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Effects of orally administered *Lactobacillus casei* DN-114 001 on the composition or activities of the dominant faecal microbiota in healthy humans

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The composition and activities of the faecal microbiota in twelve healthy subjects analysed in a single open study were monitored before (1-week baseline step), during (10 d supplementation step) and after (10 d follow-up step) the ingestion of a fermented milk containing *Lactobacillus casei* DN-114 001. Fluorescent *in situ* hybridisation with group-specific DNA probes, real-time PCR using *L. paracasei* group-specific primers and temporal temperature gradient gel electrophoresis (TTGE) using group-specific primers were carried out, together with bacterial enzyme activity and metabolite analyses to monitor the structure and activities of the faecal microbiota. *L. casei* DNA was detected in the faeces of all of the subjects by TTGE after 10 d supplementation. Its quantification by real-time PCR showed a 1000-fold increase during the test step compared with initial levels. No major modification in either the dominant members of the faecal microbiota or their activities was observed during the trial. In conclusion, the short-term consumption of a milk product containing *L. casei* DN-114 001 was accompanied by a high, transient increase in the quantity of this strain in the faeces of all of the subjects without markedly affecting biochemical or bacteriological factors.

Human faecal microbiota: Probiotic: *Lactobacillus casei*: Real-time PCR: Fluorescent *in situ* hybridisation

A large and complex microbial community inhabits the human intestinal tract. This community includes a wide variety of bacterial species (Tannock, 2002). The intestinal microbiota is of major importance in maintaining human health and well-being. These bacteria, predominantly anaerobes, are involved in the fermentation of undigested carbohydrates, the metabolism of endogenous and exogenous compounds, the prevention of colonisation by pathogens and the stimulation of the immune system (Gibson *et al.* 1995; Salminen *et al.* 1998). Various disease states are associated with an imbalance of the intestinal microbiota. These can include susceptibility to pathogens such as *Clostridium difficile*, chronic diseases such as Crohn's disease and ulcerative colitis, acute gastroenteritis, food hypersensitivity and allergies, and colon cancer (Gibson *et al.* 1999). The intake of probiotics as a dietary supplement has been used to reinforce or modify the microbiota.

Probiotics are living micro-organisms that, upon ingestion in sufficient numbers, exert health benefits beyond basic nutrition (Fuller, 1989). The use of probiotics has been associated with the prevention, alleviation or cure of diverse intestinal disorders such as lactose intolerance, viral and bacterial diarrhoea, constipation, inflammatory bowel diseases and food allergy (Marteau *et al.* 2001). Lactic acid bacteria and other micro-organisms are currently being used as probiotics, either singly or in association. The most common species of probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, which have a long history of safe use in the manufacture and human consumption of dairy products (Vaughan *et al.* 1999).

The evidence supporting their efficacy in the treatment or prevention of intestinal disorders is rapidly increasing. Numerous members of the genus *Lactobacillus* are used as probiotics. *Lactobacillus delbrueckii* (ssp. *bulgaricus)* from yoghurt appeared particularly effective in increasing lactose digestion (Marteau *et al.* 1990). *Lactobacillus rhamnosus* GG was shown to be significantly more effective than a placebo in decreasing the risk of diarrhoea in healthy subjects receiving antibiotics (Siitonen *et al.* 1990). Milk fermented with *L. casei* DN-114 001 reduced the duration of diarrhoeal episodes (Pedone *et al.* 1999) and the incidence of diarrhoea in children (Pedone *et al.* 2000). Interestingly, this strain was also able to significantly modify β-glucuronidase and β-glucosidase activity in the intestinal microbiota of young infants (Guérin-Danan *et al.* 1998).

Abbreviations: Cy5, indodicarbocyanine; D, day; FISH, fluorescence *in situ* hybridisation; FITC, fluorescein isothiocyanate; TTGE, temporal temperature gradient gel electrophoresis.

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To apply lactic acid bacteria as probiotics, it is important to determine whether these bacteria survive in the gastrointestinal tract during consumption by humans. The survival rate in the faeces varies greatly between species and strains. Strains that survive generally comprise between 1 % and 5 % of the quantity ingested (Drouault & Corthier, 2001). Most of the probiotic strains are excreted in faeces during ingestion and for several days after the end of the supplementation step. Recent studies have, however, shown that lactobacilli and bifidobacteria persist longer in the faeces of some individuals (Alander *et al.* 1999; Fujiwara *et al.* 2001; Collins *et al.* 2002).

Our present knowledge of the intestinal microbiota has been obtained using bacteriological culture techniques and microscopy, but, according to direct cell counts, 60–70 % of faecal bacteria remain uncultured (Suau *et al.* 1999; Tannock, 2002). The application of culture-independent molecular techniques based on 16S rRNA sequences has resulted in more detailed investigations of the intestinal microbiota. Denaturing gradient gel electrophoresis of 16S rRNA gene amplicons has allowed the detection of bacterial species and changes in community structure (Zoetendal *et al.* 1998, 2001). On the other hand, PCR techniques with universal, group-specific or species-specific primers have been developed for detecting bacteria in intestinal ecosystems (Wang *et al.* 1996; Matsuki *et al.* 2002; Ott *et al.* 2004), and the quantification of single cells within complex ecosystems is accomplished by fluorescent *in situ* hybridisation (FISH) with specific 16S rRNA-targetted oligonucleotide probes directed at different phylogenetic levels.

The aims of the present study were to study the orofaecal persistence of amplifiable DNA from *L. casei* DN-114 001 upon consumption in a fermented milk product and to investigate the effects of its consumption on the composition and metabolic activities of the intestinal microbiota in healthy human subjects. Persistence of the probiotic amplifiable DNA was assessed using temporal temperature gradient gel electrophoresis (TTGE), a qualitative method, and real-time PCR, which made it possible to quantify the probiotic equivalents.

Subjects and methods

Subjects

Twelve healthy subjects, seven women and five men, aged 23–44 years were selected for this study. Enrolled subjects had a Western European diet. Inclusion criteria were a lack of history of digestive pathology, no current medication affecting the intestinal microbiota, and a moderate consumption of fermented milk products (fewer than two fermented milk products per week). Informed consent was obtained from all subjects, and the study was approved by the Ethics Committee of Necker Hospital. Subjects were included and completed the study without any major deviation, and with a good compliance with the study product.

Dairy product

The test product was fermented milk, delivered in a 100 ml bottle. The milk was fermented with yoghurt cultures (*Streptococcus thermophilus* and *L. bulgaricus*) and *L. casei* DN-114 001. Each bottle contained 10^8 colony-forming units/ml *L. casei* DN-114 001. The product is usually marketed under the name Actimel. At the expiry date, platings were performed using Man Rogosa Sharp medium (VWR International, Strasbourg, France) and no loss of viability was observed. The product was supplied by Danone Vitapole (Palaiseau, France).

Study design and faecal samples

During the 4-week study step, there were no restrictions with regard to diet, except for the consumption of fermented dairy products, which had to be fewer than two fermented milk products per week. The single-centre, open study was divided into three steps: a 1-week baseline step (D−7 to D0), a 10 d supplementation step (D0–D10), and a 10 d follow-up step (D10–D20). During the supplementation step, each subject ingested three bottles of 100 ml of the fermented milk product per day. Faecal samples were collected in sterile containers under anaerobic conditions using an Anaerocult A (Merck, Nogent sur Marne, France) before (D0), during (D10) and after (D20) the supplementation step. The samples were stored at 4°C, sent to the laboratory in refrigerated containers within 3–12 h of collection and processed within 6 months of conservation for analysis, as described below.

DNA extraction

Total DNA was extracted as described in Seksik *et al.* (2003) from 0·2 g faecal samples, from pellets obtained with 5 ml liquid culture of *L. casei* in Man Rogosa Sharpe broth, and from pellets obtained with 2 ml Actimel in 2·2 ml screw-cap tubes (Sarstedt, Ursay, France). The DNA of *L. bulgaricus* was extracted from traditional yoghurt cultures. The concentration and integrity of the nucleic acids were determined visually by electrophoresis on 1 % agarose gel. DNA was extracted from varying quantities of faeces (from 0·05–0·4 g) to ascertain that there was a linearity between the quantity of faecal material and the quantity of DNA extracted up to the 0·2 g faeces used in this study (data not shown).

Temporal temperature gradient gel electrophoresis analyses

TTGE was applied to 16S rRNA genes amplified by PCR from total bacterial DNA using two different sets of primers. *Bifidobacterium* genus-specific PCR was performed using Bif 164-f and Bif 662-GC-r primers (Satokari *et al.* 2001) and *Lactobacillus–Pediococcus–Leuconostoc–Weissella* group-specific PCR with primers Lac1 and Lac2GC (Walter *et al.* 2000). Bif- and Lac-specific primers produced 520 bp and 400 bp PCR amplicons, respectively. PCR conditions were as described in Seksik *et al.* (2003) using the Hot Start *Taq* polymerase (Qiagen Courtaboeuf, France). PCR amplicons obtained with primers Bif 164-f and Bif 662-GC-r were separated using a temperature range of 66–70°C with a ramp rate of 0·2°C/h and a voltage of 64 V. For amplicons obtained with primers Lac1 and Lac2GC, the temperature range was 63·8–70°C with a ramp rate of 0·4°C/h and a voltage of 66 V.

Electrophoresis was run for 16 h with 15 min at 20 V at the beginning to increase the resolution. For each gel, we loaded three lanes with a marker consisting of the PCR products of seven cloned rRNA genes obtained in our laboratory (Suau *et al.* 1999). Gels were stained with SYBR-Green I Nucleic Acid Gel Stain (Roche Diagnostics, Meylan, France) and read on a Storm system (Molecular Dynamics Bonoloufle, France). TTGE profiles were analysed using GelCompar software (version 2.0; Applied Maths, Kortrijk, Belgium). Analysis included interpattern comparisons using the Pearson coefficient calculated as a measure of the degree of similarity.

Lactobacillus paracasei group-specific SYBR-Green real-time PCR

The *L. paracasei* group contains the species of the probiotic strain *L. casei* DN-114 001. *Lactobacillus paracasei* group-specific SYBR-Green real-time PCR was performed using 16S rRNA gene-targetted primers Lp1 5′-GTGCTTGCACCT-AGATTCAACATG-3′ and Lc2 5′-TGCGGTTCTTGGATCT-ATGCG-3′ designed using PrimerExpress 1·0 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) (Furet *et al.* 2004). The oligonucleotides were synthesised by MWG-Biotech (Ebersberg, Germany).

Gene quantification was performed on ABI Prism 7700 sequence Detection System (Perkin-Elmer Applied Biosystems). Quantitative PCR reactions were performed in 10 μl of a ten-fold dilution of 300 μl DNA extracted from 0·2 g of each faecal sample as template and 15 μl of 1X PCR mix (SYBR-Green I PCR core kit, Applied Biosystems), with optimum concentrations of primers. Some samples contained inhibitors (data not shown), as evidenced using the IPC kit (Perkin Elmer Applied Biosystems). For these samples, higher dilutions of DNA extracts (1/100 or 1/1000) were used for the PCR to circumvent inhibition. The efficiency of PCR amplification was checked for various primer concentrations. Thermal cycling conditions were as follows: 10 min at 95°C followed by 45 repeats of 15 s at 95°C, and 1 min at 60°C.

During each run, a standard dilution of the PCR fragment with a known quantity (determined by spectrophotometry) was included to estimate gene quantification using Excel linear regression. In each run, a negative control (distilled water) was included according to the manufacturer's instructions. For each genomic DNA quantification, measurements of the copy number were taken three times (triplicates), and the mean value was used for analysis. Calculation of bacterial equivalents of the *L. paracasei* group was based on the presence of five rRNA operons in the genome of *L. casei*, formerly estimated by restriction of genomic DNA and Southern blots (Furet *et al.* 2004). DNA quantifications determined by real-time PCR were calculated using the results of triplicates.

Fluorescence in situ *hybridisation and flow cytometry*

The probes used in FISH targeted the small subunit rRNA. The targeted phylogenetic groups, sequences and references of the control and group-specific probes are presented in Table 1.

Stools were homogenised and fixed using 4 % paraformaldehyde in PBS as previously described (Rochet *et al.* 2001). After overnight fixation at 4°C, fixed suspensions were stored at −70°C until use for hybridisation (Rochet *et al.* 2001). A EUB338 probe was used as a positive control probe. Conversely, a NON338 probe designed by Wallner *et al.* (1993) was used as a negative control probe. These

Table 1. 16S rRNA-targeted oligonucleotide probes used in fluorescent *in situ* hybridisation experiments

Probe	Sequence 5′ → 3′	Targeted phylogenetic group	Oligonucleotide probe database nomenclature	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Domain bacteria	S-D-Bact-0338-a-A-18	Amann *et al.* 1990
NON338	ACATCCTACGGGAGGC	Negative control	S-D-Bact-0338-a-S-16	Amann *et al.* 1990
Ato291	GGTCGGTCTCTCAACCC	*Atopobium*	S-*-Ato-0291-a-A-17	Harmsen *et al.* 2000
Bac303	CCAATGTGGGGGACCTT	*Bacteroides/Prevotella*	S-*-Bac-303-a-A-17	Manz *et al.* 1996
Bif164	CATCCGGCATTACCACCC	*Bifidobacterium* genus	S-G-Bif-0164-a-A-18	Langendijk *et al.* 1995
Erec482	GCTTCTTAGTCARGTACCG	*Eubacterium rectale–Clostridium coccoides*	S-*-Erec-0482-a-A-19	Franks *et al.* 1998
Fprau645	CCTCTGCACTACTCAAGAAAAAC	*Fusobacterium prausnitzii* and related	S-*-Fprau-0645-a-A-23	Suau *et al.* 2001
Lab158	GGTATTAGCAYCTGTTTCCA	*Lactobacillus–Enterococcus*	S-G-Lab-0158-a-A-20	Harmsen *et al.* 1999
Enter1432	CTTTGCAACCCACT	Enterobacteria	S-*-Ent-1432-a-A-15	Sghir *et al.* 2000

R is an A or G; Y is a C or T.

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two control probes were covalently linked at their 5′ end to either fluorescein isothiocyanate (FITC) or indodicarbocyanine (Cy5) (Interactiva, St Malo, France). The group-specific probes were labelled at their 5′ end with Cy5. The fixed suspensions were permeabilised and hybridised as described in Rigottier-Gois *et al.* (2003). Aliquots of hybridised suspensions were added to 1 ml FACS FLOW (Becton Dickinson, Pont de Claix, France) for flow cytometry acquisition.

Data were obtained with a FACS Calibur flow cytometer (Becton Dickinson) equipped with an air-cooled Ar ion laser providing light at 488 nm combined with a 635 nm red-diode laser. Analyses were made using CellQuest Software (Becton Dickinson) as previously described (Rigottier-Gois *et al.* 2003). The enumeration of cells was done by combining, in the same hybridisation tube, one group-specific Cy5 probe with the EUB338 FITC probe. An FL1 histogram (green fluorescence) was used to evaluate the total number of bacteria hybridising with the EUB338 FITC probe. A gate was designed in this histogram gathering total bacterial cells in the sample and was used to build an FL4 histogram (red fluorescence) to directly estimate the proportion of cells targeted by the Cy5-labelled group probe among the total bacterial cells in the sample. The proportion of cells labelled with the group probe was corrected by a subtraction of background fluorescence measured using the negative control NON338 Cy5 probe. Results were expressed as cells hybridising with the Cy5-labelled group probe as a proportion of total bacteria hybridising with the general EUB338 FITC probe. Cell proportions estimated by FISH were calculated using the result of duplicates.

Biochemical analyses

Analyses of bacterial metabolism were performed on D0 and D10, before and during fermented milk consumption. Faecal enzyme activities were measured in a thermoregulated anaerobic chamber (H_2, CO_2, N_2; 10:10:80 v/v/v). Faecal samples were diluted 1/20 in pre-reduced PBS (pH 6·7). The activities α- and β-galactosidases, β-glucosidase, β-glucuronidase, α- and β-N-acetyl-galactosaminidase, β-N-acetyl-glucosaminidase, and α-L-fucosidase were measured by determining the rate of *p*-nitrophenol release from *p*-nitrophenyl-glycosides, as previously described (Andrieux *et al.* 2002). Azoreductase activity was determined using amaranth (5 mmol/l) as a substrate. Neuraminidase activity was measured using 4-methylumbelliferyl-N-acetylneuraminic acid as a 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) using a 1/500 faecal dilution in Na_2CO_3 (2 %) and NaOH (0·1 mol/l). Bovine serum albumin was used as the standard. SCFA were analysed in duplicate using gas chromatography (Perkin-Elmer Saint-Quentin-en-Yvelines, France 1020 GC) after water extraction of the acidified samples, as described in Andrieux *et al.* (2002). Lipids were extracted in faecal samples with ethanol over 24 h in a Soxhlet apparatus (VWR International, Strasbourg, France). The composition of bile acid and neutral steroids was determined by GLC, as previously described by Boehler *et al.* (1999).

Statistical methods

The summary data have been reported with means or medians and standard deviations. Microbial composition and biochemical parameters were compared between D0 and D10 on the one hand and between D10 and D20 on the other hand using the Wilcoxon test with statistical software (StatGraphics, Manugistics, Rockville, MD, USA and SAS, SAS Institute, Cary, NC, USA). Statistical analyses were performed by a two-tailed test with $\alpha = 0{\cdot}05$.

Results

Examination of faecal samples by PCR-TTGE

The PCR-TTGE profiles obtained with Lac1 and Lac2GC primers from the faecal samples of five subjects (A, B, C, D, E) are shown in Fig. 1. The profile obtained with the fermented milk (Ac) showed two main bands, one migrating at the same level as the one observed with the probiotic strain *L. casei* DN-114 001, and the second located in the upper part of the gel and co-migrating with the band observed with the *L. bulgaricus* strain. At D0, each subject harboured a specific *Lactobacillus–Pediococcus–Leuconostoc–Weissella* species diversity. A faint but distinct band co-migrating with the band obtained with DNA from *L. casei* DN-114 001 was observed in faecal samples from four subjects (B, F, I, J) at D0. At the end of the test step (D10), a high-intensity band co-migrating with the band obtained with DNA from *L. casei* DN-114 001 was observed in faecal samples from the twelve subjects. This band was not detected in the samples of the subjects 10 d after the end of supplementation (D20), except in three subjects (A, B, L). Modifications in the PCR-TTGE profiles obtained with Lab primers were visualised for each subject between D0 and D10, showing modifications of the *Lactobacillus–Pediococcus–Leuconostoc–Weissella* species diversity. A band that could be attributed to the *L. bulgaricus* strain was retrieved in half of the subjects (A, B, C, E, H, L) on D10.

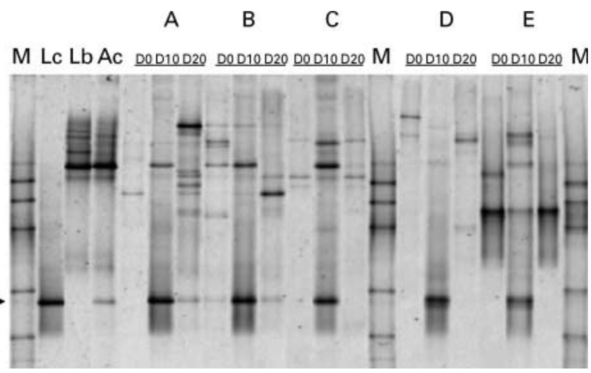

Fig. 1. Representative temporal temperature gradient gel electrophoresis (TTGE) electrophoregram of the dynamics of *Lactobacillus*-like community for five individuals (A, B, C, D, E) during the trial. TTGE analysis of amplicons generated by *Lactobacillus*-group-specific PCR with primers Lac1 and Lac2GC from faecal samples collected at day (D) 0 before *Lactobacillus casei* DN-114 001 consumption (lane D0), at D10, at the end of the study product supplementation step (lane D10), and at D20, 10 d after the end of the supplementation step (lane D20), respectively. M, Marker; Lc, *Lactobacillus casei* DN-114 001, Lb, *Lactobacillus bulgaricus*; Ac, fermented product: Actimel (Danone Vitapole, France).

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PCR-TTGE profiles were obtained using *Bifidobacterium*-specific primers (Satokari *et al.* 2001) for the samples collected before and at the end of supplementation (D0 and D10; from four subjects: B and F, I, J (data not shown); in three subjects: A, B and L (data not shown); of subjects A, B, C, E and H, L (data not shown)). PCR-TTGE profiles presented between one and ten different bands and were different between individuals (similarities below 85 % on D0). They were not affected by supplementation, as illustrated by similarities between the D0 and D10 profiles of on average 94·7 % SD 5·2 % (78·6–98·0 %).

Quantification of bacteria of the Lactobacillus paracasei *group by real-time PCR*

The test product ingested contained 10^8 *L. casei* colony-forming units/ml, corresponding to $9{\cdot}5 \times 10^9$ bacterial equivalents of the *L. paracasei* group/ml, as assessed by real-time PCR. The results of real-time PCR quantification for bacteria from the *L. paracasei* group, which contains the species of the probiotic strain *L. casei* DN-114 001, are presented in Fig. 2. Real-time PCR generated threshold cycle values that were used to calculate bacterial equivalents of the *L. paracasei* group in the faecal samples from the twelve subjects during the trial.

At D0, eleven of the twelve subjects harboured initial levels of bacterial equivalents of the *L. paracasei* group ranging from $3{\cdot}6 \times 10^4$/g to $1{\cdot}5 \times 10^8$/g faeces. One subject (F) harboured a higher initial level of bacterial equivalents of the *L. paracasei* group, of $2{\cdot}6 \times 10^9$/g faeces. The mean value obtained from the twelve subjects at D0 was $6{\cdot}3 \times 10^6$ bacterial equivalents of the *L. paracasei* group/g faeces. A significant increase ($P < 0{\cdot}01$) in bacteria from the *L. paracasei* group was observed after 10 d daily ingestion of $3{\cdot}0 \times 10^{10}$ CFU *L. casei* DN-114 001. Bacteria from the *L. paracasei* group were quantified in all faecal samples at levels ranging from $1{\cdot}8 \times 10^8$ (subject K) to $8{\cdot}3 \times 10^{10}$ (subject A) bacterial equivalents of the *L. paracasei* group/g faeces. Seven out of the twelve subjects (A, C, D, E, F, J, L) exhibited probiotic levels superior to 10^{10} bacterial equivalents of the *L. paracasei* group/g faeces. The mean value obtained from the twelve subjects at D10 was $7{\cdot}9 \times 10^9$ bacterial equivalents of the *L. paracasei* group/g faeces. Increases in the number of bacterial equivalents of the *L. paracasei* group/g faeces between D0 and D10 ranged from 6·3 to $2{\cdot}5 \times 10^6$, with a mean increase of 1260-fold.

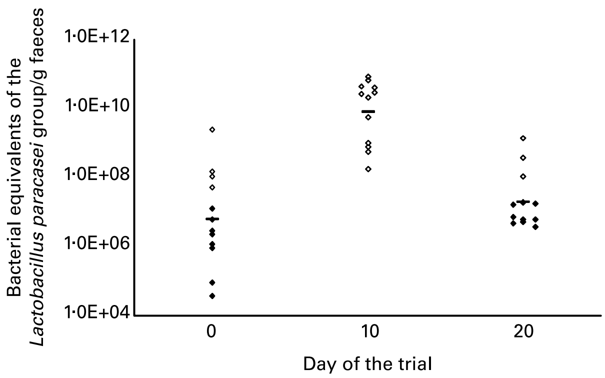

Fig. 2. Recovery of bacterial equivalents of the *Lactobacillus paracasei* group in faeces of the twelve subjects during the trial. *Lactobacillus paracasei* group-specific SYBR-Green real-time PCR was performed using primers Lp1 and Lc2 at day (D) 0, D10 and D20.The bar indicates mean values obtained at each measurement time for the twelve subjects. (◇) Samples in which *L. casei* was detected by temperature gradient gel electrophoresis (TTGE); (◆) samples in which *L. casei* was not detected by TTGE.

At D20, 10 d after the end of the supplementation step, bacteria from the *L. paracasei* group were still detected in the faeces of all of the subjects. The mean value obtained from the twelve subjects at D20 was $2{\cdot}0 \times 10^7$ bacterial equivalents of the *L. paracasei* group/g faeces. Three of these samples (A, B, L) exhibited quantities of *L. paracasei* of over $1{\cdot}0 \times 10^8$ bacterial equivalents of the *L. paracasei* group/g faeces. Nine samples (A, B, C, D, E, G, H, K, L) had final (D20) levels of bacterial equivalents of the *L. paracasei* group that were higher than their own corresponding initial (D0) levels, whereas three other subjects had final levels of *L. paracasei* lower than their own corresponding initial levels.

Composition of the faecal microbiota of the subjects during the study

The set of seven group probes was used in FISH adapted for detection by flow cytometry to enumerate the proportion of specific groups in the faecal samples from the twelve healthy subjects before, during and after the ingestion of a dairy product containing 10^8 colony-forming units *L. casei*/ml. The dynamics of the faecal microbiota from the twelve subjects was analysed during the study (Table 2). Results were expressed as the means for the twelve subjects for each phylogenetic group during the different steps of the study.

At baseline, the *Clostridium coccoides* group was the most abundant group in the twelve subjects, representing 22·9 % of the bacteria. The two following phylogenetic groups *Faecalibacterium prausnitzii* (the second most abundant group in five subjects) and *Bacteroides* (the second most abundant group in three subjects) accounted for 8·2 % and 8·6 % of the microbiota, respectively. The *Bifidobacterium* genus (the second most abundant group in four subjects) was the fourth major group overall and represented, at baseline, 6·4 % of the bacteria. The three other phylogenetic groups – *Atopobium, Lactobacilli–Enterococci* and Enterobacteria – together represented 6 % of the microbiota.

At the end of the supplementation step, a slight but not significant increase was observed for the *Bacteroides* group (12 % on D10 compared with 8·6 % on D0) and for the sum of the proportions of bacterial cells hybridised with the set of seven group-specific probes (52·9 %), compared with the sum of the proportions of bacteria hybridised at baseline (48·9 %) and 10 d after the end of the intake (46·1 %). No significant difference was observed in the proportion of the seven phylogenetic groups between the two steps (D0 compared with D10, and D10 compared with D20).

Biochemical analyses

Enzyme activities, pH and SCFA, together with bile acids and neutral steroids, were determined at D0 and D10 in faecal samples from all the subjects (Table 3). No significant modification of bacterial enzyme activities occurred as a result of

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Table 2. Mean proportions of *Atopobium*, *Bacteroides-Prevotella*, *Bifidobacterium*, *Clostridium coccoides*, *Faecalibacterium prausnitzii*, enterobacteria and *Lactobacillus–Enterococcus* groups in the microbiota from the twelve subjects before (day (D0), during (D10) and after (D20) the ingestion of *L. casei* DN-114 001 assessed with fluorescence *in situ* hybridisation combined with flow cytometry detection using Ato 291, Bac 303, Bif 164, Erec 482, Fprau 645, Enter 1432 and Lab 158 probes, respectively

	Bacterial group (%)															
	Ato 291		Bac 303		Bif 164		Erec 482		Fprau 645		Enter 1432		Lab 158		Total	
Day	Mean*	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
D 0	3·1	1·7	8·6	6·6	6·4	3·6	22·9	12·0	8·2	5·9	0·3	0·5	2·6	2·6	48·9	24·4
D 10	2·7	2·3	12·0	12·4	8·0	6·0	22·2	10·9	8·1	6·4	0·2	0·5	2·4	2·1	52·9	20·0
D 20	2·5	1·8	8·2	6·1	6·7	4·3	20·9	12·0	7·2	4·5	1·0	1·9	2·2	2·0	46·1	19·7

*For each measurement time and each probe, the mean was calculated from the twenty-four values obtained from duplicates from the twelve subjects. No significant difference was observed for any specific bacterial group between the three steps.

fermented milk consumption. A slight but not significant decrease in β-glucuronidase activity was observed between baseline at D0 (median 4·15 IU/g protein) and D10 (median 3·35 IU/g protein) (P=0·065). The concentrations of acetate, propionate, butyrate, caproate, valerate, isobutyrate and isovalerate did not change between D0 and D10. Although no significant difference was shown in the SCFA profile, there was an upward trend in total SCFA concentration at D10 (median 112 μmol/g faeces) compared with D0 (median 60 μmol/g faeces). The faecal sterol proportions varied widely between subjects, and no statistical difference was observed between D0 and D10. However, the proportion of chenodeoxycholate was increased between D0 and D10 (median 0·0 compared with 2·7), although this increase was not significantly different.

Table 3. Enzyme activities, pH, SCFA, bile acids and neutral sterols in faeces before (day (D0) and at the end of the 10 d supplementation step with the study product (D10)

(Median and standard deviation for the twelve subjects)

	D0		D10	
Enzyme activities*	Median	SD	Median	SD
α-Galactosidase	31·0	6·3	34·5	11·9
β-Galactosidase	74·5	21·5	77·5	31·5
β-Glucosidase	10·1	4·1	7·7	4·1
β-Glucuronidase	4·15	1·5	3·35	1·1
Neuraminidase	7·0	2·0	17·0	6·6
α-N-Acetylgalactosaminidase	0·8	0·7	0·8	0·5
β-N-Acetylgalactosaminidase	2·2	1·7	2·2	1·4
α-Fucosidase	2·3	0·9	2·4	1·2
Reductase	0·1	0·5	0·0	0·4
Azoreductase	1·0	0·9	1·2	0·4
β-N-Acetylglucosaminidase	12·5	6·6	14·0	6·2
Parameters of the colic environment				
pH	6·3	0·5	6·5	0·6
Total SCFA (μmol/g)	60·0	63·9	112·0	56·8
Acetate (%)	58·5	5·2	56·8	3·4
Propionate (%)	17·0	4·0	17·8	6·2
Butyrate (%)	18·6	4·3	14·8	5·9
Caproate (%)	1·9	0·7	2·1	0·8
Valerate (%)	0·4	0·5	0·8	0·6
Isobutyrate + isovalerate (%)	4·6	2·9	5·0	2·5
Bile acids and neutral sterols*				
Cholic	0·0	2·2	0·0	5·2
Deoxycholic	39·7	11·4	39·7	11·3
Chenodeoxycholic	0·0	1·8	2·7	2·9
Litocholic	40·7	14·1	43·5	11·5
Ketonic compounds	14·8	7·3	12·9	5·0
Primary bile acids	0·0	3·1	3·1	6·8
Secondary bile acids	100·0	3·1	96·9	6·8
Cholesterol	20·3	35·7	22·8	35·7
Coprosterol	77·1	34·7	72·9	35·1

*Enzyme activities are expressed as μmol metabolised substrate per min and per g protein; bile acids and neutral sterols are expressed as % of total bile acids and total neutral sterols, respectively.

Discussion

In this study, molecular and biochemical approaches were applied to assess the recovery of amplifiable DNA from the probiotic strain *L. casei* DN-114 001 and modifications in the composition and activities of the gut microbiota in healthy adults before, during and after the daily ingestion of $3·0 \times 10^{10}$ colony-forming units *L. casei* DN-114 001.

We demonstrated that the short-term consumption of *L. casei* DN-114 001 resulted in a consistent increase of amplifiable DNA from this strain after 10 d supplementation compared with initial levels. TTGE analysis evidenced a high-intensity band revealing the presence of amplifiable DNA from the probiotic strain 10 d after ingestion in all of the subjects. These results were supported with real-time PCR, which allowed a quantification of amplifiable DNA from the probiotic strain, although differences were observed between cultivable cell counts and quantitative PCR of the probiotic in the product, which could be explained by the amplification of DNA from non-cultivable or dead bacteria. The mean increase of amplifiable DNA from *L. casei* DN-114 001 after 10 d supplementation was transient and reverted to initial levels when assessed 10 d post-feeding, with three subjects harbouring lower final levels of *L. paracasei* compared with their own initial levels. The average bacterial equivalents of the *L. paracasei* group measured per g faeces corresponded to a recovery of 33 % of the bacterial equivalents of the *L. paracasei* group ingested daily, on the basis of an average excretion of 120 g faeces per subject per day. This is only an end point measurement that indicates a rather high DNA recovery. It does not account for fluxes resulting from bacterial growth or DNA degradation, and merely suggests good survival of the corresponding bacterial cells, at least in the upper part of the intestine.

On a methodological standpoint, when we compared the results of real-time PCR and TTGE, we observed that when

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we had more than $5{\cdot}0 \times 10^7$ bacterial equivalents of the *L. paracasei* group/g faeces in a sample, we had a band in the PCR-TTGE profile co-migrating with the band of the *L. casei* DN-114 001 DNA. The detection limit of the amplifiable DNA from the probiotic strain using the group-specific primers Lac1 and Lac2GC and PCR-TTGE was between $2{\cdot}0 \times 10^7$ (no detection) and $5{\cdot}0 \times 10^7$ (detection of a band in the PCR-TTGE profile) bacterial equivalents of the *L. paracasei* group/g faeces.

In a 6-month feeding trial in which healthy subjects ingested $1{\cdot}6 \times 10^9$ *L. rhamnosus* DR20 daily, Tannock *et al.* (2000) were not able to detect the probiotic strain DR20 during the test step by PCR-Denaturing Gradient Gel Electrophoresis using the universal primers HDA1GC and HDA2, although the strain was cultivated from the faeces of nine subjects out of ten. Walter *et al.* (2000) tested two faecal samples collected during the test step of the study of Tannock *et al.* (2000) with the group-specific primers Lac1 and Lac2GC. They detected the *L. rhamnosus* DR20-specific band in both samples. The group-specific primers lowered the detection limit so that *L. rhamnosus* DR20 could be detected in one faecal sample giving a negative result by the culture technique (Tannock *et al.* 2000). These primers are useful tools in tracking bacteria belonging to subdominant groups of the faecal microbiota and lactic acid bacteria, commonly associated with foods.

Although the methods used here may evidence persistence of DNA rather than the actual survival of live culturable bacteria, the transient passage of bacterial DNA may itself provide benefits, as recently evidenced for immunostimulatory DNA sequences also known as CpG-DNA from probiotics in the context of chemically induced inflammation (Rachmilewitz *et al.* 2004).

No major alterations in the bacteriology or biochemistry of the faecal microbiota in the twelve healthy human subjects were observed with the methods used. The FISH analysis with the set of seven group-specific probes provided quantitative data on the relative proportions of the dominant bacterial groups along the trial. In this study, we demonstrated that a short-term consumption of *L. casei* DN-114 001 did not affect the communities of obligate anaerobes, which are the numerically dominant members of the faecal microbiota. The average level of *L. paracasei* equivalents observed at the end of the supplementation step ($7{\cdot}9 \times 10^9$/g faeces) was not accompanied by an increase in the *Lactobacillus–Enterococcus* group in the total faecal microbiota assessed by FISH. This transient increase was furthermore accompanied by fluctuations in the species distribution within lactobacilli, as evidenced in the TTGE profiles.

Similar results have been obtained in other probiotic feeding trials (Tannock *et al.* 2000; Collins *et al.* 2002). Using a set of five group-specific probes targeting dominant members of the faecal microbiota and FISH detection, Tannock *et al.* (2000) observed that the consumption of *L. rhamnosus* DR20 did not affect the populations of obligate anaerobes that represent the dominant faecal microbiota. Nevertheless, they evidenced transiently increased counts of *Lactobacillus* and *Enterococcus* in the majority of consumers. Collins *et al.* (2002) showed significantly increased concentrations of faecal enterococci and total lactobacilli, although *Bifidobacteria*, coliforms and *Bacteroides* were not significantly modified after 21 d consumption of a fermented milk containing 10^{10} *L. salivarius* strain UCC118/d. Overall, the absence of alteration of the dominant faecal microbiota based on the FISH data presented can be viewed as a beneficial outcome.

As observed in our study, probiotic strains incorporated into fermented milk products can be detected in the faeces of human subjects only during the phase of intake (Goldin *et al.* 1992; Tannock *et al.* 2000). However, more recent studies have shown that some strains of lactobacilli or bifidobacteria could still be excreted in faecal samples up to 4 weeks after the end of intake (Fujiwara *et al.* 2001; Collins *et al.* 2002). Furthermore, the study of faecal samples may underestimate the survival of probiotic strains when performed during limited steps of ingestion. Alander *et al.* (1999) observed that, 1 week after the discontinuation of strain *L. rhamnosus* GG intake, the strain persisted in the colonic mucosa even after its disappearance from faecal samples. This suggests that strain GG can survive in high numbers in the colonic mucosa despite its rapid turnover in the gut lumen. However, 2 weeks after the end of intake, even this mucosa-associated strain was gradually eliminated.

Although probiotic supplementations have not correlated well with an impact on faecal microbiota, continual feeding will probably be required for most individuals. Other criteria, such as specific growth rate and the ability to associate and persist on intestinal mucosal surfaces, could favour probiotic effects (Lee *et al.* 2004). In turn, optimal dose and duration of consumption are likely to be strain-specific parameters. For this reason, each probiotic strain should be evaluated individually in specific nutritional studies to conclude on its recovery and potential health benefits (Agence Française de Securité Sanitaive des Aliments, 2003).

As has previously been observed in children (Guérin-Danan *et al.* 1998), fermented milk containing yoghurt starters and *L. casei* DN-114 001 did not significantly modify the bacterial enzyme activities of β-galactosidase and α-glucosidase or the fermentative metabolite profile. However, the potentially harmful enzyme activity of β-glucuronidase decreased, especially when this activity presented initially high values. In adults, other studies reported a decrease in β-glucuronidase activity when *Lactobacillus* or *Bifidobacterium* were consumed (Bouhnik *et al.* 1996; Goldin, 1998), especially in subjects who initially had high β-glucuronidase activity because of a diet rich in meat. On D10, the 7α-dehydroxylation of chenodeoxycholate was significantly reduced ($P < 0{\cdot}05$). This effect may be considered beneficial for health as secondary bile acids may be carcinogenic (Panda *et al.* 1999). Large interindividual differences in FISH and enzyme activities for the twelve subjects of this study could certainly have obscured clear fluctuations between the three steps of the trial.

The use of molecular techniques to detect and quantify endogenous bacteria and probiotic strains will offer new opportunities for culture-independent studies of human intestinal microbiota and for analyses of the persistence of amplifiable DNA from ingested probiotic strains during nutritional trials. In the future, the results of long-term control studies involving a larger number (e.g. 50–100) of subjects could potentially provide a better understanding of the effect of probiotic on digestive microbiota.

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