancer preventive mechanisms are modulation of the activities of phase I (cytochrome P450s) and phase II (glutathione-S-transferase, UDP-glucuronosyltransferase and quinone reductase) biotransformation enzymes, redox regulation antiproliferation and induction of cell cycle arrest and by increasing the rate of apoptosis in cancer cells (21–23). For more details according to these anticarcinogenic mechanisms the reader is referred to the review papers of Hecht (24), Musk (25), Mithen et al. (10), Talalay and Fahey (26) and Verhoeven et al. (6).

Upon plant tissue disruption during food processing (e.g. by cutting), glucosinolates presumably stored in the cell vacuole are released and hydrolysed by the enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1.), which is located in the cytoplasm. Myrosinase hydrolytically cleaves off the glucose, resulting in an unstable intermediate (aglycone). This

Abbreviations: AITC, allyl isothiocyanate; BITC, benzylisothiocyanate; GC, gas chromatography; GSH, glutathione; GST, glutathione-S-transferase; HPLC, high performance liquid chromatography; ITC, isothiocyanate; SPME, solid-phase microextraction.
glycine spontaneously rearranges into the potential cancer-protective ITCs, nitriles or other products, such as thiocyanates (8). Which breakdown products will be formed, depends on the glucosinolate substrate as well as the reaction conditions, such as pH and the presence of Fe²⁺ or epithiospecifier protein (10). The chemical structure of a glucosinolate and the breakdown products formed on myrosinase activity are shown in Figure 1.

If cruciferous vegetables are cooked, whereby the myrosinase enzyme is inactivated, intact glucosinolates can reach the large intestine, where they can be degraded by the resident microflora (27). The ability to degrade glucosinolates is widely distributed among bacterial species in the human intestine, e.g. Bacteroides, Peptostreptococcus, Enterococcus, and Escherichia (28). Until recently, few data were available with regards to the amounts and identities of the total glucosinolates and the breakdown products produced by the colonic bacterial enzymes. Elfoul et al. showed that 2-propenyl glucosinolate (sinigrin), a common glucosinolate in Brassica vegetables, can be hydrolysed by a Bacteroides thetaiotaomicron strain of human origin to yield allyl isothiocyanate (AITC) in the large bowel of gnotobiotic rats inoculated with this bacterium (29).

More information on the bioaccessibility of glucosinolates in the digestive tract, including the microbial availability of parent compounds and their breakdown products, is required to evaluate properly the potential role of glucosinolates and ITCs in the prevention of human colon cancer; such information would be particularly helpful to understand underlying mechanisms (30).

The aim of this study is to investigate the microbial metabolism of glucosinolates in a dynamic in vitro large-intestinal model, closely resembling the human colon (31). Previous applications of this large-intestinal model have proven the model to be a useful tool to study the fate of (undigested) compounds and their effects on microbial metabolism and ecology (32). The use of this model is considered a powerful alternative for studies in experimental animals.

The production of AITC from sinigrin was investigated in the dynamic in vitro large-intestinal model, which was inoculated with faecal microflora (comparable with 100 g fresh material) at 37°C with 80 ml gastric juice with lipase (500 mg/l) and pepsin (150 mg/l) at pH 4.0 for 1 h and at pH 2.0 for another 2 h. Subsequently, the mixture was incubated with 40 ml bile solution (2%) and pancreatic juice (7%) at pH 6.5 for 1.5 h and continued at pH 7.0 for another 1.5 h. The digested Brussels sprout mixture was stored overnight at 4°C.

Microflora
A human microflora was prepared by pooling fresh faeces from 10 healthy volunteers, six women and four men, mean age 26 and 28 years, respectively. The mixture was cultured in a fermenter and stored at −80°C as described by Minekus et al. (31). The large-intestinal model was inoculated with 45 g inoculum mixed with 90 ml of an artificial ileal delivery medium, the composition of which is described elsewhere (33). In a control experiment the model was inoculated with an inactivated microflora, achieved by irradiating the pooled human microflora with gamma rays at 100 kGy for 10 h.

Individual human microflora were derived from the fresh faeces of four different healthy volunteers, three women and one man, mean age 31 (range 23–38). Fresh faeces (45 g) collected and transported in an anaerobic bag, was mixed with ~45 g of artificial ileal delivery medium in an anaerobic glove box and introduced into the model within 1 h. The model was filled up to 135 ml with the artificial ileal delivery medium.

The dynamic in vitro large-intestinal model
The in vitro large-intestinal model is a dynamic, computer-controlled system that mimics the physiological processes in the human large intestine, especially the proximal colon (schematically presented in Figure 2 and for more information see also http://www.voeding.tno.nl/PS.cfm?PNR=voc288e). This model consists of glass units with flexible walls inside. Water of 37°C is pumped into the space between the glass jackets and walls, which squeezes the walls at regular intervals. Its compression and relaxation ensure mixing and transport of the chyme by peristaltic movements. The peristaltic movements force the chyme to circulate through the tubular, loop-shaped system. The model is inoculated with human microflora and fed with an artificial ileal delivery medium at a rate of 3.5 ml per 45 min. The luminal environment is kept strictly anaerobic by flushing with nitrogen to allow the metabolic activity and growth of a high-density, complex microflora. The pH is continuously measured and adjusted to pH 5.8 by the computer-controlled addition of 2 M NaOH.

Dialysis fluid is pumped through semi-permeable hollow-fibre-membranes, placed inside the lumen of the model, to remove water and microbial fermentation products. The flow of the dialysis fluid through the hollow fibre membranes is 1 ml/min. The amount of chyme in the system is measured by a volume sensor and kept constant at 135 ml by the removal of dialysis fluid.

Experimental design
To set up the system for the metabolism of glucosinolates, a first series of experiments was performed with a pooled human microflora. After adaptation of the human microflora for 16 h, sinigrin was added to the system. Samples from the luminal and dialysis fluids were collected before the addition of sinigrin and subsequently at 3 h intervals until 18 h after the addition, and thereafter at 24 and 36 h. Degradation of sinigrin was investigated at an initial concentration of 1 mM (56 mg/135 ml), and 15 mM (810 mg/135 ml). Experiments were performed in duplicate. To determine the recovery and
stability of the substrate in the system a control experiment was performed in which the model was inoculated with an inactivated pooled human microflora (sinigrin final concentration: 12 mM).

Subsequently, various factors that might influence the microbial degradation of sinigrin were investigated, such as the variation in individual microflora and the effect of a cruciferous vegetable matrix. Four experiments were performed in which the microflora of four different individuals were tested, at an initial concentration of sinigrin of 12 mM.

In a fifth experiment the large-intestinal model, inoculated with the pooled human microflora, was fed with the myrosinase-inactivated Brussels sprouts. The digested Brussels sprouts (as described under Substrates) were mixed 1:1 with the artificial ileal delivery medium and added to the model during 14 h. Thereafter, the model was fed with the artificial ileal delivery medium without the Brussels sprouts.

Sample collection
At each time point of collection, samples of 8 ml were retrieved from the lumen: four ml were immediately placed into 10 ml glass vials, given a headspace volume equal to 6 ml. Vials were tightly closed with butyl-rubber stoppers, sealed with aluminium caps and stored below –18°C until AITC analysis. The remaining volume of the samples was centrifuged (10 min at 10 000 x g) and the supernatant was stored below –18°C until sinigrin analysis. The total volume of the collected dialysis fluid was measured and 8 ml were prepared and stored as described above pending AITC and sinigrin analysis.

Chemical analyses of sinigrin and AITC
All samples collected from the lumen and dialysis fluid were analysed for non-degraded sinigrin by HPLC as described by Bjerg and Sorensen (34), except in the experiment with Brussels sprouts, where non-degraded sinigrin was measured by HPLC analysis of desulfsinigrin (35). After thawing and mixing, 50 µl of the luminal samples were diluted with 50 µl acetonitrile and 400 µl phosphate buffer (50 mM, pH 7.0). Subsequently, samples were centrifuged and the supernatant was filtered. The dialysis samples were thawed, vortexed and filtered through a 0.2 µm filter (Waters Acrodisc). From the filtrate 100 µl were injected into the HPLC system. The retention time of sinigrin was ~8.2 min. For analysis of desulfsinigrin, samples were added to a column filled with an ion-exchange resin and sinigrin was enzymatically desulfated on the column. Of the samples eluted from the resin, 100 µl were injected into the HPLC system. The retention time of desulfsinigrin was ~5.1 min.

Solid phase microextraction (SPME) used in conjunction with gas chromatography (GC) has been shown to be an effective method for analysis of volatile organic compounds (36,37) including volatile isothiocyanates (29,38). Our analytical method involved exposing an SPME fibre to the headspace of the glass vials containing lumen or dialysis samples and desorbing the volatile AITC extracted into the fibre coating in the injection port of a gas chromatograph (29). The sample vials were thawed at 4°C and 40 µl of a 2.5 mM methyl isothiocyanate solution (100 nmol) was injected through the butyl-rubber stopper as an internal standard. The vials were thoroughly vortexed to obtain a homogenous solution and warmed to 35°C while being magnetically stirred. After 10 min stirring, a carbobox/polydimethylsiloxane-coated silica fibre (film thickness 75 µm, Supelco, Saint-Quentin-Fallavier, France) fitted into a protective stainless-steel needle was introduced through the stopper and exposed to the vial headspace for 10 min to allow adsorption of the volatile isothiocyanates. The adsorbed compounds were thermally desorbed in the splitless injection port of a gas chromatograph (Carlo Erba HRGC 5380, Milano, Italy) during 20 s at 250°C. Separation of compounds was achieved on a non-polar CP-Sil 8 CB capillary column (25 m x 0.53 mm i.d.; film thickness, 2 µm, Chrompack, Les Ulis, France) with nitrogen as the carrier gas (inlet pressure 70 kPa). The temperature gradient was programmed as follows: basal temperature was stable at 35°C for 2 min, then temperature increased to 50°C at 2°C/min, the rate increased to 5°C/min until temperature reached 70°C, then the temperature was rapidly increased at 20°C/min to 200°C and held for 1 min. Detection was by flame ionisation and detector temperature was set at 250°C. Data were collected and peaks integrated using a Shimadzu C-R6A integrator. Identification was based on the identity of retention times with those of the authentic standards, 3.98 and 10.57 for methyl isothiocyanate and AITC respectively. Calibration curves were plotted using equimolar amounts of 0 to 200 nmol of AITC and methyl isothiocyanate which were injected in glass vials of identical capacity to sample vials and containing 4 ml of sterile artificial ileal delivery medium. In these conditions, quantification limit was 2 nmol (0.5 µM) and precision of the method expressed as the relative standard deviation of three measurements ranged from 2.3% to 11.2%. The mean response factor obtained from the ratio of peak areas of methyl isothiocyanate and AITC amounts of AITC present in sample vials was quantified by comparing the peak area of AITC to the peak area of methyl isothiocyanate in the sample corrected by the response factor.

Identification of AITC was confirmed on one sample of each experiment using GC-mass spectrometry analysis run on a Fisons 800 GC coupled to a quadrupole Fisons QMS 8000 mass spectrometer equipped with an acquisition data system INCONS (Finngan). GC separation was achieved on a Q2 capillary column (25 m x 0.25 mm i.d.; film thickness, 0.25 µm, Quadrex) and the oven temperature was programmed as described above for the isothiocyanate analysis. Injection port and interface temperature were set at 250°C and source temperature was 200°C. Ionization was performed by electron impact (70 eV; emission current, 0.5 mA) and masses were scanned from 30 to 300 uma.

Bacteriological analysis
In the experiments with the pooled human microflora, samples from the lumen of the large intestinal model at the start and at the end of the experiment were used to evaluate the stability of the pooled human microflora with respect to the composition and the number of microorganisms. Serial 10-fold dilutions were made in peptone (1 g/l) physiological salt (8.5 g/l NaCl) solution. Anaerobic bacteria were enumerated on pre-reduced media in an anaerobic glove box (31). Total anaerobic bacteria, Bifidobacterium and Bacteroides were counted on Reinforced Clostridium Blood Agar-Chinabue, and (sulphite-reducing) Clostridium on Perfringens Agar Base with Bacillus-cereus selective supplement (Oxoid, Haarlem, The Netherlands). Plates were incubated at 37°C under anaerobic conditions for 1 to 4 days, depending on the species. Lactobacillus spp. were incubated anaerobically after plating aerobically on LAMVAB Agar (39). Enterobacteriaceae were incubated under aerobic conditions on Violet Red Bile Glucose Agar and Enterococcus spp. on Slanetz and Bartley Agar (Oxoid).

Results
Bioconversion of sinigrin by the pooled human microflora
The bacterial composition of the pooled human microflora was steady over the experiment, comparable with what was observed in previous studies in the large intestinal model, and remained within the physiological range (Table I) (31).

The results of the control experiment, in which the model was inoculated with the inactivated microflora, showed the absence of the formation of AITC in the lumen. The sinigrin concentration slowly decreased in the lumen, and had mostly disappeared after 36 h (0.3% sinigrin left) (Figure 3). A large part of it, ~74%, was recovered in the dialysis fluid. This part of the initial sinigrin dose was considered as the part available
Table I. Composition of the microflora in the lumen of the dynamic in vitro large-intestinal model, inoculated with a pooled human faecal microflora compared with the reference data in the human faeces (57,58)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Start of experiment ((t = 0 \text{ h}))</th>
<th>End of experiment ((t = 36 \text{ h}))</th>
<th>Composition in the human colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus spp.</td>
<td>(1.9 \times 10^7)</td>
<td>(2.4 \times 10^7)</td>
<td>(10^5-10^{10})</td>
</tr>
<tr>
<td>Enterobacteriaceae spp.</td>
<td>(1.4 \times 10^5)</td>
<td>(3.6 \times 10^7)</td>
<td>(10^5-10^{10})</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>(3.1 \times 10^7)</td>
<td>(2.7 \times 10^7)</td>
<td>(10^8-10^{10})</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>(4.6 \times 10^3)</td>
<td>(1.0 \times 10^9)</td>
<td>(10^8-10^{10})</td>
</tr>
<tr>
<td>Bacteroides spp.</td>
<td>(3.0 \times 10^9)</td>
<td>(6.0 \times 10^8)</td>
<td>(10^8-10^{10})</td>
</tr>
<tr>
<td>Clostridium spp. (sulfitreducing)</td>
<td>(1.3 \times 10^6)</td>
<td>(2.2 \times 10^6)</td>
<td>(10^6-10^{11})</td>
</tr>
</tbody>
</table>

Fig. 3. Amounts of sinigrin in the lumen (squares) of the dynamic in vitro large-intestinal model harbouring an inactivated pooled human microflora and the cumulative amount of sinigrin in the dialysis fluid (circles).

Fig. 4. Kinetics of sinigrin degradation (squares) and AITC formation (closed circles) in the lumen and the cumulative amount of AITC (open circles) in the dialysis fluid in the dynamic in vitro large-intestinal model harbouring a pooled human microflora after the addition of 15 mM sinigrin \((n = 2)\). Insert is a magnification of curve representing the amount of AITC in the lumen (closed circles).

for microbial degradation. The other part is probably bound to the membranes in the model or to compounds in the artificial ileal delivery medium. In subsequent experiments performed with active flora, the percentage of sinigrin converted into AITC was therefore corrected for these availability data.

The experiments with this pooled active human microflora and an initial concentration of 1 mM sinigrin showed that the amount of sinigrin totally disappeared from the lumen within 9 h (Figure 4). A part of it, 14 ± 3% of the initial dose, was recovered as such in the dialysis fluid (not shown).

AITC was quickly removed from the lumen via the dialysis membrane; therefore only small amounts of AITC were present in the lumen from 3 to 24 h, reaching a maximum concentration at 6 h. The mean overall production of AITC was 0.8 \(\mu\text{mol}\), which accounts for 1.0% of the degraded sinigrin (after correction for percentage of availability).

The results of the experiments with 15 mM sinigrin showed that the amount of sinigrin in the lumen decreased steadily to zero within 12 h after feeding (Figure 5). 20.3 ± 0.2% of the initial sinigrin was recovered in the dialysis fluid (not shown).

The kinetics of distribution of formed AITC in the lumen and the dialysis fluid were comparable, irrespective of the initial sinigrin concentration 1 or 15 mM. With the latter concentration, however, the quantity of AITC in the luminal and dialysis fluids increased more slowly and AITC production continued for a longer period; it was still present in the lumen after 24 h. Overall, compared with the addition of 1 mM sinigrin, AITC production was higher, namely 41.9 \(\mu\text{mol}\), which accounts for 4.0% of sinigrin degradation (after correction for percentage of availability).

Bioconversion of sinigrin by individual human microflora

In Figure 6A–D, the results of four experiments are presented in which the model was inoculated with the microflora of different human individuals. Sinigrin (initial concentration 12 mM) disappeared from the lumen of the model within 12 h after feeding in all experiments. On the average of the four different individuals, 21.9 ± 4.9% of the initial amount of sinigrin was recovered in the dialysis fluid (not shown). AITC was detected in the lumen at 3 h, reaching its maximum at 12 h, and was still present at 24 h. The cumulative amount of AITC recovered in the dialysis fluid increased from 3 h until 36 h to ~40 \(\mu\text{mol}\) (except for person A, where it was around 10 \(\mu\text{mol}\)). Not all the AITC produced in the lumen was recovered in the dialysis fluid, because the amount of AITC measured in the lumen was higher compared with the total cumulative amount of AITC collected in the dialysis fluid. The overall production of AITC varied between 82.5 \(\mu\text{mol}\) and 245.4 \(\mu\text{mol}\), which accounts for 9.6 to 30.4% of sinigrin degradation (19.0% ± 8.6%). This is much higher than the <5% of sinigrin conversion to AITC obtained with the pooled human microflora.

Bioconversion of sinigrin in Brussels sprouts by the pooled human microflora

As shown in Figure 7 the concentration of sinigrin increased in the lumen, during the time that the ileal delivery medium
Metabolism of sinigrin by human colonic microflora

Experiments performed with the dynamic in vitro large-intestinal model inoculated with a pooled human microflora indicated that the system closely simulates the human physiological conditions in the proximal colon; the composition and concentration of the microflora were within the physiological range.

Large differences with respect to the quantity of AITC present in the lumen were observed between the experiments with the pooled, cultured human microflora and with the individual, fresh microflora. The amounts of AITC remaining in the lumen were 12- to 40-fold higher with the individual microflora. In contrast, the amounts of AITC recovered in the dialysis fluids are approximately the same with both types of microbial inoculum. The overall production of AITC was remarkably higher with the individual human microflora compared with the pooled human microflora. This phenomenon is probably due to the fermentation, storage conditions and the mixing of the pooled human microflora, which might have resulted in the loss of bacteria capable of converting sinigrin into AITC or the pooled human microflora has degraded sinigrin into other metabolites than AITC. Alternatively, the pooled human microflora has converted AITC further into other, not measured, metabolites.

Elfoul et al. studied the conversion of sinigrin in gnotobiotic rats harboring Bacteroides thetaiotaomicron isolated from human faeces after an oral dose of 50 µmol sinigrin (29). They showed that ~50% of the sinigrin dose were actually available for microbial degradation in the large intestine of rats. Although the conversion rate of sinigrin into AITC could not be estimated in that study, the kinetics of sinigrin degradation and AITC production appear to be similar in the large-intestinal model inoculated with a complex pooled human microflora and in the rat caecum harbouring a single bacterial strain of human origin. However, large differences in the absolute amounts of AITC produced occur; this is probably due to the fact that a significant quantity of AITC was presumably absorbed through the rat intestinal mucosa and, therefore, was not quantified. Other explanations for the difference in the amount of AITC could be a flora effect (a complex ecosystem in the large-intestinal model versus a single strain in the rat caecum) or a pH effect (5.8 ± 0.2 in the large-intestinal model versus 6.5–7.0 in the rat caecum), factors that will probably result in the formation of other metabolites.

Whether a pooled or individual human microflora was used, AITC production did not account for the overall sinigrin that was degraded. Various mechanisms could explain the partial recovery of AITC. Firstly, the conjugation of AITC to, for example, glutathione (GSH) or cysteine. Rapid conjugation to GSH is unlikely in this model, because glutathione-S-transferases (GST) are missing. Spontaneous conjugation has been reported by Zhang et al. (40). However, reasonable extrapolation to the large-intestinal conditions, such as in humans or in the model is doubtful, because the reaction described in the paper of Zhang occurred under very precise experimental conditions, while the content of the large-intestinal model is a complex mixture at a pH and temperature, which is different compared with the conditions described in the paper by Zhang. Furthermore the content of the large-intestinal model is a complex mixture of microorganisms and metabolites under dynamic conditions as found in humans, which means release of AITC in gas phase and absorption of AITC through the membranes into the dialysis fluid. Although cysteine is present in the model cysteiny1-conjugation is not expected, because cysteinyl conjugation is very slowly. Moreover, Mennicke et al. (41) reported that mercapturic acids are unstable compounds that decompose at pH values higher than 5.

Secondly, the microflora might have produced other compounds directly formed from sinigrin, such as allylthiocyanate and allylcyanide, or arisen from a further metabolic conversion of AITC. This hypothesis is supported by other microbiological studies showing that bacteria can transform glucotropaeolin
into benzyl isothiocyanate (BITC), which can be further hydrolysed spontaneously into benzylamine (42). Recently, we have shown that AITC can be hydrolysed spontaneously to allylamine (the same phenomenon as with BITC) (43). The rate of transformation and the nature of metabolites of the glucosinolates in that study (43) were not modified by the presence of glucose, indicating that a simple source of energy is not an important factor in the degradation of isothiocyanates (ITCs). This hydrolyse reaction occurred in simple experimental conditions (phosphate buffer, 37°C, pH 7.0) and cannot be simply extrapolated. Whether these amines will be produced in the dynamic in vitro large-intestinal model and in vivo from dietary glucosinolates remains to be established. However, investigating this way of degradation of ITCs should receive as much attention as the conjugation; actually the reactions may compete with one another.

Palop et al. (44) reported that AITC accounted only for 55% of degraded sinigrin by Lactobacillus agilis strain R16 and could prove with complementary experiments that AITC degraded spontaneously in another, yet unknown, compound. The same group (45) has found that incubation of sinigrin with Fusarium cells leads to the degradation of the substrate without detection of AITC. They used only one bacterial or fungal strain, whereas in the large-intestinal model a complex mixture of bacteria was present. A combination of spontaneous degradation of AITC and of a bacteria-mediated degradation could have occurred, and it is therefore not surprising that, compared with the above mentioned studies, less AITC was found in our investigation.

In all experiments AITC was still present in the lumen, after the disappearance of sinigrin, suggesting that a fraction of the sinigrin was taken up or adsorbed extracellularly to the membranes of the bacteria. The mechanisms by which glucosinolates and ITCs interact with microorganisms are not well known. Delaquis and Mazza (46) reported that ITCs have different abilities to interact with bacteria, depending on their chemical structure and on the bacterial species. As far as we know, no data have been published on the uptake and intracellular accumulation of ITCs by bacterial cells, which will probably be dependent on their membrane structure. Zhang (47,48) has reported rapid and very high levels of intracellular accumulation of ITCs in cultured mammalian cells. However, it is difficult to extrapolate these data obtained in hepatoma cells (which may overexpress enzymes like GST and have developed strategies to accumulate substrates for this enzyme) to bacteria, because the physiology and nutritional requirements of eucaryotic cells, especially in vitro cultures of carcinogenic cells, and of prokaryotic cells are fundamentally different.

The addition of the food into the model and the release of sinigrin from the food matrix is a continuous process leading to a steady formation of low amounts of AITC. Conjugation of AITC, interaction of AITC with bacteria, spontaneous degradation or further degradation into other metabolites could be explanations for the fact that no AITC was detected in the lumen after the addition of Brussels sprouts as a source of sinigrin.

It is very important to investigate interactions between bacteria and isothiocyanates at a cellular or molecular level. These interactions, further conversions to other compounds such as allylamine and/or spontaneous conjugation probably explain why the conversion rate of sinigrin into AITC is not equimolar. However, specific investigations, possibly under other experimental conditions, are required for that purpose.

In general, the partial conversion of sinigrin into AITC in our large-intestinal model does reflect a real biological process, because various researchers using different methods obtain conversion rates in the same order of magnitude as we found. Getahun and Chung reported that upon incubation of cooked watercress juice with human faeces, ~5.7% of glucosinolates was hydrolysed to ITCs (49). The same authors found that the total urinary excretion of ITCs conjugates following ingestion of cooked watercress juice by humans ranged from 1.2 to 7.3% of the total dose of glucosinolates ingested (50). Different experiments in humans following the ingestion of cruciferous vegetables which myrosinase had been inactivated by cooking, have resulted in 10–24% recovery of excretion of dithiocarbamates in urine (51–53). Furthermore, in the study of Rouzaud (54) it was pointed out that there were great variations in the recovery of allyl mercapturic acid following ingestion of cooked vegetables compared to raw vegetables. This suggests that different colonic flora have different abilities to hydrolyse glucosinolates to ITCs. The data derived from our study also suggest this idea. If we consider that 50–80% of ITCs present in the digestive tract are recovered as mercapturic acids in urine (55,56) the conversion rates of glucosinolates to ITCs by the colonic microflora ranges from ~10–40% in the studies discussed above, which is in accordance with our results.

In conclusion, the experiments presented in this paper strongly suggest that sinigrin is converted into AITC in the human large intestine. They also support the use of the dynamic in vitro large-intestinal model as an appropriate and reliable tool to investigate bacterial glucosinolate metabolism occurring in the digestive tract. This includes factors that modulate the process of glucosinolate metabolism in relation to the composition and activity of the colon microflora. Measurements of glucosinolates and their metabolites in the lumen and in the dialysis fluid (which mimics the absorption compartment) of the large-intestinal model allow for the possibility to determine the kinetics and mass balance more easily than with animal models. The large-intestinal model can therefore also be useful in reducing reliance upon laboratory animals.

Future experiments will focus on the influence of environmental variables such as dietary fibres, non-digestible oligosaccharides, and minerals as modulating agents for microbial degradation of sinigrin or other glucosinolates, e.g. glucotropaeolin. Furthermore, the use of radioactive sinigrin or analysis of metabolites with GC-MS or HPLC-MS-MS can help to identify other, unknown, metabolites. These unknown metabolites might be of high importance with respect to anticarcinogenic properties of glucosinolates. The large-intestinal model in combination with genotoxicity assays can also be used for mechanistic studies to investigate the antimutagenic potential of AITC (or other glucosinolate metabolites) of bacterial origin in the colon.

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