

Brussels sprouts, inulin and fermented milk alter the faecal microbiota of human microbiota-associated rats as shown by PCR-temporal temperature gradient gel electrophoresis using universal, *Lactobacillus* and *Bifidobacterium* 16S rRNA gene primers

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(Received 16 May 2004 – Revised 9 November 2004 – Accepted 11 November 2004)

We investigated the effect of Brussels sprouts, inulin and a fermented milk on the faecal microbiota diversity of human microbiota-associated (HMA) rats by PCR-temporal temperature gradient gel electrophoresis (PCR-TTGE) using universal and group-specific 16S rRNA gene primers. The HMA rats were submitted to a control diet for 10 d (initial time), then switched to the experimental diets for 4 weeks (final time). Using universal primers, the mean degree of similarity between all faecal samples at initial time was 80.8%. In the group consuming the control diet throughout the experiment, the mean degree of similarity between the PCR-TTGE profiles at initial v. final time was 76.8%, reflecting a spontaneous temporal variation. The mean degree of similarity between control and experimental groups at final time was lower, 72.4%, 74.4% and 75.6% for inulin, Brussels sprouts and fermented milk, respectively, indicating a dietary effect on the predominant populations. Using specific primers, bifidobacteria could be detected only in those rats that had consumed inulin, showing a specific increasing effect of this dietary compound. The *Lactobacillus* population was very heterogeneous at initial time but tended to homogenize within each dietary group. At final time, caecal contents were collected for analysis of SCFA and β -glucuronidase activity. Inulin and Brussels sprouts increased the butyrate and acetate proportion, respectively, while the fermented milk did not modify the caecal biochemistry. This experiment shows for the first time that cruciferous vegetables are able to alter the diversity and the metabolic activities of the digestive microbiota in HMA rats.

Human intestinal microbiota: Prebiotic: Probiotic: *Brassica* vegetables: TTGE

Epidemiological studies implicate heterocyclic amines (HA), mutagenic/carcinogenic compounds commonly found in fried meats and fish, as risk factors in the aetiology of human colon cancer (Felton *et al.* 2002). For several years, strong efforts have been made to identify dietary constituents that could protect against the genotoxic and carcinogenic effects of HA. Among them, oligosaccharides, fermented milks containing lactic acid bacteria and *Brassica* vegetables have shown chemoprotection in laboratory rodents (Reddy & Rivenson, 1993; Schwab *et al.* 2000; Kassie *et al.* 2002, 2003; Tavan *et al.* 2002; Humblot *et al.* 2004) and different mechanisms have been proposed to explain their protective effect. Chemoprotection by *Brassica* vegetables such as Brussels sprouts, red cabbage and garden cress is often paralleled with a significant increase of hepatic UDP-glucuronosyl transferase (EC 2.4.7.17) activity (Kassie *et al.* 2002, 2003). Since conjugation with glucuronic acid is the main detoxification pathway of HA, this increase could account for the protective effect of *Brassica* vegetables. On the other hand, it has been hypothesized that hydrolysis of glucuronidated HA by

bacterial β -glucuronidase (EC 3.2.1.31) in the colonic lumen leads to the release of reactive metabolites that can cause DNA damage in the colonic mucosa or in the liver, which they can reach via the enterohepatic circulation. Therefore, the decreasing effect of fructose polymers such as inulin on the β -glucuronidase activity of intestinal microbiota may contribute to their protective effect against the genotoxicity of HA (Rowland *et al.* 1998; Humblot *et al.* 2004). With regard to fermented milks, their protective action cannot be related to any modification of mammalian or bacterial enzymes involved in the activation and detoxification of HA (Humblot *et al.* 2004); hence, direct binding of these compounds by lactic acid bacteria, which has been described in several *in vitro* studies, is still put forward as the most likely explanation (Knasmüller *et al.* 2001).

Nevertheless, data on the impact of the intestinal microbiota on the genotoxicity of HA *in vivo* are still scarce. Kassie *et al.* (2001) have shown that the extent of HA-induced DNA damage in colonocytes and hepatocytes, measured using the comet assay, was 2–3-fold higher in human microbiota-associated rats (HMA

Abbreviations: DGGE, denaturing gradient gel electrophoresis; HA, heterocyclic amine; HMA rat, human microbiota-associated rat; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MRS, De Man, Rogosa and Sharpe; TGGE, temperature gradient gel electrophoresis; TTGE, temporal temperature gradient gel electrophoresis.

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rats), and 4–5-fold higher in conventional rats, than in germ-free companions. These findings highlight the crucial impact of intestinal microbiota on the genotoxicity of HA and suggest the hypothesis that fermented milks, but also other dietary constituents, could be protective through an alteration of the intestinal microbial ecology. In a recent study (Humblot *et al.* 2004), we have shown that Brussels sprouts, inulin and a fermented milk were capable of reducing up to 70% the extent of DNA damage induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the colonic mucosa and in the liver of HMA rats. In this context, the aim of the present study was to investigate the influence of these dietary constituents on the phylogenetic diversity of the faecal microbiota of HMA rats.

Our present knowledge of the intestinal microbiota is largely based on cultivation studies but, according to estimates, up to 85% of the entire microbial population in the human intestine might be uncultured (Suau *et al.* 1999). In order to overcome the limitations associated with culturing techniques, molecular biological methods are increasingly being applied to study the intestinal microbial ecology (Vaughan *et al.* 2000). One of the most widely used approaches in ecological studies has been the use of 16S rRNA and its encoding genes as target molecules (Amann *et al.* 1995). Specific PCR primers and probes can be designed based on the variable regions of this molecule to detect predominant bacterial populations as well as specific genera (Zoetendal *et al.* 1998; Satokari *et al.* 2001; Walter *et al.* 2001). The amplified fragments of 16S rRNA are then separated in a sequence-specific manner in temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.* 1993; Muyzer & Smalla, 1998). These techniques have already been successfully applied to monitor bacterial populations in human faecal samples (Zoetendal *et al.* 1998; Satokari *et al.* 2001; Walter *et al.* 2001) and in the faeces of gnotobiotic rats harbouring human intestinal microbiota (Gérard *et al.* 2004).

In the experiment reported here, we applied PCR-temporal temperature gradient gel electrophoresis (TTGE) analysis to monitor the predominant population and specific subpopulations of the faecal microbiota of HMA rats, before and after consumption of Brussels sprouts, fermented milk and inulin. For this purpose, we used a combination of universal and group-specific (targeting the genus *Bifidobacterium* and the *Lactobacillus* group) 16S rRNA gene primers. In parallel, intestinal microbiota-associated metabolic biomarkers of colon cancer, namely β -glucuronidase and SCFA, were measured.

Material and methods

Chemicals

Polypeptone and papaic digest of soyabean meal were purchased from AES Laboratoire (Combourg, France), yeast extract and De Man, Rogosa and Sharpe (MRS) culture medium from Difco (Le Pont de Claix, France), and sodium glycerophosphate and ascorbic acid from VWR (Fontenay-sous-Bois, France). The agarose and ethidium bromide came from Euromedex (Mundolsheim, France), the Tris(hydroxymethyl)aminomethane-borate-ethylenediaminetetraacetic acid from Gibco (Invitrogen, Cergy-Pontoise, France), the acrylamide from Eurobio (Les Ulis, France), the extract of meat dry and the urea from Merck (Paris, France). Tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetic acid was purchased from Bio-Rad (Marnes-la-Coquette, France) and SYBR

Green I Nucleic Acid Gel Stain was from Roche Diagnostics (Mannheim, Germany). All other chemicals came from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Animals

Forty-eight germ-free male Fischer 344 rats, aged 3 months at the start of the experiment, were provided by the breeding facilities of the Unit on Ecology and Physiology of the Digestive Tract of INRA (Jouy-en-Josas, France). Throughout the study, animals were housed three per cage in flexible-film isolators (La Calhène, Vélizy, France) and were given free access to their diet and to sterilized tap water. The room harbouring the isolators was maintained at constant temperature and humidity ($21 \pm 1^\circ\text{C}$, $50 \pm 5\%$) with a 12 h light/dark cycle.

Diets

The diets were semi-synthetic designed to simulate a human-type diet. Therefore, lipids and proteins of both animal and vegetable origins, as well as saccharose and cooked starch, were included. The control diet consisted of 28.985% (w/w) maize starch, 29% mashed potato, 5% saccharose, 5% casein, 12% soya isolate, 3% maize oil, 3% lard, 0.015% cholesterol, 6% cellulose and 8% mineral and vitamin additives (Lhoste *et al.* 2003). Analytical compounds of DM were crude proteins 18%, crude fat 8%, ash 6%, carbohydrates 68% (energy: 19.33 MJ/kg DM; Eurofins Scientific Analytics, Nantes, France). In the inulin diet, saccharose and 5% maize starch were replaced by 10% Raftilose[®] Synergy 1 (Orafti, Tienen, Belgium): 100 g Raftilose[®] Synergy 1 provided 97 g carbohydrates (energy 693 kJ), among which 89 g were inulin; protein and fat were absent and vitamin and mineral content was negligible. In the Brussels sprouts diet, 4% maize starch, 4% mashed potato and 2% soya isolate were replaced by 10% freeze-dried Brussels sprouts (*Brassica oleracea* variety Cyrus; Novartis Seeds, Enkhuizen, the Netherlands) containing 24 μmol glucosinolates/g DM (Rouzaud *et al.* 2003). Brussels sprouts are usually cooked before consumption; this process denaturates plant myrosinase (EC 3.2.1.147) so that glucosinolates reach the colon, where they are hydrolysed by the intestinal microbiota into bioactive isothiocyanates (Johnson, 2002). Therefore, Brussels sprouts were treated to denature myrosinase before inclusion in the diet as described by Krul *et al.* (2002) and Rouzaud *et al.* (2003). The fermented milk diet was a control diet supplemented with the fermented milk Actimel[®] purchased from a local market (1 ml fermented milk for 1 g dietary powder). Actimel[®] contains three species of lactic acid bacteria, namely *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Streptococcus thermophilus*. Five batches of Actimel[®] were used throughout the experiment; each batch was numbered for *L. bulgaricus* on acid MRS (55 g/l, pH 5.4), for *L. casei* on MRS (55 g/l, pH 6.5) and for *S. thermophilus* on M17 saccharose (polypeptone 5 g/l, papaic digest of soyabean meal 5 g/l, extract of meat dry 4 g/l, yeast extract 2.5 g/l, saccharose 5 g/l, sodium glycerophosphate 19 g/l, ascorbic acid 0.5 g/l and magnesium sulphate 0.25 g/l). The results are 2.2 (SEM 1.0) $\times 10^8$, 5.0 (SEM 0.2) $\times 10^7$ and 9.0 (SEM 0.4) $\times 10^7$, respectively for *L. bulgaricus*, *L. casei* and *S. thermophilus*. Actimel[®] typically contains (per 100 ml): 2.8 g proteins, 1.6 g fat and

14.3 g carbohydrates (energy 350 kJ); saccharose, lactose and glucose accounted for 68, 21 and 2% of carbohydrates, respectively.

All dietary powders were manufactured by U.A.R. (Villemoisson-sur-Orge, France), packed in double-vacuum bags and sterilized by γ -irradiation at 45 kGy (U.A.R.). Diets were prepared freshly every 2 d in the isolators by mixing thoroughly 50 g dietary powder with 100 ml water (control, inulin and Brussels sprouts diets) or with 50 ml fermented milk and 50 ml water (fermented milk diet).

Experimental design

All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals.

The germ-free rats were randomly allocated to four dietary groups (control, Brussels sprouts, fermented milk and inulin groups) housed in four distinct isolators (twelve animals per group, three animals per cage). On day 1, all rats were inoculated orally with the whole faecal microbiota of a healthy adult man as described previously (Roland *et al.* 1996) and offered the control diet. On day 10, one faecal pellet was freshly collected from each HMA rat; pellets were pooled by cage and stored at -80°C until analysis of the microbiota by PCR-TTGE (initial time). HMA rats of the Brussels sprouts, fermented milk and inulin groups were thereafter switched to the experimental diets. Four weeks later, faecal pellets were again collected, pooled by cage and stored at -80°C until PCR-TTGE analysis (final time). HMA rats were then killed by CO_2 inhalation, the caecal pH was measured, and caecal contents were weighed and distributed into several vials stored at -80°C for SCFA and β -glucuronidase analyses.

DNA extraction, PCR and temporal temperature gradient gel electrophoresis analysis of the faecal samples and the fermented milk

Total DNA was extracted from the faecal samples and from the fermented milk using an amalgamator with glass beads (Mini-beadbeater-8, Biospec products, VWR) as described by Godon *et al.* (1997). The V6 to V8 regions of the 16S rRNA genes were amplified by PCR using the *HotStar Taq* Master Mix Kit (Qiagen, Courtabœuf, France) in a PCT 100 thermocycler (MJ Research, USA) (Gérard *et al.* 2004). The universal primers U968-GC and L1401 originally described by Zoetendal *et al.* (1998) were used to amplify non-selectively the bacteria; the primers Lac 352 and Lac 679 initially designed by Walter *et al.* (2001) were used to amplify selectively the lactobacilli; in order to examine the bifidobacteria we used the primers Bif 164 and Bif 662 initially designed by Satokari *et al.* (2001). TTGE analysis of the amplicons was performed using the Dcode™ Universal Mutation Detection System (Bio-Rad, Paris, France) as described by Seksik *et al.* (2003). Electrophoresis was run at a fixed voltage of 76 V for 16 h with an initial temperature of 66°C for universal and *Bifidobacterium* amplicons and of 64°C for *Lactobacillus* amplicons, and a ramp rate of $0.2^{\circ}\text{C}/\text{h}$. For better resolution, the voltage was fixed at 20 V for 15 min at the beginning of electrophoresis (Zoetendal *et al.* 1998; Satokari *et al.* 2001; Walter *et al.* 2001; Seksik *et al.* 2003). After the completion of the electrophoresis, gels were stained in the dark by immersion in a solution of SYBR Green I Nucleic Acid Gel Stain and read on a Storm system (Molecular Dynamics).

Analysis of SCFA and β -glucuronidase activity in the caecal contents

All analyses were performed in duplicate. β -Glucuronidase activity was measured spectrophotometrically ($\lambda = 400\text{ nm}$) by the rate of release of *p*-nitrophenol from the *p*-nitrophenylglucuronide as described by Andrieux *et al.* (1998). Enzymatic activity was expressed as μmol of product formed per min and per g of wet caecal content. SCFA were analysed after water extraction of acidified samples, 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 100°C and detector temperature 280°C . 2-Ethylbutyrate was used as the internal standard (Rabot *et al.* 2000).

Calculations and statistical analyses

Inter-individual variability and the effects of time and diet on the faecal microbiota diversity were investigated. To determine the inter-individual variability, TTGE profiles of all faecal samples ($n = 16$) were compared at initial time; the effect of time was consequently assessed by comparing the TTGE profiles of control samples ($n = 4$) at initial *v.* final time; eventually, the dietary effect was examined by comparing at final time the control TTGE profiles ($n = 4$) with those of rats consuming the experimental diets. Comparisons of TTGE profiles were performed using the GelCompar II™ software (version 2.0, Applied Maths, Kortrijk, Belgium). Analysis included 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 the samples are identical.

The effect of diet on caecal SCFA and β -glucuronidase activity was analysed using a one-way ANOVA. When ANOVA indicated significant differences, groups receiving the inulin, Brussels sprouts and fermented milk diets were each compared with the group fed on the control diet using the Dunnett's test. Statistical significance was set at $P < 0.05$. Calculations were performed using the Statview® software (version 5.0, SAS Institute, Cary, NC, USA). All data were expressed as mean values and standard errors of the means.

Results

Effect of the diets on the PCR-temporal temperature gradient gel electrophoresis profiles of human microbiota-associated rats' faecal microbiota using universal primers

PCR-TTGE analysis of the faecal microbiota using universal primers results in complex profiles that represent the predominant microbial communities, and in which each band represents at least a single bacterial 16S rDNA. Fig. 1 shows an example of the TTGE profiles obtained at the initial time of the experiment, i.e. 10 d after all initially germ-free rats had been inoculated with the human faecal microbiota and fed on the control diet. At this time, the mean Pearson correlation coefficient between all samples was 80.8 (SEM 1.0)%; this figure represents the inter-individual variability at initial time. To monitor the spontaneous evolution of the predominant species-pattern over time, we

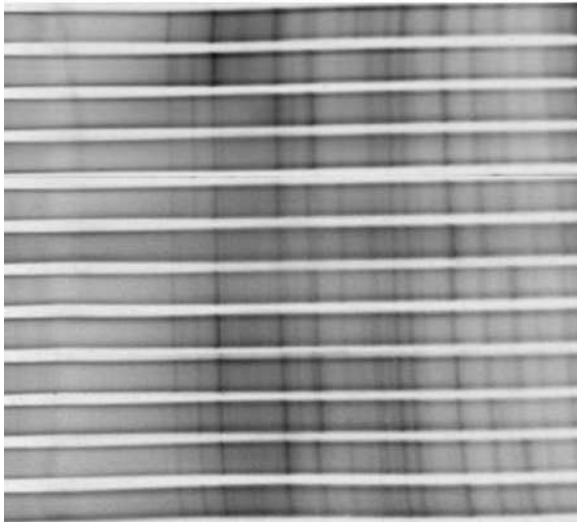


Fig. 1. Example of faecal PCR-temperature gradient gel electrophoresis profiles obtained with universal primers in human microbiota-associated rats. Samples were collected 10 d after initially germ-free rats had been inoculated with the human faecal microbiota and fed continuously on the control diet (initial time). For details of diets and procedures, see p. 678.

compared the TTGE profiles of the control group at initial time with those of the same group at final time, i.e. after 4 weeks consumption of the control diet. The mean Pearson correlation coefficient was 76.8 (SEM 2.5)% (Fig. 2). At final time, the mean Pearson correlation coefficients between the control group and each of the experimental groups were 72.4 (SEM 1.0)%, 74.4 (SEM 1.0)% and 75.6 (SEM 2.0)%, respectively, for inulin, Brussels sprouts and fermented milk (Fig. 2). These data indicate that the faecal microbiota diversity was more altered after consumption of the experimental diets than after continuous feeding on the control diet. In conclusion, consumption of experimental diets for 4 weeks modified the pattern of the predominant populations in the faeces of the HMA rats.

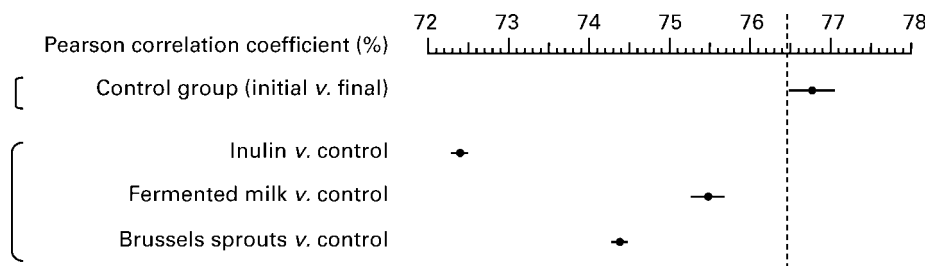


Fig. 2. Pearson correlation coefficients between PCR-temperature gradient gel electrophoresis profiles obtained with universal primers (means (bullets) with their standard errors). At initial time, the mean Pearson correlation coefficient between all samples was 80.8 (SEM 1.0)% (inter-individual variability). The control group was compared at initial v. final time to monitor the spontaneous evolution of the pattern with time (effect of time). Groups receiving the inulin, fermented milk and Brussels sprouts diets were compared with the control group at the final time (effect of the experimental diets). For details of diets and procedures, see p. 678.

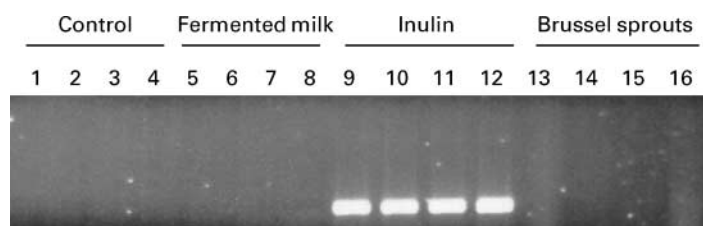


Fig. 3. PCR products of amplification using *Bifidobacterium* specific primers. Lanes 1–4, 5–8, 9–12 and 13–16 are PCR products from human microbiota-associated rats fed on the control, the fermented milk, the inulin and the Brussels sprouts diet, respectively (final time). For details of diets and procedures, see p. 678.

Effect of the diets on the PCR-temporal temperature gradient gel electrophoresis profiles of human microbiota-associated rats' faecal microbiota using Bifidobacterium specific primers

No PCR amplicon of *Bifidobacterium* 16S rDNA could be obtained at the initial time, regardless of dietary groups (data not shown), and at the final time in the groups consuming the control, Brussels sprouts and fermented milk diets (Fig. 3). On the contrary, faecal samples collected at the final time from rats receiving the inulin-diet contained *Bifidobacterium* species at a predominant level, as shown by the PCR products obtained with *Bifidobacterium* specific primers (Fig. 3). TTGE separation of these PCR products led to a similar pattern in all samples with one band, representing at least one species of *Bifidobacterium*, being particularly stimulated by the inulin consumption (Fig. 4).

Effect of the diets on the PCR-temporal temperature gradient gel electrophoresis profiles of human microbiota-associated rats' faecal microbiota using Lactobacillus specific primers

Fig. 5 shows for each dietary group the TTGE profiles obtained with *Lactobacillus* specific primers at initial v. final time. At the start of the experiment, all faecal samples exhibited various one to three band-patterns, apart from one sample of the control group that presented no band at all. Four weeks later, all control samples shared a common two to three band-pattern that differed from those observed at the initial time. In the inulin group, a new band, common to all samples, appeared after 4 weeks of consumption of the experimental diet, while the initial bands had almost totally disappeared. Similarly, an extensive reorganization of the *Lactobacillus* population occurred in the Brussels sprouts group as the initial one to three band-pattern totally disappeared, while two new bands appeared. In the fermented milk group as well, a new band appeared in all samples whereas several initial bands were not visible anymore.

The fermented milk profile is composed of two bands corresponding to the species *L. bulgaricus* and *L. casei*. A band



Fig. 4. PCR-temperature gradient gel electrophoresis profiles obtained with *Bifidobacterium* primers in human microbiota-associated rats fed on the inulin diet for 4 weeks (final time). For details of diets and procedures, see p. 678.

co-migrating with *L. bulgaricus* was present at initial time in several faecal samples; at final time, this band persisted only in the faeces of HMA rats fed on the fermented milk diet. Interestingly, no band co-migrating with *L. casei* could ever be seen, whatever the sample and the time of the experiment.

On the whole, the *Lactobacillus* population was remarkably heterogeneous at the beginning of the experiment and a trend towards homogenization was observed with time, with specific features within each dietary group.

Effect of the diets on caecal weight, pH, β-glucuronidase activity and fermentative metabolites

None of the diets modified the caecal pH (Table 1) and the caecal weight increased only after inulin consumption (136% of the control value; $P < 0.05$; Table 1). The β-glucuronidase activity and the total SCFA concentration were similar regardless of the diet while the SCFA profile differed significantly in the groups fed on the inulin and the Brussels sprouts diets compared with the control group. Inulin increased the proportion of butyrate (179% of the control value; $P < 0.05$; Table 1) at the expense of acetate and branched-chain fatty acids (respectively 89% and 44% of the control values; $P < 0.05$; Table 1). Brussels sprouts increased the proportion of acetate (108% of the control value; $P < 0.05$; Table 1) at the expense of branched-chain fatty acids (41% of the control value; $P < 0.05$; Table 1).

Discussion

HMA rats are now a well-validated model for experimental studies aimed at evaluating the effects of functional foods in gastrointestinal physiology or in carcinogenesis (Djouzi *et al.* 1997; Kleessen

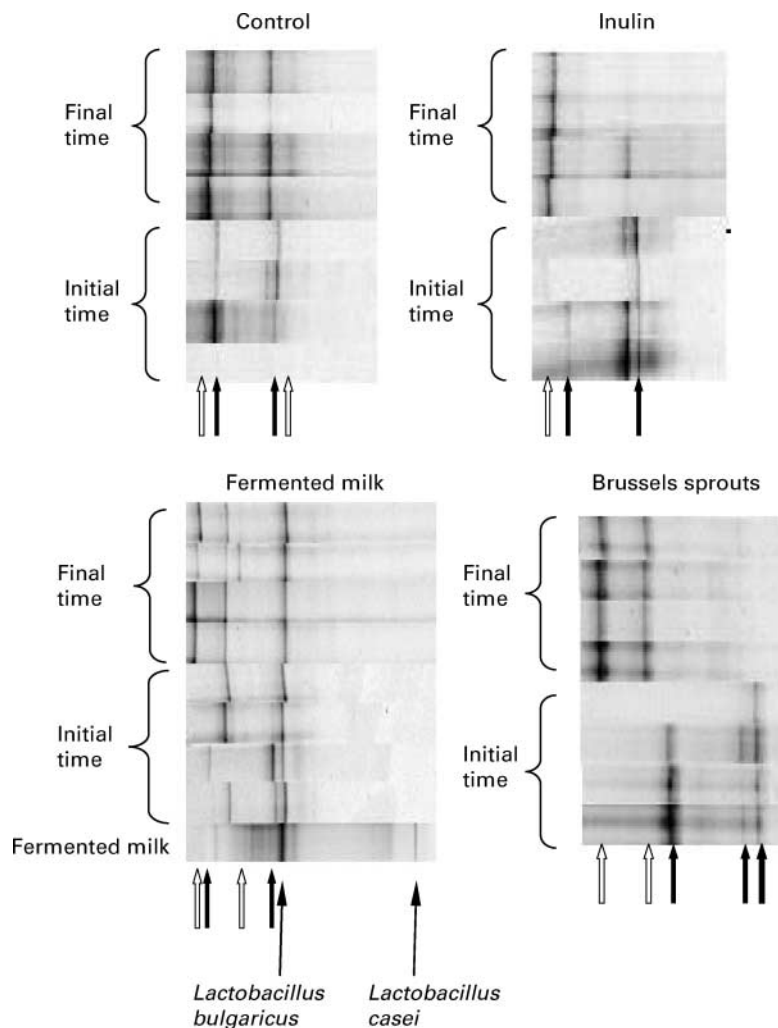


Fig. 5. PCR-temperature gradient gel electrophoresis profiles obtained with *Lactobacillus* primers from the fermented milk and from human microbiota-associated rats at initial time (consumption of the control diet for 10 d) and at final time (consumption of the experimental diets for 4 weeks). ↑ the bands present at the initial time but absent at the final time; † the new bands detected at the final time. For details of diets and procedures, see p. 678.

Table 1. Effect of the Brussels sprouts, fermented milk and inulin diets on the weight and pH of the caecal content, and on the caecal β -glucuronidase activity and SCFA concentration and profile in human microbiota-associated rats†

(Mean values with their standard errors of the means)

	Control diet		Brussels sprouts diet		Fermented milk diet		Inulin diet	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Caecal weight (g)	5.36	0.25	5.69	0.25	5.90	0.31	7.28*	0.62
Caecal pH	6.77	0.14	6.64	0.10	6.58	0.16	6.51	0.11
β -Glucuronidase ($\mu\text{mol}/\text{min}/\text{g}$)‡	0.31	0.04	0.28	0.04	0.27	0.04	0.34	0.08
SCFA ($\mu\text{mol}/\text{g}$)‡	70.17	10.87	87.51	4.50	66.28	7.44	87.03	7.19
SCFA profile ($\mu\text{mol}/100 \mu\text{mol}$)								
Acetate	67	1	72*	1	67	1	60*	1
Propionate	15	1	13	1	15	2	11	1
Butyrate	15	1	13	1	15	2	27*	1
Valerate+caproate	1	0	1	0	1	0	1	0
BCFA	2	0	1*	0	2	0	1*	0

BCFA, branched-chain fatty acids.

* Mean values were significantly different from the control group (Dunnett's test, * $P < 0.05$).

† For details of diets and procedures, see Material and methods.

‡ Results are expressed as g caecal wet weight. All analyses were made in duplicate.

et al. 2001; Humblot *et al.* 2004; Imaoka *et al.* 2004). In particular, this model facilitates the investigations on the interactions between food compounds and the human gut microbiota, since it is easier to control and alter animal diets than those of human subjects. Furthermore, it enables there to be several repetitions of the same microbiota for one experimental diet, thus avoiding the fluctuations observed by several authors in human experiments (Tannock, 2001; Zoetendal *et al.* 2001). A major proportion of the gut microbiota is currently uncultivable but the use of molecular techniques allows this difficulty to be overcome (Tannock, 2001). In this respect, this study is, to our knowledge, the first one that exploits PCR-TTGE analysis to examine the effect of functional foods on the faecal microbial population of HMA rats, using universal and group-specific primers.

Among the three foodstuffs that were studied here, i.e. inulin, Brussels sprouts and a fermented milk, inulin was undoubtedly the most effective with regard to modifications of the predominant faecal bacterial populations. The mean degree of similarity between inulin and control samples was 72.4%, a value noticeably distant from 76.8%, i.e. the mean coefficient between control samples at initial *v.* final time that reflects the spontaneous evolution of the predominant microbiota-profile with time. The marked bifidogenic effect of inulin probably accounts, at least partly, for this reshaping of the overall PCR-TTGE profile. Fructose polymers with short chains (oligofructose) or long chains (inulin) have been extensively investigated for their selective stimulation of *Bifidobacterium* (Roberfroid, 2001; Cummings & MacFarlane, 2002). Campbell *et al.* (1997) showed by enumeration on selective media that oligofructose increases the *Bifidobacterium* in the caecum of conventional rats; more recently, Kleessen *et al.* (2001) have used fluorescent *in situ* hybridization to demonstrate that inulin and oligofructose are both able to increase the *Bifidobacterium* population in the digestive tract of HMA rats. Using PCR-TTGE, we confirm this increase and we show that several bands accounting for different *Bifidobacterium* species become predominant following 4 weeks of inulin consumption, one of them being particularly stimulated. Bifidobacteria have been studied by several authors for their ability to detoxify HA (Knasmüller *et al.* 2001). Most of the studies take place *in vitro*, and the most important mechanism appears to be direct binding of HA to the wall of the bacterial cells (Zhang & Ohta, 1993;

Orrhage *et al.* 1994; Lankaputhra & Shah, 1998). Recently, Tavan *et al.* (2002) have reduced by 96% the frequency of HA-induced colonic aberrant crypt foci by feeding rats with a fermented milk containing the species *Bifidobacterium animalis* (Tavan *et al.* 2002); similarly, Zsivkovits *et al.* (2003) have obtained a reduction of HA-induced DNA damage in the colon of rats after gavage with *Bifidobacterium longum* (Zsivkovits *et al.* 2003). Because of the heterogeneousness of the *Lactobacillus* group at the start of the experiment and its temporal variation in the control group, it is difficult to conclude whether the shifts observed in the inulin group arise from a specific dietary effect or from a spontaneous temporal instability. These observations are consistent with those of Vanhoutte *et al.* (2004), who have lately investigated the temporal stability of different populations of the human faecal microbiota using DGGE; they concluded that the *Lactobacillus* group tends to show strong temporal variations whereas other autochthonous groups, e.g. the genus *Bifidobacterium*, do not undergo major population shifts in time. We confirm otherwise the butyrogenic effect of inulin (Andrieux *et al.* 1991; Kleessen *et al.* 2001). Since butyrate has been consistently found to cause apoptosis of cancerous cells (Avivi-Green *et al.* 2002), this inulin property supports the current insights on its chemopreventive potential against the development of colon cancer. We did not find any modification of the β -glucuronidase activity. Actually it must be emphasized that the modulating effect of inulin on this enzyme activity, which is considered to be a luminal biomarker of colon cancer, is not consistently reported throughout the literature (Djouzi & Andrieux, 1997; Rowland *et al.* 1998; Hughes & Rowland, 2001; Humblot *et al.* 2004).

Similar to inulin, Brussels sprouts modified the predominant populations of the faecal microbiota of HMA rats. Indeed, the mean degree of similarity between the PCR-TTGE profiles of control rats and those of companions fed for 4 weeks on the Brussels sprouts diet was 74.4%, a figure below the 76.8% threshold value. No increasing effect could be observed on the *Bifidobacterium* population and, once again, because of a temporal variation background, the shifts of the *Lactobacillus* group could not be confidently ascribed to a dietary effect. To our knowledge, this study is the first evidence of the effect of a cruciferous vegetable on the composition of faecal microbiota of human origin. Besides these modifications, the consumption of Brussels sprouts led to an

alteration of the SCFA pattern, increasing acetate at the expense of branched-chain fatty acids, but did not change the β -glucuronidase activity. In a previous study, we had shown that Brussels sprouts reduce the DNA damage induced by IQ in the colon of HMA rats (Humblot *et al.* 2004). The chemoprotection of cruciferous vegetables against the genotoxicity of HA is usually attributed to an induction of the UDP-glucuronosyl transferase, a key enzyme of the detoxification pathway of HA (Kassie *et al.* 2002, 2003). The present study gives a new insight into the biological activities of cruciferous vegetables. However, whether these modifications of the predominant bacterial community and its fermentation pattern play a role in the chemopreventive effect of these foodstuffs remains to be established.

Unlike inulin and Brussels sprouts, the fermented milk Actimel[®] did not induce a noticeable modification of the predominant bacterial population pattern (mean degree of similarity between the control and treated groups: 75.6%). Again, the heterogeneousness of the *Lactobacillus* population at initial time and the temporal variation observed in the control group prevent any firm conclusion on a possible dietary effect. A band co-migrating with the *Lactobacillus acidophilus* present in the fermented milk was observed in the faeces of several HMA rats at the start of the experiment, when all animals consumed the control diet. This band was present in all the samples collected from HMA rats fed for 4 weeks on the fermented milk diet. Nevertheless, and because of its sporadic presence at initial time, it is impossible to assign this DNA band to the *Lactobacillus acidophilus* of the fermented milk. This difficulty has already been noticed by certain authors in the course of human studies, where the initial presence of a band co-migrating with the *Lactobacillus* strain of the fermented milk disturbs interpretation of the data (Kimura *et al.* 1997; Tannock *et al.* 2000). On the whole, in our experimental conditions, the consumption of the fermented milk Actimel[®] resulted in a slight modification of the predominant faecal microbiota, without any marked influence on *Bifidobacterium* and *Lactobacillus* populations. In the same way, no consistent alterations of the caecal biochemistry could be observed following consumption of the fermented milk. This result contrasts with other studies in which consumption of the same fermented milk by HMA rats resulted in a decrease of the β -glucuronidase activity and a modification of the SCFA profile (Djouzi *et al.* 1997). However, consumption of this fermented milk by healthy infants remained ineffective on the SCFA profile and reduced the faecal β -glucuronidase activity mainly in those subjects whose basic activity was unusually high (Guérin-Danan *et al.* 1998). Therefore, it seems that the effect of functional foods on bacterial β -glucuronidase may depend on the basic activity of this enzyme in a given faecal microbiota.

The origin of the microbiota strongly influences the extent of the genotoxic effects of HA. For instance, DNA damage in the colonic mucosa following IQ treatment is twice greater in rats harbouring their natural microbiota than in HMA companions (Kassie *et al.* 2001). Focusing on the human colonic ecosystem, a recent study has shown that rats harbouring human microbiota from vegetarian subjects have 35% less IQ-induced DNA damage than rats inoculated with meat eaters' faecal microbiota (Kassie *et al.* 2004). In a previous study, we have demonstrated that diets containing inulin, Brussels sprouts or the fermented milk Actimel[®] protected HMA rats against the intestinal genotoxicity of IQ, with a 55% (Brussels sprouts) to 70% (inulin) reduction in the damage intensity. In the present study, inulin and, to a lesser extent, Brussels sprouts alter the profile

of the predominant bacterial community of the human faecal ecosystem and, in the case of inulin, of the *Bifidobacterium* group. Therefore, it is possible that changes in the intestinal microbiota mediated by these foodstuffs contribute to their protective effects against HA genotoxicity.

Acknowledgements

The authors thank Rosa Durao for breeding the germ-free rats. Many thanks also to Dr Ruud Verkerk (University of Wageningen, the Netherlands) for the gift of the freeze-dried Brussels sprouts and to Dr Jan Van Loo (Orafti) for the Raftilose[®] Synergy 1. Dr Gérard Corthier, Dr Maria-José Flores-Sanabria and Dr Philippe Langella of INRA made helpful comments on the draft manuscript. This research was supported by the European Community under the RTD programme "Quality of Life and Management of Living Resources", QLK1-CT99-01 197, entitled "Heterocyclic Amines in Cooked Foods – Role in Human Health". Christèle Humblot acknowledges a PhD grant from the French Ministry of Education and Research.

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