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Influence of Camembert consumption on the composition and metabolism of intestinal microbiota: a study in human microbiota-associated rats

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The objective of the present study was to evaluate the consequence of Camembert consumption on the composition and metabolism of human intestinal microbiota. Camembert cheese was compared with milk fermented by yoghurt starters and *Lactobacillus casei* as a probiotic reference. The experimental model was the human microbiota-associated (HM) rat. HM rats were fed a basal diet (HMB group), a diet containing Camembert made from pasteurised milk (HMCp group) or a diet containing fermented milk (HMfm group). The level of micro-organisms from dairy products was measured in faeces using cultures on a specific medium and PCR–temporal temperature gradient gel electrophoresis. The metabolic characteristics of the caecal microbiota were also studied: SCFA, NH_3, glycosidase and reductase activities, and bile acid degradations. The results showed that micro-organisms from cheese comprised 10^5–10^8 bacteria/g faecal sample in the HMCp group. *Lactobacillus* species from fermented milk were detected in HMfm rats. Consumption of cheese and fermented milk led to similar changes in bacterial metabolism: a decrease in azoreductase activity and NH_3 concentration and an increase in mucolytic activities. However, specific changes were observed: in HMCp rats, the proportion of ursodeoxycholic resulting from chenodeoxycholic epimerisation was higher; in HMfm rats, α and β-galactosidases were higher than in other groups and both azoreductases and nitrate reductases were lower. The results show that, as for fermented milk, Camembert consumption did not greatly modify the microbiota profile or its major metabolic activities. Ingested micro-organisms were able to survive in part during intestinal transit. These dairy products exert a potentially beneficial influence on intestinal metabolism.

Cheese: Camembert: Human microbiota-associated rats: Intestinal microbiota: Probiotics

There is growing evidence that the balance of gastrointestinal microbiota and microbiota functions play an important role in maintaining health and preventing diseases. The colonic microbiota is a complex ecosystem, which until recently was identified using cultures of specific medium and phenotypic characteristics. During the last 10 years, molecular methods have revealed that 70–80 % of micro-organisms escape analysis by bacteriological culture methods (Suau *et al.* 1999). The molecular techniques based on the detection of nucleotide sequence of 16S rRNA and the amplification of 16S rDNA sequences, coupled with separation by denaturing gel electrophoresis, allow a better assessment of the microbiota composition and dominant species complexity (Blaut *et al.* 2002).

Functional foods contain ingredients that beneficially affect the health of consumers (Salminen *et al.* 1998). Among these functional foods, yoghurt and fermented milks provide live bacteria called probiotics, belonging mainly to *Lactobacillus* and *Bifidobacterium* species, although some other species or other micro-organisms, such as yeast, have been a matter of interest (De Roos & Katan, 2000; Heyman & Ménard, 2002). In human subjects, several studies have shown that these micro-organisms resist endogenous digestion during intestinal transit and are found at high levels in the stools during fermented milk consumption (Marteau *et al.* 1994; Bezkorovainy, 2001). There is also some evidence that probiotics improve the intestinal metabolism, modulate immunity and prevent intestinal diseases such as diarrhoea (Goldin, 1998; Marteau *et al.* 2002). However, experimental studies suggest that each strain and each association of strains lead to specific effects (Sanders, 1993; Reid, 1999).

One of the possible mechanisms of action of probiotics is an effect on the composition and the metabolism of the intestinal microbiota. In children, we have previously shown that fermented milk containing *Lactobacillus casei* DN-114 001 and yoghurt symbiosis have positive effects on diarrhoea and regulate some deleterious bacterial activities, such as reductase and β-glucuronidase (Guérin-Danan *et al.* 1998). Similar observations have been obtained in

Abbreviations: HM, human microbiota-associated; HMB, group fed on basal diet; HMCp, group fed on diet containing Camembert made from pasteurised milk; HMfm, group fed on diet containing fermented milk; Psc, Pearson similarity coefficient; TTGE, temporal temperature gradient gel electrophoresis.

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human microbiota-associated (HM) rats (Djouzi *et al.* 1997).

In an effort to expand the products providing probiotics, food such as Cheddar cheese, Gouda cheese, cottage cheese or ice-cream have been studied as food-carriers of both *Bifidobacterium* and *Lactobacillus* probiotic strains (Stanton *et al.* 1998). However, nothing is known about the effect of flowered-rind soft cheese containing live bacteria such as Camembert, which is traditionally consumed in France and many other countries worldwide (Lenoir *et al.* 1995).

The objective of the present study was to evaluate the consequence of Camembert on intestinal microbiota composition and metabolism. The model used is the HM rat, as described by Djouzi *et al.* (1997). The influence of Camembert consumption on dominant intestinal microbiota was evaluated using PCR–temporal temperature gradient electrophoresis (TTGE) analysis. The concentration of common cheese micro-organisms was measured in faecal samples using both cultures on specific medium and PCR–TTGE analysis with specific primers. The metabolic characteristics of the faecal microbiota were studied: SCFA as markers for glycolytic fermentation, NH_3 and iso-acids as markers for proteolytic fermentation, and glycosidases involved in carbohydrate hydrolysis (α- and β-galactosidase, α- and β-glucosidase) and in mucin degradation (neuraminidase, β-*N*-acetyl-galactosaminidase and α-L-fucosidase). The β-glucuronidase, nitrate reductase and azoreductase activities and bile acid conversions leading to the release of toxic substances were also investigated. Milk fermented by yoghurt starters and *Lactobacillus casei* was used as a probiotic-containing reference product.

Methods

Animals and diets

Germ-free male Fischer rats (2·5 months old, *n* 30; UEPSD, Jouy en Josas, France) were reared in three Texler-type isolators (La Cahlène, Vélizy, France). The rats were fed the basal diet B (Table 1) for 1 week. They were then inoculated through a single oral gavage with 1 ml of a 10^{-2} dilution of a faecal sample from a 30-year-old healthy female volunteer, who had not received antibiotics for at least 3 months before stool collection. The faecal dilution was performed from a frozen 10^{-1} dilution of the faecal sample (−80°C into glycerol) in an anaerobic cabinet (N_2–H_2–CO_2 (85:10:5, by vol.)) in (g/l): NaCl 5·0, glucose 2·0, cysteine hydrochloride 0·3. For the following 3 weeks, rats were maintained on the basal diet. At t_0, rats received the Cp diet (Table 1) containing Camembert made from pasteurised milk (HMCp rats), the fm diet (Table 1) containing fermented milk (HMfm rats) or were kept on the basal diet B (HMB rats) for 6 weeks. The basal diets was sterilised by γ-irradiation at 45 kGy in plastic vacuum bags and offered as a paste prepared daily (described in Table 1).

Table 1. Composition of the diets

Diet...	B	Cp	fm
Basal diet components (g/kg)			
Casein	50	0	50
Soyabean-protein isolate	120	120	120
Maize starch	280	280	280
Mashed potato	280	280	280
Sucrose	40	40	40
Maize oil	30	30	30
Lard	50	0	50
Cellulose	70	70	70
Mineral and vitamins	80	80	80
Diet preparation (g/d per rat)			
Basal diet	15	15	15
Cheese*	0	50	0
Fermented milk	0	0	50
Water	20	20	15

* Protein 200 g/kg; fat 220 g/kg.

The microbial compositions of Camembert and fermented milk are shown in Tables 2 and 3. They were conditioned in sterile pots sealed with a double cover. The pots were placed daily in the isolators using peracetic acid (100 ml/l).

Sample collection

Several samples of faeces were collected from the rectum of each individually identified rat before (t_0), at 3 weeks (t_3) and 6 weeks (t_6) under the three dietary conditions. Fresh faecal samples collected at t_6 were pooled for enumeration of bacteria of dairy-product origin. The other samples were frozen at −80°C until analyses. For molecular analyses, faecal samples from three rats (no. 1, no. 2, no. 3) of each dietary group collected at t_0, t_3 and t_6 were used individually and in pools. For steroid analysis, faeces were collected at t_6 over two consecutive 24 h periods from each cage and stored at −80°C until analysis. The two sequential 24 h samples were pooled to give a single sample per cage (five rats per dietary group).

Rats were killed using CO_2 and their caeca were removed and weighed. The caecal contents were immediately frozen in liquid N_2 and stored at −80°C until determinations of enzymic activities and metabolites. The caecal samples from the three rats, no. 1, no. 2 and no. 3 of each group, were used for PCR–TTGE analysis.

All procedures were conducted in accordance with the Institute's Guide for the Care and Use of Laboratory Animals.

Micro-organism enumeration

The dairy products were provided from regular factory products every 2 weeks during the experiment. They were maintained at 4°C and introduced in isolators three times per week. Microbiological analyses were performed twice on each of the three batches of products: at the beginning and at the end of their use (after 2 weeks storage at 4°C). The crust and the inside of Camembert were analysed separately. Pimaricine (25 g/l water; 1 ml/l medium) (Sigma-Aldrich, Saint Quentin Fallavier, France) was added to analyse the crust of cheese in order to eliminate fungal micro-organisms.

The dairy products were analysed using culture in specific medium. Samples were diluted from 10^{-1} to

Table 2. Microbiological analyses (log colony-forming units/g) of Camembert and of faecal samples*

Micro-organism	Camembert†	Faeces HMCp‡	Faeces HMB‡
Streptococcus thermophilus	7·8–8·2	7·5	ND
Lactococcus	8·1–9·1	ND	ND
Leuconostoc mesenteroides	6·0–7·5	ND	7·5
Thermophilic *Lactobacillus*	0–4	ND	ND
Mesophilic *Lactobacillus*	0–7·3	7·7	ND
Hafnia alvei	4·7–8·6	ND	ND
Corynebacteria	0–5·1	6·1	ND
Micrococcaceae	2·7–6·8	6·8	ND
Geotrichum	7·7–7·9	7·0	ND
Yeast	3·7–6·8	5·3	ND
Penicillium	5·5–6·5	ND	ND
Enterobacteria	ND	6·8	5·75
Enterococcus	ND	7·5	9·0

ND, not detected.
* Rats were fed the basal diet (HMB group) or Camembert diet (HMCp group) for 6 weeks; for details of diets and procedures, see Table 1 and p. 430.
† Range from six analyses.
‡ From a pool of faecal samples collected at t_6 (after 6 weeks feeding of experimental diets).

Table 3. Microbiological analyses (log colony forming units/g) of fermented milk and of faecal samples*

Micro-organism	Fermented milk†	Faeces HMfm‡	Faeces HMB‡
Streptococcus thermophilus	8·8–8·3	ND	ND
Lactobacillus casei	8·2–8·5	8·3	ND
Lactobacillus delbruecki bulgaricus	6·3–7·1	ND	ND

ND, not detected.
* Rats were fed the basal diet (HMB group) or fermented milk diet (HMfm group) for 6 weeks; for details of diets and procedures, see Table 1 and p. 430.
† Range from six analyses.
‡ From a pool of faecal samples collected at t_6 (after 6 weeks feeding of experimental diets).

10^{-9} in medium containing tryptone (1 g/l; Biokar, Beauvais, France) and NaCl (9 g/l; Merck, Nogent sur Maine, France) and then surface-plated (0·1 ml) in duplicate on selective agars.

For lactic acid bacteria, De Mann–Rugosa–Sharpe medium (Difco, Becton Dickinson, Le Pont de Clair, France) was incubated at 30°C (mesophile) or 45°C (thermophile) for 48 h in anaerobiosis (Anaerocult; Merck). For *Streptococcus thermophilus,* M17 medium (Biokar) was used and plates were incubated at 45°C for 48 h in aerobiosis. For *Leuconostoc*, medium containing tomato juice (Difco) with 5 g calcium citrate/l (Merck) and 6 mg vancomycine/l (Sigma) was incubated for 3 d at 22°C. Enterococci were enumerated after 24 h incubation at 37°C using citrate azide Tween carbonate (Merck).

Micrococcaceae, Enterobacteriaceae and Coryneform bacteria were determined using tryptone soyabean agar (Oxoid, Dardilly, France) with and without NaCl (30 g/l). Bacteria were enumerated after 3 d incubation at 30°C.

Geotrichum and yeast were enumerated using modified Czapeck medium (g/l): $(NH_4)_2SO_4$ (Panreac, Lyons, France) 2, KH_2PO_4 (Merck) 1; KCl (Merck) 0·5, $MgSO_4.7H_2O$ (Merck) 0·5, $FeSO_4.7H_2O$ (VWR, Fontenay sous Bois, France) 0·01, glucose (Merck) 10, yeast extract (Difco) 2, agar (Biokar) 20. Plates were incubated for 6 d at 25°C. For *Penicillium* analysis, medium contained (g/l): glucose (Merck) 30, NaCl (Merck) 80, $NaNO_3$ (Merck) 3, KCl (Merck) 0·5, $MgSO_4.7H_2O$ (Merck) 0·5, K_2HPO_4 (Merck) 1, $FeSO_4$ (VWR) 0·01, yeast extract 5, agar (Biokar) 20. Plates were incubated for 6 d at 25°C.

Faecal samples were analysed at the end of the experiment using pools from each group. Because the objective was to analyse the microbiota from the dairy products ingested, nalidixic acid (30 g/l; Sigma) and pimaricine (25 g/l water, 1 ml/l medium; Sigma), were added to inhibit Gram-negative bacteria.

Gram staining, catalase production and API® plates were also used (BioMérieux, Marcy l'Etoile, France) to improve bacterial identification.

DNA isolation

Total DNA was extracted from 0·2 g faecal samples, 1·5 g cheese or 2 ml fermented milk (pellet from centrifugation at 15 000 ***g*** for 15 min) in 2·2 ml screw-capped tubes (Sarstedt International, Orsay, France), as described previously (Godon *et al.* 1997). The concentration and integrity of the nucleic acids were determined visually by electrophoresis on agarose (10 g/l) gel containing ethidium bromide.

PCR amplification

Primers U968-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401 (5′-GCG TGT GTA CAA GAC CC-3′) were used to amplify the V6 to

V8 regions of the bacterial 16S rDNA (Zoetendal *et al.* 1998). Primers Lac1 or Lac 352-f (5′-AGC AGT AGG GAA TCT TCC A-3′) and Lac 2GC or Lac 679-GC-r (5′-CGC CCG CCG CGC GCG CGG CGG GCG GGG GCG GGG GCG GGG CAC GGG GGG ATT YCA CCG CTA CAC ATG-3′) (Walter *et al.* 2001) were used for specific amplification of the 16S rDNA sequences of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* genera, belonging to the *Lactobacilli* group. These two sets of primers produced approximatively 433 and 327 bp PCR amplicons respectively. All primers were purchased from Qbiogene Research Services (Evry, France).

PCR was performed using HotStar Taq DNA polymerase (Qiagen, Courtaboeuf, France). The PCR mix (50 μl) contained 1 × PCR buffer, 1 mM-$MgCl_2$, 200 μM each dNTP, 20 pmol each primer, 2·5 U Hot Star Taq polymerase and approximately 2 ng DNA. The samples were amplified in a PCT 100 thermocycler (MJ Research, Inc., Albertville, MN, USA) using the following program: 95°C for 15 min, thirty cycles of 97°C for 1 min, 58°C for 1 min and 72°C for 90 s, and finally 72°C for 15 min for the first set of primers. Hybridation temperature was 61°C for 1 min for the primers Lac1 and Lac 2GC. PCR products were analysed by electrophoresis on agarose (10 g/l) gel containing ethidium bromide to check their size and estimate their concentration.

Temporal temperature gradient gel electrophoresis

PCR amplification allowed generation of DNA fragments that were of the same size, but variable in sequence. TTGE allows separation of the various DNA sequences as discrete bands by migration in a denaturing polyacrylamide gel containing urea, with a concomitant increase in temperature during electrophoresis.

The DCode Universal Mutation Detection System (Bio-Rad, Paris, France) was used for sequence-specific separation of PCR products. Electrophoresis was performed, as previously described (Seksik *et al.* 2003), through a 1 mm thick, 160 × 160 mm polyacrylamide (80 g/l) gel–acrylamide/Bis (37·5:1·0, v/v), 7 M-urea, 1·25 × Tris–acetate–EDTA, and 55 μl and 550 μl TEMED (Eurobio, Les Ulis, France) and ammonium persulfate (100 g/l) respectively using 7 litres 1·25 × Tris–acetate–EDTA as the electrophoresis buffer.

At the beginning of electrophoresis, for better resolution the voltage was fixed at 20 V for 15 min. For the first set of amplicons, electrophoresis was run for 16 h at 64 V with an initial temperature of 66°C and a ramp rate of 0·2°C per h. For amplicons obtained with primers Lac1 and Lac 2GC, the temperature range was 63·8–70·0°C with ramp rate of 0·4°C per h and at 66 V.

Each well was loaded with 100–200 ng amplified DNA plus an equal volume of 2 × gel loading dye (Bromophenol Blue (0·5 g/l)–xylene cyanol (0·5 g/l)–glycerol (700 g/l)). For each gel, three lanes were loaded with a marker obtained by mixing PCR products of seven rDNA clones isolated from one molecular inventory of one human faecal sample (Suau *et al.* 1999). Clones were related to members of the *Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides* group and *Bifidobacterium* group. After the completion of electrophoresis, the gels were stained in the dark by immersion for 30 min in a solution of SYBR Green I Nucleic Acid Gel Stain (Roche Diagnostics, Meylan, France) and read on a Storm system (Molecular Dynamics, Amersham Biosciences, Orsay, France).

Biochemical analyses

Enzyme activities were measured in caecal samples using a thermo-regulated anaerobic chamber (H_2–CO_2–N_2 (10:10:80, by vol.)), as previously described (Andrieux *et al.* 2002). Samples were diluted 1:20 using pre-reduced PBS (pH 6·7). α- and β-galactosidase, α- and β-glucosidase, β-glucuronidase, β-*N*-acetyl-galactosaminidase and α-L-fucosidase activities were measured by determining the rate of *p*-nitrophenol released from *p*-nitrophenyl glycosides. Azoreductase activity was determined using amaranth (5 mM) as substrate. Neuraminidase activity was measured using 4-methylumbelliferyl-*N*-acetylneuraminic acid as substrate. Nitrate reductase was determined by the generation of nitrite. Enzyme activities were expressed as μmol metabolised substrate per min and per g protein.

Protein concentration was determined in triplicate by the method of Lowry *et al.* (1951) on 1:500 faecal dilution in Na_2CO_3 (20 g/l) and NaOH (0·1 M). Bovine serum albumin was used as the standard.

SCFA concentrations in caecal samples were analysed in duplicate after water extraction of acidified samples using GC (Perkin-Elmer 1020 GC; Saint Quentin, France) (Andrieux *et al.* 2002). NH_3 was determined using the Berthelot method adapted by Dropsy & Boy (1961).

For bile acid analysis in faeces, 4 g faeces was thoroughly homogenised in a minimal volume of distilled water; lipids were extracted with 100 ml ethanol under reflux for 48 h in a Soxhlet apparatus. Ethanolic extracts (4 ml) were then saponified (120°C, 3 h, under pressure) in the presence of 1 ml 10 M-NaOH. Neutral steroids were removed from the saponified mixture, with 3 × 10 ml light petroleum (40–60°C) and bile acids were extracted from the remaining acidified (pH 2) aqueous phase with 3 × 10 ml diethyl ether. After evaporation of the solvent, bile acids were redissolved in 2 ml methanol. For chromatographic analysis, free bile acids were methylated with diazomethane, then sylilated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide–triethylchlorosilane and trimethylsilylimidazole–pyridine (1:4, v/v) (Supelco, Saint Quentin Fallavier, France) in dichloromethane. Trimethylsilyl derivates were analysed using a GC Peri 2000 (Perichrom, Saulx-les-Chartreux, France) equipped with a Ross injector and an OV-1 column (30 m × 0·32 μm × 0·2 μm; Perichrom). Respective proportions of the different molecular species of bile acids were determined using calibration lines obtained from the analysis of standard mixtures and 5α-cholestan as external standard.

Statistical analyses

PCR–TTGE profiles were compared using GelCompar software (GelComparII™, version 2.0; Applied Maths, Kortrigk, Belgium). Analyses take into account number,

intensity and position of PCR–TTGE bands (PCR-amplified 16S rDNA fragments) in the gel. Pearson correlation provided similarity based upon densitometric curves. Similarity coefficients of Pearson were calculated to generate a similarity matrix. The dendrogram was obtained from this matrix by using the algorithm UPGMA (Unweighted Pair Group Method using Arithmetic averages).

Results of biochemical analyses were expressed as mean values with their standard errors of the mean. Data were analysed using ANOVA (StatView; Abacus Concepts, Berkeley, CA, USA). Where ANOVA indicated significant treatment effects, mean values were compared using the Newman–Keuls procedure. Statistical significance was accepted at $P<0{\cdot}05$.

Results

Bacterial PCR–temporal temperature gradient gel electrophoresis analysis of faecal samples

At t_0, 3 weeks after the human microbiota inoculation and before the dairy products were given, faecal samples of three rats per group were analysed individually and in pools. The PCR–TTGE profiles of faecal pools were representative of the profile of individual samples within the pool (Pearson similarity coefficient (Psc) $>94\,\%$) (Fig. 1). Faecal microbiota profiles were similar between the three experimental groups (Psc $>95{\cdot}9\,\%$).

In the control group (HMB), the comparison between PCR–TTGE profiles of pools of faecal samples at different times (3 weeks (t_0), 6 weeks (t_3) and 9 weeks (t_6) after human faecal inoculation) indicated a high stability of the microbiota over time for the period of 9 weeks.

As in the control group, in HMCp and HMfm rats fed the dairy products the profiles of faecal pools were representative of the individual profile, over time (Psc >94 to $96\,\%$). In HMCp rats, however, profiles were quite similar at t_0 and t_3 (Psc $>93\,\%$), some bands appeared or disappeared during Camembert consumption (Fig. 2). No differences were observed between profiles obtained at t_3 and t_6 (Psc $>95\,\%$)

In HMfm rats, Psc was only $90\,\%$ between t_0 and t_3 and $96\,\%$ between t_3 and t_6 (Fig. 3).

No bands specific to the PCR–TTGE profiles of dairy products were observed in the profiles of HMfm and HMCp rats during the experimental diet when bacterial PCR primers were used (Figs 2 and 3).

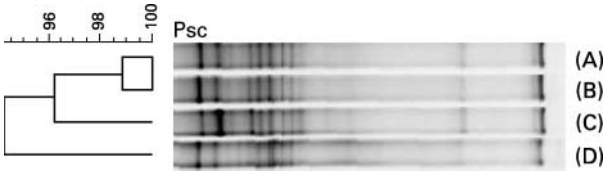

Fig. 1. Comparison between temporal temperature gradient gel electrophoresis profiles of PCR products of the V6 to V8 regions from DNA of pooled and individual faecal samples from human microbiota-associated (HM) rats. (A), faecal sample from HM rat no. 1 at t_0; (B), faecal sample from HM rat no. 2 at t_0; (C), faecal sample from HM rat no. 3 at t_0; (D), pool of faeces from HM rats no. 1, no. 2 and no. 3 at t_0. For details of procedures, see p. 430.

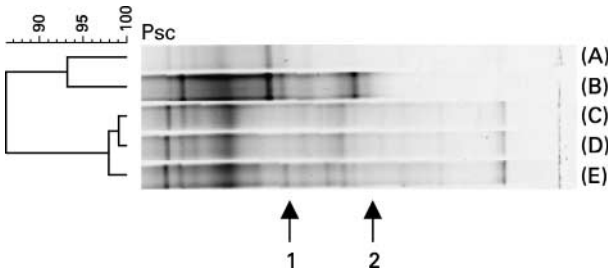

Fig. 2. Comparison between temporal temperature gradient gel electrophoresis profiles of PCR products of the V6 to V8 regions from DNA of the Camembert and faecal samples from a group of human microbiota-associated rats fed on diet containing Camembert made from pasteurised milk (HMCp). (A), Camembert (inside); (B), Camembert (crust); (C), pool of faeces (HMCp group, n 3) at 6 weeks (t_6); (D), pool of faeces (HMCp group, n 3) at 3 weeks (t_3); (E) pool of faeces (HMCp group, n 3) at 0 weeks (t_0). For details of diets and procedures, see Table 1 and p. 430. Alteration of the profile during Camembert consumption is illustrated by arrow 1 showing the disappearance of a band at t_3 and t_6 and by arrow 2 showing the appearance of a new band at t_3 and t_6.

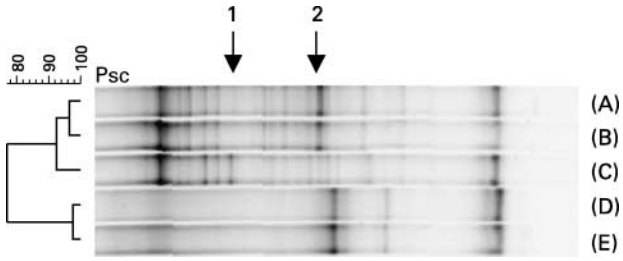

Fig. 3. Comparison between temporal temperature gradient gel electrophoresis profiles of PCR products of the V6 to V8 regions from DNA of the fermented milk and faecal samples from a group of human microbiota-associated rats fed on diet containing fermented milk (HMfm group). (A), pool of faeces (HMfm group, n 3) at 3 weeks (t_3); (B), pool of faeces (HMfm group, n 3) at 6 weeks (t_6); (C), pool of faeces (HMfm group, n 3) at 0 weeks (t_0); (D), fermented milk (one sample); (E), fermented milk (other sample). For details of diets and procedures, see Table 1 and p. 430. Alteration of the profile during fermented milk consumption is illustrated by arrow 1 showing the disappearance of a band at t_3 and t_6 and by arrow 2 showing the appearance of a new band at t_3 and t_6.

At the end of the experiment, caecal and faecal PCR–TTGE profiles were similar in all groups (Psc $>99\,\%$).

Lactobacilli *group-specific PCR–temporal temperature gradient gel electrophoresis analysis of faecal samples*

With *Lactobacilli* group-specific PCR primers, specific bands from fermented milk were obtained in faecal profiles of HMfm rats (Fig. 4). No bands were obtained for the cheese sample. However, in faecal samples from HMCp rats, *Lactobacilli* group-specific bands were obtained at t_6, whereas no band was found at t_0.

Enumeration in faecal samples of micro-organisms from Camembert and fermented milk

Results obtained in cheese samples and in faecal samples are reported in Table 2. Camembert contained mainly lactic acid bacteria *Lactococcus* (8·1–9·1 log colony forming units/g),

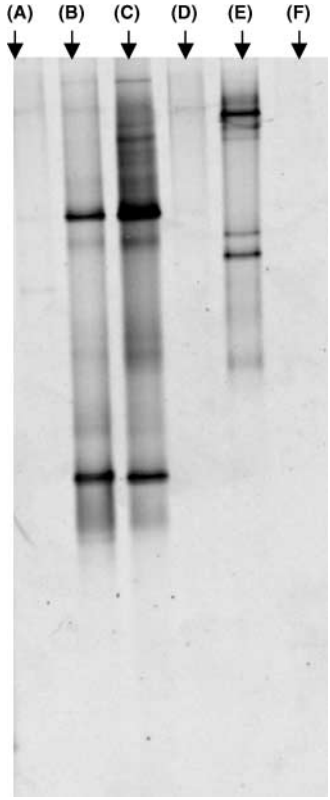

Fig. 4. Temporal temperature gradient gel electrophoresis profiles of *Lactobacilli* group-specific PCR product from DNA of the fermented milk, Camembert and faecal samples from human microbiota-associated rats fed diets containing fermented milk (HMfm) or Camembert (HMCp) at t_0 (before ingestion of dairy products) and after 6 weeks consumption of dairy products (t_6). (A), HMfm sample at t_0; (B), HMfm sample at t_6; (C), fermented milk; (D), HMCp at t_0; (E), HMCp at t_6; (F), Camembert.

Streptococcus thermophilus (7·8–8·2 log colony forming units/g), *Leuconostoc* (6·0–7·5 log colony forming units/g). Non-starter mesophilic and thermophilic *Lactobacillus* were found occasionally (0·0–7·3 log colony forming units/g). The ripening microbiota was mainly composed of associated *Hafnia alvei*, coryneform bacteria, *Micrococcaceae, Geotrichum*, *Penicillium* and yeast.

Several bacterial species from cheese *Streptococcus thermophilus,* mesophilic *Lactobacillus*, *Micrococcaceae* and *Geotrichum* were found at levels of 5·3–7·7 log colony forming units/g faecal samples of HMCp rats feeding on Camembert for 6 weeks. *Lactococcus, Leuconostoc*, *Penicillium* and *Hafnia alvei* were not detected (Table 2).

Fermented milk contained high levels of *Streptococcus thermophilus*, *Lactobacillus casei* and *Lactobacillus delbrueckii bulgaricus* (Table 3).

In faecal samples of rats fed the fermented milk for 6 weeks, *Lactobacillus casei* was found at 8·3 log colony forming units/g, whereas *Streptococcus thermophilus* and *Lactobacillus delbrueckii bulgaricus* were not detected (Table 3).

Caecal bacterial metabolism

Comparison between rats fed the basal diet (HMB) and rats fed Camembert (HMCp) revealed that in caecal samples of rats fed Camembert, azoreductase activity was significantly lower and mucolytic activities (neuraminidase, *N*-acetylgalactosaminidase and fucosidase) were higher (Table 4). SCFA caecal concentration and SCFA profile were not significantly modified, but NH_3 caecal concentration was lowered (Table 5).

In caecal samples from the HMfm rats fed fermented milk, α and β-galactosidase were higher than in other groups. Neuraminidase and fucosidase activities were higher than in the HMB rats, but *N*-acetyl galactosaminidase was not modified. Azoreductase and nitrate reductase activities were both reduced (Table 4). NH_3 concentration was lower in HMCp and HMfm rats compared with HMB rats, without change in SCFA profile (Table 5).

Faecal bile acids

As illustrated in Table 6, only ursodeoxycholic and ω-muricholic acids, resulting from chenodeoxycholic and β-muricholic microbial epimerisation respectively were significantly affected by diet. When expressed as % composition of bile acids in faeces, ursodeoxycholic acid, which was not detected in faeces from control rats

Table 4. Enzymic activities (μmol/min per g protein) in caecal contents of human microbiota-associated rats*

(Mean values with their standard errors for ten rats per group)

Group...	HMB		HMCp		HMfm	
	Mean	SEM	Mean	SEM	Mean	SEM
β-Galactosidase	1·54[b]	0·14	1·50[b]	0·17	2·00[a]	0·10
α-Galactosidase	0·66[b]	0·03	0·63[b]	0·04	0·78[a]	0·04
β-Glucosidase	0·26	0·04	0·17	0·03	0·26	0·03
β-Glucuronidase	0·22	0·01	0·20	0·02	0·27	0·02
Neuraminidase	0·05[b]	0·01	0·12[a]	0·03	0·10[a]	0·02
N-acetylgalactosaminidase	0·061[b]	0·008	0·098[a]	0·006	0·063[b]	0·005
α-L-Fucosidase	0·058[b]	0·005	0·071[ab]	0·005	0·097[a]	0·041
Nitrate reductase	0·083[a]	0·020	0·090[a]	0·020	0·024[b]	0·006
Azoreductase	0·065[a]	0·010	0·025[b]	0·008	0·024[b]	0·003

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Rats were fed the basal diet (HMB group), the diet containing Camembert (HMCp group) or fermented milk (HMfm group) for 6 weeks; for details of diets and procedures, see Table 1 and p. 430.

Table 5. Concentrations of SCFA (μmol/g caecal contents), lactic acid and ammonia in caecal contents of human microbiota-associated rats*

(Mean values with their standard errors for ten rats per group)

Group...	HMB		HMCp		HMfm	
	Mean	SEM	Mean	SEM	Mean	SEM
SCFA	76·1	7·2	69·1	3·0	64·1	7·1
Acetate	52·9	4·8	49·1	2·3	45·9	5·0
Propionate	12·6	1·5	9·3	0·4	8·8	1·0
Butyrate	8·4	0·9	8·5	0·4	7·2	0·9
Valerate + caproate	1·56	0·12	1·36	0·30	1·66	0·15
Iso-acids	0·62	0·40	0·83	0·22	0·89	0·13
Lactic acid	4·75	0·17	3·35	0·10	3·74	0·15
NH_3	3·25[a]	0·23	2·08[b]	0·08	2·28[b]	0·22

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Rats were fed the basal diet (HMB group), the diet containing Camembert (HMCp group) or fermented milk (HMfm group) for 6 weeks; for details of diets and procedures, see Table 1 and p. 430.

(HMB), significantly and similarly increased with both dairy products. However, when expressed as μg bile acids/g faeces, this increase remained significant in HMfm rats only. HMCp rats was intermediate between HMB and HMfm rats. ω-Muricholic acid, whether expressed as % composition or faecal concentration, was also significantly increased in HMfm rats. Ursocholic acid, bacterial epimer of cholic acid, was not detected in the HMB rats, and occasionally appeared in faeces of rats fed either dairy product, but the increase did not reach statistical significance. Neither dairy product affected the other secondary bile acids, deoxycholic and lithocholic acids, resulting from 7α-dehydroxylation of cholic and chenodeoxycholic respectively. Last, total bile acid concentration in faeces was the lowest in HMB rats and the highest in the HMfm rats. It was intermediate in the HMCp rats, and did not significantly differ from both other groups.

Discussion

The soft cheese Camembert contained a complex microbiota mainly composed of *Streptococcus thermophilus*, *Lactobacillus, Lactococcus, Leuconostoc, Hafnia alvei* and *Geotrichum,* each reaching at least 7–8 log colony forming units/g. Our present approach is the first experimental study on the impact of cheese consumption on the intestinal microbiota. The objectives were: first, to determine the capacity of the cheese micro-organisms to survive through the gastrointestinal transit; second, to study the influence of these bacteria on a human microbiota established in our rat model; third, to study the influence of

Table 6. Distribution and concentration of bile acids in faeces from human microbiota-associated rats*

(Mean values with their standard errors for five rats per group)

Group...	HMB		HMCp		HMfm	
	Mean	SEM	Mean	SEM	Mean	SEM
Bile acids (mass %)						
Cholic	8·86	0·62	7·42	0·48	7·19	0·37
Deoxycholic	43·10	3·02	42·35	1·12	40·06	1·33
Ursocholic	0·00	0·00	0·89	0·89	1·69	1·04
Chenodeoxycholic	17·15	8·83	5·10	0·76	5·44	0·23
Lithocholic	13·57	5·54	16·74	1·60	11·32	1·36
Ursodeoxcholic	0·00[a]	0·00	3·08[b]	1·33	3·74[b]	0·39
β-Muricholic	9·62	2·58	13·10	1·84	13·69	0·54
ω-Muricholic	7·70[a]	2·49	11·32[a]	1·05	16·88[b]	1·06
Bile acids (μg/g faeces)						
Cholic	0·86	0·19	0·85	0·05	1·32	0·17
Deoxycholic	4·34	1·25	5·03	0·76	7·59	1·39
Ursocholic	0·00	0·00	0·10	0·10	0·30	0·18
Chenodeoxycholic	1·36	0·52	0·59	0·09	1·04	0·20
Lithocholic	1·10	0·45	1·87	0·02	1·96	0·07
Ursodeoxcholic	0·00[a]	0·00	0·38[ab]	0·18	0·69[b]	0·14
β-Muricholic	1·01	0·35	1·66	0·51	2·62	0·54
ω-Muricholic	0·91[a]	0·46	1·34[a]	0·21	3·26[b]	0·68
Total bile acids	9·58[a]	1·93	11·82[ab]	1·64	18·78[b]	3·06

[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Rats were fed the basal diet (HMB group) or diets containing Camembert (HMCp group) or fermented milk (HMfm group) for 6 weeks; for details of diets and procedures, see Table 1 and p. 430.

cheese consumption on intestinal microbiota metabolism. The results were compared with those obtained with the fermented milk containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii bulgaricus* and *Lactobacillus casei* strains. This fermented milk has been previously studied with our model (Djouzi *et al.* 1997).

It has been shown that when human faecal microbiota is inoculated into rats, the microbiota maintains its major metabolic characteristics (Andrieux *et al.* 1991; Rumney & Rowland, 1992). However, little is known on the reproducibility of the microbial populations implanted in intestine of rats. PCR–TTGE analyses showed a high reproducibility and stability over time of the faecal microbiota profiles between groups in our present model. The HM rat model was therefore very useful to compare the effects of dietary components on the microbiota established in the intestinal ecosystem. Moreover, the similarity of faecal and caecal microbiota profiles suggested that the bacterial metabolism assessed in faecal samples could be related to metabolic variables analysed in caecal contents. SCFA were analysed in the caecum rather than in faeces because of their rapid absorption through the colonic epithelium (Macfarlane *et al.* 1991).

In HM rats fed the diet containing Camembert for several weeks, many microbial species from cheese were found at high levels in faecal samples, i.e. *Streptococcus thermophilus*, mesophilic *Lactobacillus*, yeast and *Geotrichum*. These micro-organisms were not found in rats fed the basal diet, demonstrating their capacity to resist during the gastrointestinal transit. The results obtained with the fermented milk confirmed the survival of *Lactobacillus casei*, but not that of *Lactobacillus delbrueckii bulgaricus* and *Streptococcus thermophilus.* Although lactic acid bacteria were found at high concentrations when culture analyses were used, they were not detected when using universal PCR primers and TTGE, suggesting that the transient bacteria constitute only a minor part of the total microbiota. Similarly, Tannock *et al.* (2000) did not detect *Lactobacillus rhamnosus* DR20 in human faecal samples using PCR–denaturing gradient gel electrophoresis and universal primers, whereas *Lactobacillus rhamnosus* was detected when group specific primers Lac1 and Lac2GC or culture analysis were used (Walter *et al.* 2001). In our present study, the use of the Lac1 and Lac 2GC primers lowered the detection limit so that *Lactobacilli* could be detected by PCR–TTGE in faecal samples in HMfm and HMCp groups during consumption of the dairy products. *Lactobacillus* species could be detected even in the presence of DNA from the predominant members of the faecal microbiota (about 10^{11} cells/g) in the PCR. With the same approach, using specific primers, Satokari *et al.* (2001) detected *Bifidobacterium lactis* Bb12 in human subjects during the ingestion of Bb12 in fermented milk.

Bacterial PCR–TTGE detects 90–99 % of the numerically dominant bacterial species in faecal samples (Zoetendal *et al.* 1998). We observed only slight variations of the PCR–TTGE profiles during dairy product consumption, suggesting that the dominant microbiota was not greatly modified especially when rats were fed Camembert. This agrees with previous studies in gnotobiotic rats (Djouzi *et al.* 1997) and in human subjects, thus showing the stability of the dominant autochthonous faecal microbiota during consumption of fermented milks containing yoghurt starters, *Lactobacillus casei* or *Bifidobacterium* (Bartram *et al.* 1994; Guérin-Danan *et al.* 1998; Tannock *et al.* 2000; Satokari *et al.* 2001). However, with group-specific PCR primers we were able to monitor the variations of *Lactobacilli* at sub-dominant level.

It is difficult to prove that bacteria from cheese exert metabolic activity when they pass through the intestinal tract of HM rats. Caecal SCFA concentration was not significantly affected by cheese ingestion. However, several changes were observed in rats fed Camembert. The increase of mucolytic activities in HMCp rats may be related to the mucolytic activities of the cheese bacteria. The decrease in azoreductase activity suggests that the cheese bacteria, which do not produce reductase, were able to influence the reductase activity of the human microbiota. This beneficial influence of cheese was also observed with fermented milk. In addition, with fermented milk we observed a significant increase of β-galactosidase activity that could be due either to the β-galactosidase activity of bacteria provided by fermented milk or to the lactose contained in fermented milk used as substrate by the autochthonous microbiota.

Bile acid modifications in faeces are also a sensitive marker of microbial activity in the hindgut, since bile acid molecular profile and different bile acids exhibit distinct biological effects. To our knowledge, the effect of dairy products on bile acid profile in faeces has not been investigated before. We demonstrated an increase in bacterial epimerisation of bile acids under cheese and fermented milk consumption. This effect was more pronounced with fermented milk. As for other enzyme activities investigated in our present study, the question arises whether this was carried out by microbial enzymes from the dairy products or from the indigenous microbiota as modified by dairy product consumption. The first hypothesis might be the right one, since we also observed epimerisation of bile acids in germ-free rats fed Camembert (results not shown). Microbial epimerisation of bile acids is not direct, but proceeds in two steps catalysed by two hydroxysteroid dehydrogenases. Epimerisation of cholic and chenodeoxycholic acids to ursocholic and urosdeoxycholic acids respectively requires a 7α- and a 7β-hydroxysteroid dehydrogenase, whereas epimerisation of β-muricholic to ω-muricholic acid requires a 6α- and a 6β-hydroxysteroid dehydrogenase. These enzymes would therefore be present in dairy products and would operate in the digestive tract of a HM rat model. This is of particular interest for health, since hydrophilic bile acids, such as ursodeoxycholic and muricholic acids, prevent cholestasis and liver damage (Milkiewicz *et al.* 2002). Ursodeoxycholic acid has been shown to be a chemopreventive agent (Earnest *et al.* 1994) and inhibits cell proliferation but does not induce apoptosis (Martinez *et al.* 1998). Interestingly, faecal deoxycholic and lithocholic acids, which are considered as tumour promoters in the colon (Cohen *et al.* 1980; Milovic *et al.* 2001), were not significantly affected by feeding on either dairy product, so that the overall faecal bile acid profile was displaced towards selective

enrichment in beneficial bile acids. This of course should be further evaluated in human subjects.

The results obtained here show that, as fermented milk, Camembert consumption did not greatly modify the dominant intestinal microbiota or the major metabolic activities. But, we observed that micro-organisms from cheese microbiota were able to partly survive the intestinal transit. Moreover, cheese consumption led to a potentially beneficial influence on the intestinal metabolism such as a decrease in azoreductase activity, a decrease in NH_3 concentration and an increase of the proportion of ursodeoxycholic.

Because great variability was observed between human subjects (Andrieux *et al.* 2002), we cannot generalise on the results obtained with a single microbiota. Nevertheless, several animal and human studies have observed the decrease of deleterious activities or metabolite production during probiotic administration (Bezkorovainy, 2001).

More information should be obtained in human volunteers to confirm our present results and identify the micro-organisms from cheese able to survive in the human intestinal tract and exert metabolic activities. Moreover, other investigations will be necessary to investigate mechanisms underlying potentially beneficial effects of cheese.

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