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Interrelationships between dairy product intake, microflora metabolism, faecal properties and plasmid dissemination in gnotobiotic mice

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We previously described the effects of intake of dairy products on plasmid dissemination in the digestive tract of gnotobiotic mice associated with human faecal flora (HFF) and found that yoghurt, heat-treated yoghurt (HTY) and milk reduced population levels of transconjugants compared with findings in mice fed a standard mouse diet. In the case of lactose intake, transconjugants were not detected. The aim of the present study was to assess the possible interrelationships between these observations and other variables (bacterial ecology, pH, moisture, enzyme activities, short-chain fatty acid (SCFA) contents, lactic acid contents). Much of the interest of the present comparison lies in the fact that the animals were homogeneous in terms of age, gender, food and intestinal microflora, owing to the gnotobiotic mouse model maintained in sterile isolators. We observed no variation in SCFA and lactic acid contents or in the population levels of strictly anaerobic strains of *Bacteroides* and *Bifidobacterium*, and of the facultative anaerobic recipient *Escherichia coli* PG1 strain. The main modifications were the reduction of population levels of transconjugants in mice receiving yoghurt, HTY and milk, and concomitantly an increase of β-galactosidase and a decrease of β-glucosidase activities, compared with control mice fed a standard diet. Total inhibition of plasmid transfer was observed in HFF mice consuming lactose, and concomitantly the two enzyme activities (β-glucosidase and β-galactosidase) were increased, compared with the findings in control mice fed a standard diet. In axenic mice consuming lactose, plasmid transfer occurred, β-galactosidase was not detected and β-glucosidase was decreased. It is therefore proposed that these two enzyme activities influence plasmid transfer and persistence of transconjugants in the digestive tract of HFF associated mice. When both activities were increased there was a total inhibition of plasmid transfer (case of lactose intake). When β-galactosidase increased and β-glucosidase decreased (case of yoghurt, HTY and milk), plasmid transfer occurred at a lower efficiency than in the control group, resulting in lower population levels of transconjugants.

Yoghurt: Milk: Lactose: Intestinal metabolism: Plasmid transfer

Plasmid transfer occurs between bacterial strains in the digestive tract of man and animals (Duval-Iflah *et al.* 1980, 1994; Duval-Iflah & Chappuis, 1984; Lester *et al.* 1990). The survival of strains carrying plasmids in the environment depends on many factors. Antibiotic selective pressure is responsible for widespread dissemination of resistance genes in the environment. In the absence of any selective pressure there is an equilibrium between the loss of plasmid (curing) and the plasmid's ability to transfer itself to the cured strains (Duval-Iflah *et al*. 1980; Duncan *et al.* 1995). Transconjugants which are formed in the digestive tract are generally at a disadvantage and are rapidly eliminated by intestinal transit, unless antibiotic is administered (Duval-Iflah *et al*. 1980). In some instances the transconjugants are capable of colonising the digestive tract of gnotobiotic mice associated with human digestive microflora (Duval-Iflah *et al.* 1994; Garrigues-Jeanjean *et al.* 1999). In such experimental mouse models we have shown that some plasmids and transposons, in association with specific recipient strains, give rise to transconjugants able to establish themselves in the digestive tract at high levels in the absence of any selective pressure. The same exceptional animal models were used to assess the effect of dairy products and probiotics on plasmid transfer and on the persistence of transconjugants in the digestive tract. It was shown that consumption of yoghurt lowered the population

Abbreviations: CFU, colony-forming units; HTY, heat-treated yoghurt; SCFA, short-chain fatty acids.

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levels of transconjugants by a magnitude of three logs, consumption of milk totally inhibited the establishment of transconjugants, and lactose added to the standard diet prevented their formation (Maisonneuve *et al.* 2000, 2001).

Beneficial effects of yoghurt and fermented milks are widely described, and in some instances the mechanisms of their action have been proposed. The decrease of diarrhoeic symptoms has been associated with the lowering of intestinal transit, and modification of the composition and metabolism of the microflora (Hotta *et al.* 1987; Pochart *et al.* 1989; Marteau *et al.* 1990; Kaila *et al.* 1992; Shermak *et al.* 1995). Antimutagenic effects of dairy products are explained by a decrease in some enzymic activities (Goldin *et al.* 1980, 1992; Goldin & Gorbach, 1984*a*,*b*; Lidbeck & Nord, 1991; Bartram *et al.* 1994), and hypocholesterolemic properties have been correlated with their ability to metabolise cholesterol and bile salt (Gilliland *et al.* 1985; Gilliland & Walker, 1990; Walker & Gilliland, 1993; Buck & Gilliland, 1994).

The aim of the present work was to study some of the factors known to be modified by dairy products, such as intestinal transit time, composition, metabolic and enzymic activities of the microflora, and moisture and pH of faeces, in order to detect which of them could be correlated with the lowering of transconjugant densities and the inhibition of plasmid transfer after consumption of yoghurt, milk and lactose.

Materials and methods

Gnotobiotic mice associated with human faecal flora

Mice and protocols were those described previously (Maisonneuve *et al.* 2001). Twenty male adult germ-free C3 H/He mice (axenic) were obtained from our own experimental facilities and reared in sterile Trexler plastic isolators (La Calhène, Vélizy, France). They were inoculated with 500 μl of a 10^{-2} dilution of freshly passed faeces from mice colonised with human intestinal microflora containing the recipient *Escherichia coli* PG1 strain (Garrigues-Jeanjean *et al.* 1999) with an oesophageal tube, twice at 24 h intervals. They were fed a sterile standard pelleted mouse diet for 2 weeks (period 1). At the end of period 1, mice were randomised (four per treatment) and transferred to five experimental sterile isolators. Faeces were collected from each mouse in each group, and pooled to give faecal samples for period 1 measurements. Groups were randomised to eat, for 2 weeks, sterile powdered diets that were mixed (2 parts diet:1 part solution) where the solutions were one of the following: water (control); yoghurt; heat-treated yoghurt (HTY); milk; lactose. After 2 weeks of adaptation to the diet (period 2), faeces were collected as in period 1, and the animals were inoculated with *E. coli* UB1832 donor strain carrying a self-transmissible plasmid R388. They continued on the same diets for 2 months (period 3). Spores of strictly thermophilic *Bacillus subtilis* were inoculated together with a donor strain to monitor intestinal transit (Contrepois & Gouet, 1969). The effects of supplementation on transconjugant formation, on their persistence in the digestive tract and on various factors of interest were evaluated for 2 months (period 3).

Gnotobiotic mice associated with Escherichia coli

Four axenic mice were used to assess the effect of lactose supplementation in the absence of human faecal flora. Axenic mice were transferred into a sterile isolator and received a lactose diet throughout the experiment. During the first 7 d from the beginning of lactose supplementation, mice were kept axenic. A recipient strain *E. coli* PG1 and a donor strain *E. coli* UB1832 were then inoculated at 7 d intervals. The experiment lasted a further 16 d after the donor strain was inoculated. Faecal samples were collected in axenic mice: before they received a supplemented diet; 7 d after lactose adaptation; 7 d after recipient strain inoculation; 16 d after donor strain inoculation.

Supplementation

The yoghurt S85 was obtained with long-life 0 % milk (Finesse®; SMB, Paris, France), fermented with bacterial symbiosis S85 containing *Lactobacillus bulgaricus* 100 158 and *Streptococcus thermophilus* 001 158. The HTY was prepared by heating yoghurt S85 to 70°C for 10 min in a double boiler, then cooling it to 4°C. The milk was that used to prepare yoghurt S85 and HTY. Batches of yoghurt S85, HTY and milk were provided every 2 weeks by the Centre International de Recherche, Daniel Carasso, Groupe Ferments Fermentation (Le Plessis Robinson, France). For the lactose solution, 4·5 g lactose/100 ml water was filtered (filter pore size 0·45 μm; Sarstedt, Nümbrecht, Germany). All products were kept at 4°C. Throughout the administration period, 10 ml yoghurt, HTY, milk, lactose or water for the control group was mixed daily with 20 g sterile powdered standard mouse diet (D03, UAR, Villemoisson-sur-Orge, France). Animals received autoclaved water to drink *ad libitum*.

Faecal sampling

Groups of four mice were housed in separate isolators. Freshly passed faeces were collected and pooled from mice in each group. Four faecal samplings were collected at the end of period 1 (before supplementation), at the end of period 2 (before inoculation of the donor strain and spores) and at the end of period 3. Freshly passed faeces were used immediately after emission for bacterial enumeration and for measurement of pH and moisture. Other faecal samples were kept at − 80°C for further analysis of short-chain fatty acids (SCFA), lactic acid contents and enzymic activities.

pH measurement

Immediately after emission, the pooled faecal samples were diluted in distilled water (1:4, w/v) and faecal pH was determined using a glass pH electrode (pH-Meter 763 Multi-Calimatic, Knick, Germany).

Faecal moisture

Freshly passed faeces were used to determine the dry weight with a moisture autoanalyser (MA 30 Sartorius, Goetingen, Germany).

L-D-lactic acid contents

These were determined from frozen faecal samples and from dairy products, yoghurt, HTY and milk, with a Technicon autoanalyser (Technicon, Plaisir, France) using an enzymic method.

Short-chain fatty acid contents

SCFA were extracted from aliquots of frozen faeces and dairy products by two-step extraction, the first with hexane and the second, performed twice, with tert-butylmethyl-ether. SCFA were derivatised as *N*(0)-tert-butyldimethyl-silyl ester with *N*-methyl-*N*(tert-butyldimethylsilyl) trifluoroacetamide. The analyses were performed by GC (Girdel 300; Perichrom Delsi, Longjumeau, France) with a flame ionisation detector. The column is a wide-bore CP-Sil5CB column (Chrompack, Les Ullis, France; 25 m × 0·53 mm, 100 % methyl). The carrier gas (He) flow rate was 1 ml/min. The temperature of both the detector and the injector was 300°C. Oven temperature was programmed to increase from 80°C to 280°C at a rate of 4°C/min; 2-methylpentanoic was used as an internal standard. The extractions and analyses were manual procedures.

Enzymic activities in faeces and dairy products

Faecal samples were diluted in 0·9% NaCl (1:19, w/v). The faecal suspension was homogenised with Ultra Turrax (T25 basic IKA). The homogenate was used for the enzyme assay. The enzyme reaction was run at 37°C. The reaction contained 0·05 ml homogenate, 0·250 ml sodium phosphate buffer (0·1 M, pH 6·4) and 0·1 ml substrate solution (5 mM, Sigma Chemical Co., St Louis, MO, USA). Assays were carried out of α- and β-glucosidase, α- and β-galactosidase, β-glucuronidase, α-fucosidase, β-xylosidase, *N*-acetyl α- and β-galactosaminidase and *N*-acetyl β-glucosaminidase, with substrates *p*-nitrophenyl α-D- and β-D-glucopyranoside, α-D- and β-D-galactopyranoside, β-D-glucuronide, α-L-fucopyranoside, β-D-xylopyranoside, *N*-acetyl α-D- and β-D-galactosaminide and *N*-acetyl β-D-glucosaminide respectively (Andrieux *et al.* 1992). The reaction was stopped by adding 0·6 ml Na_2CO_3 (0·5 M). Readings were taken at 400 nm with a spectrophotometer (Beckman DU.70, Fullerton, CA, USA). A standard curve was constructed with *p*-nitrophenol (Sigma Chemicals). The protein concentration in each sample used to measure enzymic activities was determined using a method described by Lowry *et al.* (1951), with bovine serum albumin as the standard. Activities are expressed as nmol/min per mg protein. All these enzyme activities were also determined in dairy product samples by the same process.

Bacterial enumeration

Immediately after the mice had defecated, faecal samples were diluted in Liquid Casein Yeast extract medium (Raibaud *et al.* 1966) and spread onto selective media. For enumeration of strictly anaerobic strains, dilution and inoculated procedures were carried out in an anaerobic chamber with prereduced media. *Bacteroides* spp. counts were obtained on brain–heart infusion agar (Difco Laboratories, Difco, Becton Dickinson, Le Pont de Claix, France) containing 5 mg haemin per litre (Sigma Chemical Co.) and neomycin (140 mg/l, BHI-Hneo). Inoculated plates were kept in contact with the air for 1 h (Corthier *et al.* 1996), then reintroduced into an anaerobic chamber and incubated at 37°C for 4 d. A modified Beerens agar medium (Beerens, 1990) containing 35 g/l Brain Hart Infusion and 5 g/l yeast extract (Difco Laboratories) instead of Columbia medium was used to enumerate *Bifidobacterium* spp. Plates were incubated anaerobically at 37°C for 4 d. Total colonies on each medium were counted. Selective enumeration of *E. coli* parental strains, recipient PG1 and donor UB1832, and transconjugants carrying the self-transmissible plasmid R388 was performed as previously described (Maisonneuve *et al.* 2001).

Colony hybridisation screening

The selectivity of both media, BHI-Hneo agar and modified Beerens agar medium, for the quantification of *Bacteroides* spp. and *Bifidobacterium* spp. respectively, was examined. One hundred colonies grown on BHI-Hneo and 100 colonies grown on modified Beerens agar were picked and then placed onto BHI-H agar plates and incubated at 37°C for 2 d. All procedures were performed in an anaerobic chamber. The assay was performed in triplicate. Colonies were transferred onto nylon transfer membrane (Amersham, Life Science, Buckinghamshire, UK). The membranes were then treated by serial transfers onto filter papers soaked with denaturation solution for 7 min (1·5 M-NaCl, 0·5 M-NaOH), and then neutralisation solution twice for 3 min each time (1·5 M-NaCl, 0·5 M-Tris HCl, pH 7·2, 1 mM-EDTA). They were then washed in 2 × sodium salt citrate, placed on filter papers and fixed using a U.V. cross-linker (Amersham, Life Science). The probes used in the present study are listed in Table 1. They were synthesised by MWG-Biotech AG (Germany) and labelled at their 5′ ends using T4 polynucleotide kinase (GIBCO, BRL, Life Technologies,

Table 1. Oligonucleotide probes and washed temperature (T) used in the present study

Target group	Probe (5′–3′)	Name (OPD nomenclature)	T (°C)	Reference
Domain bacteria	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18	64	Amann *et al.* (1990)
Bifidobacterium	CGGGTGCTRCCCACTTTCATG	S-G-Bif-1412-a-A-21	59·5	Kaufmann *et al.* (1997)
Bacteroides cluster	GCACTTAAGCCGACACCT	S-*-Bacto-1080-a-A-18	60	Doré *et al.* (1998)

R, represents an A/G wobble nucleotide; OPD, oligonucleotide probe database.

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SARL, Eragny, France) and [γ-^{32}P]ATP (NEN Life Science Products Inc., Boston, MA, USA). The Bact-0338 probe served as a positive control after use of the specific probe. The membranes were prehybridised with hybridisation buffer (sodium salt citrate 6 ×, SDS 0·5%, Denhart 2·5 ×, salmon sperm 125 mg/l) for 2 h at 45°C in screw-cap hybridisation tubes. The membranes were hybridised overnight at 45°C with hybridisation buffer without Denhart and containing 1 pmol labelled probe/ml and washed twice over with sodium salt citrate 2 × and SDS 0·5% buffer at appropriate temperatures indicated in Table 1 for 30 min. The membranes were visualised by autoradiography by means of electronic radiography (Instant Imager Δ Packard; Vilbert Lourmat, Marne La Vallée, France) or at − 80°C, by Kodak X-OmatAR films (Sigma Aldrich, Steinheim, Germany).

Statistical analysis

The results are expressed as means and standard deviations. The cell counts were transformed to $\log_{10}$ numbers. We compared the average for each period 1, 2 and 3, between groups and within each group using Student's *t* test. We chose to reject the null hypothesis at the 5 % level.

Results

Bacterial populations

Bacterial counts were performed in duplicate. The total strictly anaerobic population, approximately 4×10^{10} colony-forming units (CFU)/g faeces (data not shown) remained steady in the five groups throughout the three periods. *Bacteroides* constituted the major bacterial population (10·5 (SD 0·1)) and were confirmed at 94·67 % by colony hybridisation using the *Bacteroides–Prevotella–Porphyromonas* group-specific probe. *Bifidobacteria* were at levels 100- to 1000-fold lower than those of *Bacteroides* (7·5 (SD 0·2)) and were confirmed at 81·33 % by colony hybridisation with the *Bifidobacteria*-specific probe. Neither *Bacteroides* nor *Bifidobacterium* population levels were affected by the dairy product and lactose supplementation (Table 2). The population levels of the recipient strain PG1, approximately 10^8 CFU/g faeces, remained steady throughout the experiments in all five groups. The donor strain was transient and eliminated at a faster rate than the transit marker, 24 h after inoculation. Plasmid R388 was rapidly transferred to the recipient strain PG1, and the resulting transconjugants (TC-R388) were detected at a low level, approximately 10^2 and 10^3 CFU/g faecal samples 6 h after inoculation of the donor strain, except in faecal samples from mice supplemented with lactose, where they were below the detection threshold of 10^2 CFU/g faeces throughout the experiment. During period 3 and at equilibrium, TC-R388 were significantly higher in control group mice (7·3 $\log_{10}$ CFU/g faeces) than in the groups receiving HTY or yoghurt (respectively 6·6 and 5·2 $\log_{10}$ CFU/g faeces). In mice supplemented with milk and lactose, the TC-R388 were below the detection threshold of 10^2 CFU/g faeces (Table 2).

Transit time

Spores of *Bacillus* used as an intestinal transit marker were at maximal levels 6 h after their inoculation in all groups except in the mice supplemented with milk, where the maximal level was reached at 12 h, indicating a slower transit time.

Faecal moisture, pH and lactic acid contents

No significant variation was observed in moisture, pH or lactic acid contents in mice fed the control diet, yoghurt, HTY or milk. Mean values ranged from 59·3 (SD 2·0) to 67·1 (SD 2·7) % in moisture, from 6·05 (SD 0·09) to 6·64 (SD 0·74) in pH, from 4·2 (SD 0·5) to 6·2 (SD 0·2) mmol/kg faeces in lactic L(+) acid contents and from 6·0 (SD 0·8) to 8·3 (SD 0·4) mmol/kg faeces in lactic D(−) acid contents. Lactic acid contents were similar in the yoghurt and HTY that were used in the present study. The L(+) isomer constituted 70 % (7·5 g/l L(+) isomer and 3·8 g/l D isomer). Contents in milk were 1000-fold lower.

Moisture and pH were slightly affected by lactose supplementation. The faecal pH increased from 5·99 (SD 0·15) at period 1 to 6·29 (SD 0·20) during periods 2 and 3, and the moisture decreased from 64·5 (SD 2·3) to 59·0 (SD 2·0).

Table 2. Bacterial counts in faecal samples from mice of control group and those fed yoghurt, heat-treated yoghurt (HTY) and milk during period 3 (days 30–60) following inoculation of donor strain of plasmid R388

(Mean values and standard deviations, expressed as $\log_{10}$ colony-forming units/g faeces)

	Bacteroides		*Bifidobacteria*		Recipient strain PG1		Transconjugant PG1 (R388)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	10·1	0·7	7·3	0·6	8·1	0·3	7·3	0·3
Yoghurt	10·3	0·4	7·8	1·4	8·0	0·1	5·2*	0·4
HTY	10·3	0·1	7·8	0·9	8·2	0·3	6·6*	0·6
Milk	9·7	0·8	7·7	0·8	8·2	0·2	< 2	
Lactose	10·1	0·0	7·5	0·7	8·1	0·1	< 2	

< 2, below detection threshold of 10^2 CFU/g faeces.
Mean values were significantly different from the control values: *$P < 0·05$.

Table 3. Short-chain fatty acids and lactic acid in dairy products and in faecal samples from mice of control, yoghurt, heat-treated yoghurt (HTY) and milk groups, before supplementation (baseline, period 1), during adaptation to supplementation (period 2) and during the day 30 to day 60 period following donor strain inoculation (period 3)

(Mean values, expressed as mmol/kg faeces, with their standard errors)

	Dairy products (mg/100 ml)	Period 1 (baseline)		Period 2 (adaptation)		Period 3 (day 30–day 60)	
		Mean	SE	Mean	SE	Mean	SE
Formic acid							
Control		7·6	0·6	7·2	3·8	8·3	2·1
Yoghurt	30·2	9·5	2·7	9·5	5·3	7·0	4·6
HTY	17·6	8·2	1·8	8·2	2·2	7·3	0·6
Milk	ND	8·9	3·9	7·0	1·1	7·8	1·8
Acetic acid							
Control		13·7	0·3	10·5	11·6	23·7	4·3
Yoghurt	20·5	19·3	1·7[a]	5·0	5·5[b]	16·3	5·9
HTY	18·0	16·8	2·2	16·0	3·0	22·3	1·3[b,c]
Milk	15·0	17·1	5·2	14·2	2·3	22·5	1·8[c]
Propionic acid							
Control		3·3	0·4	2·2	0·9	3·7	0·7
Yoghurt	2·4	3·0	2·4	2·9	3·2	2·5	0·2[a]
HTY	2·5	3·7	0·2	1·6	1·3[b]	4·2	0·3[c]
Milk	0·2	2·1	1·8	2·4	0·9	5·4	1·4[c]
Iso-butyric acid							
Control		0·1	0·1	0·2	0·1	0·1	0·0
Yoghurt	2·7	0·1	0·0	0·1	0·0	0·1	0·0
HTY	2·7	0·1	0·0	0·2	0·1	0·2	0·1
Milk	10·3	0·2	0·1	0·1	0·1	0·3	0·1
Butyric acid							
Control		0·2	0·0	0·4	0·2	0·2	0·1
Yoghurt	2·2	0·9	1·2	0·1	0·1	0·1	0·1
HTY	1·0	0·2	0·1	0·2	0·1	0·2	0·1
Milk	0·5	0·2	0·1	1·2	1·6	0·2	0·0
Iso-valeric acid							
Control		0·4	0·1	0·2	0·2	0·4	0·2
Yoghurt	0·3	0·2	0·2	0·1	0·2	0·3	0·4
HTY	0·2	0·3	0·2	0·5	0·3	1·0	0·6
Milk	0·3	0·2	0·1	1·0	1·1	1·2	0·8
Valeric acid							
Control		0·0	0·0	0·4	0·3	0·2	0·5
Yoghurt	0·3	0·0	0·1	ND	ND	0·2	0·3
HTY	0·3	0·1	0·0	0·1	0·1	0·1	0·1
Milk	0·3	0·5	0·3	0·0	0·1	0·1	0·0
Iso-caproic acid							
Control		0·0	0·1	0·1	0·1	0·1	0·0
Yoghurt	0·6	0·1	0·1	ND	ND	0·1	0·1
HTY	0·4	0·1	0·0	0·0	0·0	0·1	0·1
Milk	ND	0·2	0·2	0·1	0·1	0·1	0·0
Caproic acid							
Control		0·1	0·17	0·1	0·0	0·2	0·0
Yoghurt	1·2	0·2	0·2	0·1	0·0	0·2	0·0
HTY	1·3	0·1	0·0	0·1	0·0	0·2	0·0
Milk	11·4	0·1	0·0	0·1	0·0	0·2	0·0
Heptanoic acid							
Control		0·1	0·0	0·3	0·3	0·1	0·1
Yoghurt	ND	0·3	0·3	0·6	0·6	0·1	0·1
HTY	ND	0·2	0·1	0·5	0·5	0·2	0·1
Milk	ND	0·2	0·2	0·1	0·1	0·3	0·2

ND, not detected.
[a]Values significantly different from control values in the same period ($P<0·05$).
[b]Values significantly different from baseline values in the same group ($P<0·05$).
[c]Values significantly different from period 2 values in the same group ($P<0·05$).
For experimental procedures, see p. 123.

Table 4. Bacterial enzyme activities in faecal samples from mice in the control group, and groups fed yoghurt, heat-treated yoghurt (HTY) or milk before supplementation (baseline, period 1), during adaptation to supplementation (period 2) and during the day 30 to day 60 period following donor strain inoculation (period 3)

(Mean values, expressed as nmol/min per mg protein, and standard deviations)

	Period 1 (baseline)		Period 2 (adaptation)		Period 3 (day 30–day 60)	
	Mean	SD	Mean	SD	Mean	SD
α-Galactosidase						
Control	105·6	1·8	38·6	26·4[b]	60·8	21·6
Yoghurt	89·3	3·4	77·1	10·0[a]	74·8	13·3
HTY	61·6	6·9[a]	81·3	32·6	102·1	22·7
Milk	45·9	6·8[a]	57·2	11·3	102·1	65·16
β-Galactosidase*						
Control	75·3	14·8	20·6	8·1	48·3	13·5
Yoghurt	55·4	37·1	45·6	14·3	124·5	36·1[a,b]
HTY	58·4	19·7	38·9	5·9	130·7	39·2[a,b]
Milk	43·1	3·5	54·7[a]	2·6[a]	135·2[b]	39·4[a,b]
Lactose	27·6	11·0			84·3	15·4[a,b]
α-Glucosidase						
Control	24·5	18·5	20·2	4·1	39·5	23·2
Yoghurt	54·0	11·5	27·2	9·1	26·7	2·5
HTY	46·9	12·6	34·5	6·4[a]	32·2	7·0
Milk	46·5	10·6	28·4	7·8	27·2	6·6[b]
β-Glucosidase						
Control	42·2	2·0	11·9	8·1[b]	31·1	12·8
Yoghurt	30·7	3·8	21·6	8·5	12·2	4·6[a,b]
HTY	34·7	4·6	24·5	7·6	12·6	11·0
Milk	29·3	4·2	20·0	5·7	10·7	7·8[a,b]
Lactose	24·2	5·50			54·9	4·7[a,b]
β-Glucuronidase						
Control	11·4	2·6	7·3	5·3	13·8	3·6
Yoghurt	12·8	1·0	7·6	5·2	7·0	0·4[a,b]
HTY	8·8	1·6	6·7	2·8	5·8	1·4[a]
Milk	7·7	1·49	5·8	3·5	7·2	1·3[a]
β-Xylosidase						
Control	7·7	1·0	1·7	1·6[b]	7·0	2·8[b]
Yoghurt	7·0	0·0	4·7	2·3	5·2	2·5
HTY	6·2	1·7	6·0	2·4[a]	5·4	1·5
Milk	5·3	0·2	3·4	2·3	5·4	1·2
α-Fucosidase						
Control	6·1	3·1	1·0	0·6[b]	4·0	1·6[b]
Yoghurt	6·1	2·8	1·9	1·1[b]	3·0	0·4
HTY	2·8	0·0	2·9	0·8[a]	4·2	1·3
Milk	1·5	0·5[a]	1·7	0·9	4·1	1·2[b]
N-acetyl-α-galactosaminidase						
Control	0·5	0·4	0·3	0·1	0·9	0·4
Yoghurt	0·7	0·4	0·9	0·1	0·2	0·2
HTY	0·1	0·1	0·9	0·1[b]	0·4	0·5
Milk	0·2	0·3	0·6	0·2	0·5	0·0
N-acetyl-β-galactosaminidase						
Control	11·9	6·3	5·6	2·4	9·9	4·1
Yoghurt	11·9	6·7	12·3	1·7	1·0	1·9[a,b]
HTY	6·5	5·2	11·8	0·1	9·3	3·4
Milk	4·9	5·1	7·9	1·2	7·1	4·4
N-acetyl-β-glucosaminidase						
Control	107·8	13·5	35·2	191·1[b]	74·2	8·0[b]
Yoghurt	110·7	10·4	54·3	32·5	2·0	2·0[a,b]
HTY	65·0	23·6	59·4	14·7	43·3	8·5
Milk	44·4	10·3[a]	48·0	24·7	41·9	14·1

[a]Values were significantly different from control values at the same period ($P<0·05$).

[b]Values were significantly different from baseline values in the same group ($P<0·05$).

* β-Galactosidase was detected in yoghurt at 21·0 (SD 2·0). The other activities were not detected in the dairy products.

For experimental procedures, see p. 123.

Short-chain fatty acids

The mean concentrations (mmol/kg faeces) of the ten SCFA analysed were similar in all groups and were not modified after supplementation with the dairy products, during periods 2 and 3 (Table 3). The distribution and concentrations of SCFA (mg/100 ml) in yoghurt and HTY were similar. Heptanoic (C_7) acid was not detected in any of the dairy products and formic (C_1) and isocaproic (iC_6) acids were not detected in milk. Isobutyric (iC_4) and n-caproic (C_6) acids were at higher concentrations in milk.

Enzyme activities

Enzyme activities (Table 4) are expressed as nmol/min per mg protein. Modifications were observed during periods 2 and 3. The β-galactosidase activity was increased in mice of the four groups (124·5 (SD 36·1) *v.* 55·4 (SD 37·1) in the group receiving yoghurt, 130·7 (SD 39·2) *v.* 58·4 (SD 19·8) in the group receiving HTY, 135·2 (SD 39·4) *v.* 43·1 (SD 3·5) in the group receiving milk and 84·3 (SD 15·4) *v.* 27·6 (SD 11·0) in the group receiving lactose). The β-glucosidase decreased in the yoghurt, milk, and HTY groups (from 30·7 (SD 3·8) to 12·2 (SD 4·6) in the yoghurt group, from 29·4 (SD 4·2) to 10·7 (SD 7·8) in the milk group and from 34·7 (SD 4·6) to 12·6 (SD 10·9) in the HTY group), and increased in the lactose group (from 24·2 (SD 5·5) to 54·9 (SD 4·8)). The α-galactosidase activity increased in the milk and HTY groups. The *N*-acetyl-β-glucosaminidase and *N*-acetyl-β-galactosaminidase decreased in the group receiving yoghurt.

Effect of lactose intake on plasmid transfer and on β-galactosidase and β-glucosidase activities in axenic mice

Axenic mice supplemented with lactose were inoculated with recipient and donor strains at 7 d intervals. The recipient strain became established rapidly after its inoculation and reached a steady state throughout the mating experiment at a mean value of 9·1 (SD 0·2) log CFU/g faeces. The donor strain became established at 6·5 (SD 0·2) log CFU/g faeces from day 14 to day 30. Transconjugant TC-R388 appeared at 4·9 log CFU/g faeces, 3 h after inoculation of the donor strain, then its level increased progressively to reach a maximal level of 9·7 (SD 0·2) log CFU/g faeces.

There was no β-galactosidase activity in faecal material of axenic mice before or after lactose supplementation, or in mice associated with *E. coli* strains and supplemented with lactose. This activity was detected at 26·4 (SD 2·5) nmol/min per mg protein only after 30 d lactose supplementation and after transconjugants were at their maximal density.

β-glucosidase was detected at 2·4 (SD 0·3) in faeces of axenic mice before lactose supplementation, then decreased significantly to 0·5 (SD 0·1) after 7 d lactose supplementation and increased to 1·7 (SD 0·2) 7 d after the recipient *E. coli* strain was inoculated. It decreased again to 1·0 (SD 0·1) 16 d after the donor strain was inoculated and transconjugants established at a high population level.

Discussion

The main objective of the present study was simultaneously to investigate the effects of yoghurt, HTY, milk and lactose consumption on the formation of transconjugants and their persistence in the digestive tract, and look for a correlation with bacterial ecology, metabolism, enzyme activities, pH and moisture. Much of the interest of the present comparison lay in the fact that the animals were homogeneous in terms of age, gender, food and intestinal microflora, owing to the use of a gnotobiotic mouse model in sterile isolators. Use of such a procedure makes it possible to reveal any relationship between diet and potential modifications of the entities studied.

Our results are consistent with other studies performed in healthy subjects or animals exhibiting stability in terms of faecal moisture, pH and total anaerobic bacteria after the consumption of various dairy products including fermented milks, heat-treated fermented milks or milk (Goldin & Gorbach, 1984*a*,*b*; Bartram *et al.* 1994; Pedrosa *et al.* 1995; Bouhnik *et al.* 1996; Guerin-Danan *et al.* 1998; Chen *et al.* 1999). Our data showed that consuming dairy products or lactose did not result in any modification of the composition of the flora as far as the strictly anaerobic fraction (*Bacteroides* and *Bifidobacteria*) and facultative anaerobic *Enterobacteria*, represented by the recipient *E. coli* PG1 strain, were concerned (Table 2). The main modification was the reduction of the population level of transconjugants in the yoghurt, HTY and milk groups, and inhibition of transfer in the lactose group. In order for a transconjugant to establish itself in the environment (including the digestive tract), the plasmid has to maintain itself in the recipient strain. In the absence of any selective pressure, an equilibrium is established between the loss of the plasmid (curing) and its ability to transfer itself to the cured strains (Duncan *et al.* 1995). A low population level of transconjugants can result from an imbalance between these two steps (Duval-Iflah *et al.* 1980). Our observations concerning the decrease of the population densities of transconjugants after the mice had received yoghurt, HTY and milk can be explained by such an imbalance. We can speculate that the three dairy products either promoted the loss of the plasmid or reduced the efficiency of plasmid transfer, or influenced both steps. Lactose can be implicated, earlier, at the first step of the mating, that allows the formation of transconjugants, since these were not detected in faecal samples.

What can be the factors that might inhibit plasmid transfer *in vivo*? Do metabolites, pH, moisture or enzyme activities influence or inhibit plasmid transfer? These questions have not previously been answered.

Moisture and pH were shown in the present study to be exclusively modified by lactose, but to a very small extent for being considered responsible for inhibition of plasmid transfer. Lactic acid and SCFA contents were not affected by the diets.

Only enzyme activities were shown to be affected by the diets (Table 4). Yoghurt, HTY and milk increased β-galactosidase and decreased β-glucosidase. Lactose was distinguished by increasing both activities. It is not clear whether those modifications were responsible for inhibition of transfer. Data on the transfer of plasmids in axenic mice were of great interest, and showed that lactose, in the absence of complex human flora, did not inhibit plasmid transfer or the colonisation ability of transconjugants, which became established at the maximum population level, indicating an efficiency of transfer (ratio of 1 between transconjugants and recipients).

β-Galactosidase was not detected in faecal samples of axenic mice before lactose supplementation, confirming the absence of this activity in adult germ-free mice. This activity was not induced after 7 d lactose intake or after recipient, donor and transconjugant strains became established in mice consuming lactose. The β-galactosidase activity was detected only after 30 d lactose supplementation, at a low level of 26·4 (SD 2·5), which was similar to that found in the control group of mice inoculated with human faecal flora and fed the standard diet, and where transfer and colonisation with transconjugants were efficient and maximal (Tables 2 and 4). Therefore, we propose that inhibition of plasmid transfer is directly correlated with the augmentation of β-galactosidase activity in all groups of mice inoculated with human faecal flora and supplemented with yoghurt, HTY, milk or lactose (Tables 2 and 4). In mice inoculated with human faecal flora and ingesting lactose, the β-glucosidase activity was increased, whereas it was decreased in those mice receiving yoghurt, HTY or milk, and in axenic mice receiving lactose supplementation. We suggest that simultaneous augmentation of both β-galactosidase and β-glucosidase activities contributed to the total inhibition of transfer in the mice inoculated with human faecal flora and receiving lactose, and that augmentation of β-galactosidase followed by decrease of β-glucosidase partially inhibited this transfer in the groups receiving yoghurt and HTY. The case of mice supplemented with milk was particular, since the mating was observed some hours (6) after the donor was inoculated in mice and the transconjugants were around 10^2 CFU/g faeces, but they were eliminated from the digestive tract after 24 h (Maisonneuve *et al.* 2001).

The mechanisms of action of plasmid transfer and persistence of transconjugants are complex and involve multiple factors. *In vitro* studies have suggested that inhibition of plasmid transfer might be obtained by chemical agents that affect the formation of sex pili and plasmid curing, such as SDS, organic solvents, acridine dyes, high temperature and proteases (Hahn 1976; Viljanen & Boratynski, 1991). The present study indicated that some enzyme activities could affect plasmid transfer *in vivo* in the digestive tract. Further investigation is necessary to evaluate the effects of other substrates on these activities and on plasmid transfer.

Taken together, the present results indicated concomitant effects of the four dairy products on two enzymic activities (β-glucosidase and β-galactosidase) and plasmid transfer. However, it is difficult to establish a relationship between the effect of diet on faecal bacteria and their metabolism in the intestine. Most metabolites are rapidly absorbed through the colonic mucosa after their formation. Therefore, their concentrations in faecal samples do not reflect those in the caecum and proximal colon. In the present study, we monitored the kinetics of the implantation of transconjugants,

the efficiency of plasmid transfer and the evolution of biochemical and bacteriological factors in faecal samples. It would be informative to perform similar measurements in different digestive tract compartments after a long period of chronic supplementation.

Conclusion

It is probable that our observations on plasmid dissemination in the digestive tract of mice inoculated with human faecal flora were related to multiple factors, including enzyme activities. These results are of interest and should be pursued in order to find a substrate that can be used as a nutrient to prevent plasmid transfer in the digestive tract and which can compete with the selective pressure exerted by antibiotics during antibiotherapy.

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