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Survival and metabolic activity of selected strains of *Propionibacterium freudenreichii* in the gastrointestinal tract of human microbiota-associated rats

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In addition to their use in cheese technology, dairy propionibacteria have been identified as potential probiotics. However, to have a probiotic effect, propionibacteria have to survive and to remain metabolically active in the digestive tract. The aim of the present study was to investigate the survival and metabolic activity of *Propionibacterium freudenreichii* within the gastrointestinal tract of human microbiota-associated rats, and its influence on intestinal microbiota composition and metabolism. Twenty-five dairy *Propionibacterium* strains were screened for their tolerance towards digestive stresses and their ability to produce propionate in a medium mimicking the content of the human colon. Three strains were selected and a daily dose of 2×10^{10} colony-forming units was fed to groups of human microbiota-associated rats for 20 d before microbiological, biochemical and molecular investigations being carried out. These strains all reached 8-log values per g faeces, showing their ability to survive in the gastrointestinal tract. Transcriptional activity within the intestine was demonstrated by the presence of *P. freudenreichii*-specific transcarboxylase mRNA. The probiotic efficacy of propionibacteria was yet species- and strain-dependent. Indeed, two of the strains, namely TL133 and TL1348, altered the faecal microbiota composition, TL133 also increasing the caecal concentration of acetate, propionate and butyrate, while the third strain, TL3, did not have similar effects. Such alterations may have an impact on gut health and will thus be taken into consideration for further *in vivo* investigations on probiotic potentialities of *P. freudenreichii*.

Probiotics: Propionibacteria: Short-chain fatty acids: Intestinal microbiota

A probiotic is generally defined as 'a live micro-organism which, when administered in adequate amounts, confers a health benefit on the host' (Food and Agriculture Organization & World Health Organization, 2002). The main claimed effects for probiotics are improvement of lactose digestion, prevention of intestinal disturbances, treatment and prevention of antibiotic or acute diarrhoea, reduction of intestinal infection and colonisation by pathogenic bacteria, alleviation of irritable bowel syndrome and of inflammatory bowel disease and modulation of colon carcinogenesis (Marteau *et al.* 2001). The reported mechanisms that may explain the health-promoting effects of probiotics include improvement of the gut mucosal barrier, immunomodulation (immune response balance), modulation of the intestinal microbiota (allowing an equilibrium between the populations of beneficial and potentially harmful bacteria) and of the corresponding metabolic activities within the gut, leading to decreased toxigenic reactions, enhanced production of SCFA and vitamins, bile salt deconjugation or lactose hydrolysis (Ouwehand *et al.* 2002; Saarela *et al.* 2002). The criteria for probiotic selection include safety (strain, species and genus safety properties and non-pathogenicity), technological properties (tolerance towards probiotic product processing and storage), survival in the digestive tract, which is linked with stress adaptation, and at least one property beneficial to human health. Moreover, human origin and adhesion to human epithelial cells may also be considered as selection criteria for probiotics. Most of the probiotics used are lactobacilli or bifidobacteria, but some probiotic effects have also been reported for some *Bacillus* strains, a non-pathogenic strain of *Escherichia coli*, some yeasts and propionibacteria, mainly *Propionibacterium freudenreichii* (Ouwehand *et al.* 2002).

Like lactobacilli, dairy propionibacteria have a long history of safe use as starter cultures in the food industry. For the manufacture of Swiss-type cheeses, *P. freudenreichii* has received 'generally recognised as safe' (GRAS) status by the US Food and Drug Administration (21CFR133.195). Besides this application, they are also studied for their probiotic abilities. In man, dairy propionibacteria survive, at least partially, within the digestive tract (Bougle *et al.* 1999; Jan *et al.* 2002*b*) and selected strains

Abbreviations: CFU, colony-forming unit; Cy5, indodicarbocyanine; FISH, fluorescent *in situ* hybridisation; Tris, tri(hydroxymethyl)-aminomethane.

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stimulate the growth of bifidobacteria (Bougle *et al.* 1999; Satomi *et al*. 1999; Hojo *et al*. 2002). In rodents, different propionibacteria have been shown to have immunomodulatory effects (Perez Chaia *et al*. 1995; Kirjavainen *et al.* 1999). Propionibacteria also decreased activity of intestinal enzyme involved in the conversion of carcinogenic compounds, for example, β-glucuronidase (Perez Chaia *et al*. 1999), adhered to intestinal epithelium of mice (Zarate *et al*. 2002) and reduced the severity of chemically induced colitis in rats (Michel *et al*. 2005). Furthermore, propionibacteria are able to inhibit the growth of pathogenic micro-organisms (Lyon *et al*. 1993) and to produce bacteriocins and vitamin B_{12} (Holo *et al*. 2002; Hugenholtz *et al.* 2002).

Moreover, propionibacteria harbour a particular central carbon metabolic pathway, the propionic fermentation, which involves the Wood–Werkman cycle (Wood, 1981) and requires a multimeric transcarboxylase (methylmalonyl-CoA carboxyltransferase; EC 21.3.1). This peculiar metabolism allows conversion of various substrates into CO_2 and the SCFA acetate and propionate. SCFA, particularly butyrate, are known to have an important influence on colonic health (Mortensen & Clausen, 1996). This characteristic may constitute a key probiotic potential for propionibacteria, as it has been previously shown that the SCFA butyrate is a potent inducer of apoptosis in colon cancer cells *in vitro* (Scheppach *et al.* 1995). Regarding the major endproducts of dairy propionibacteria, acetate and propionate were shown to kill human adenocarcinoma cell lines by apoptosis during co-cultures with the dairy species *P. freudenreichii* and *P. acidipropionici* (Jan *et al*. 2002*a*), a property that could help colon cancer prevention or treatment.

However, to have such health effects, the potential probiotic must tolerate digestives stresses and reach significant populations within the gut. Stress tolerance is highly strain-dependent within the *P. freudenreichii* species (Jan *et al.* 2000; Anastasiou *et al*. 2006). Occurrence of propionic fermentation within the colon content also remains a key question for dairy propionibacteria probiotic application. The aim of the present study was first to screen *in vitro* a set of dairy propionibacteria, known for their technological properties, with respect to their tolerance towards digestive stresses and their production of propionate in a medium mimicking the content of the human colon. Second, after *in vitro* selection, the best candidates were administered to human microbiota-associated rats in order to evaluate propionibacteria survival and metabolic activity within the gastrointestinal tract, and to study the influence of *P. freudenreichii* on intestinal metabolism and human microbiota composition.

Material and methods

Bacterial strains and growth conditions

The *Propionibacterium* strains (Table 1) originated from our local TL propionibacteria strain collection (INRA, Agrocampus Rennes, UMR STLO). They were kept frozen (−80°C) as glycerol stocks using our standard procedure and routinely cultivated on a modified yeast extract–lactate medium containing 144 mM-sodium lactate (Malik *et al*. 1968). Growth was carried out at 30°C without shaking and monitored spectrophotometrically at 650 nm as well as by colony-forming unit (CFU) counting.

In vitro assays

Digestive stress challenge. After CFU counting, early stationary-phase cultures (3 d) were diluted 10-fold in a

Table 1. Dairy *Propionibacterium* strains used in the present study

Strain	Species	Subspecies	Origination
TL125	*Propionibacterium freudenreichii*	*shermanii*	Dairy product
TL133	*P. freudenreichii*	*shermanii*	Dairy product
TL134	*P. freudenreichii*	*shermanii*	Dairy product
TL138	*P. freudenreichii*	*shermanii*	Dairy product
TL142	*P. freudenreichii*	*freudenreichii*	Dairy product
TL144	*P. freudenreichii*	*shermanii*	Dairy product
TL160	*P. freudenreichii*	*shermanii*	Dairy product
TL165	*P. freudenreichii*	*shermanii*	Dairy product
TL166	*P. freudenreichii*	*shermanii*	Dairy product
TL162	*P. freudenreichii*	*shermanii*	Dairy product
TL1343	*P. freudenreichii*	*freudenreichii*	Human intestine*
TL1348	*P. freudenreichii*	*shermanii*	Human intestine*
TL1351	*P. freudenreichii*	*shermanii*	Human intestine*
TL1360	*P. freudenreichii*	*shermanii*	Human intestine*
TL33	*P. freudenreichii*	*freudenreichii*	Dairy product
TL34	*P. freudenreichii*	*shermanii*	Dairy product
TL2	*P. acidipropionici*	–	Dairy product
TL3	*P. freudenreichii*	*freudenreichii*	Dairy product
TL19	*P. freudenreichii*	*freudenreichii*	Dairy product
TL63	*P. freudenreichii*	*shermanii*	Dairy product
TL213	*P. freudenreichii*	*shermanii*	Dairy product
TL502	*P. freudenreichii*	*freudenreichii*	Dairy product
TL505	*P. freudenreichii*	*shermanii*	Dairy product
TL635	*P. freudenreichii*	*shermanii*	Dairy product
TL1123	*P. freudenreichii*	*shermanii*	Dairy product

* Strains isolated from human faecal samples after a 4-week wash-out period without dairy propionibacteria ingestion.

pre-warmed (37°C) acidified (pH adjusted to 3·3 using HCl) lactate broth (Jan *et al.* 2000); the mixture led to a final pH of 3·5. Propionibacteria were acid-challenged at 37°C for 1 h before centrifugation (10 min; 10 000 **g**) and re-suspended in pre-warmed (37°C) peptone water (0·1 % peptic digest of meat; Biokar Diagnostics, Beauvais, France), pH 7·0, containing 0·9 % NaCl, in order to stop acid stress. These cell suspensions were then diluted 5-fold in a pre-warmed yeast extract–lactate medium containing 0·1 % (w/v) dried ox bile (Biokar Diagnostics) and challenged at 37°C for 2 h. Bile challenge was stopped by dilution and surviving cells were enumerated on solidified yeast extract–lactate medium after 7 d of anaerobic incubation at 30°C for maximal recovery of living bacteria as described previously (Leverrier *et al*. 2003).

Short-chain fatty acid production. Early stationary-phase propionibacteria yeast extract–lactate cultures were used to inoculate (1 %) a pre-warmed medium, designed to mimic the content of the human colon (Gibson & Wang, 1994), and modified by the addition of sodium lactate to facilitate propionic fermentation (Table 2). Following incubation (37°C; 24 h), cultures were centrifuged (15 min; 10 000 **g**) and supernatant fractions diluted in 0·005 M-H_2SO_4 before filtration (0·2 μm) and chromatographic analysis. SCFA were analysed by HPLC (Gold; Beckman Coulter Corp., Fullerton, CA, USA) using UV detection at 210 nm. The anion exchange column (300 × 6 mm, Aminex A_6; Bio-Rad, Hercules, CA, USA) was operated at room temperature with 0·005 M-H_2SO_4 (0·5 ml/min) as eluent. Standard solutions of lactic acid, acetic acid and propionic acid of known concentrations were used for column calibration.

Table 2. Composition of the medium used for short-chain fatty acid production screening

Constituent	Concentration (g/l)
Pectin	0·5
Xylan	0·5
Mucin	0·5
Starch	0·5
Peptone	0·5
Tryptone	2·5
Yeast extract	0·5
Bile salts	0·05
K_2HPO_4	2
$NaHCO_3$	0·2
NaCl	4·5
$MgSO_4.7H_2O$	0·5
$CaCl_2.2H_2O$	0·45
$MnCl_2.2H_2O$	0·2
Haemin	0·05
$FeSO_4.7H_2O$ (mg/l)	5
$CoCl_2.6H_2O$	0·05
Tween 80 (ml/l)	2
Sodium lactate (60 %)	5
Thiamin HCl (μg/l)	4
Calcium pantothenate (μg/l)	10
Nicotinic acid (μg/l)	5
4-Aminobenzoic acid (μg/l)	5
Biotin (μg/l)	2
Vitamin B_{12} (μg/l)	0·5
Cysteine	0·8
PH	6·0
Distilled water	Up to 1 litre

All constituents were from Sigma (Sigma-Aldrich, Saint-Quentin-Fallavier, France) or Merck (Lyon, France), except starch which was from Labogros (Buchs, France) and peptone, tryptone, yeast extracts and bile salts which were from Oxoid (Dardilly, France).

Animals

Twenty-four adult male Fischer 344 rats were used. They were born germ-free and bred in germ-free conditions in the Germ-Free Rodent Breeding Facilities of Unité d'Ecologie et Physiologie du Système Digestif (INRA, Jouy-en-Josas, France), according to established methods (Coates, 1968). Rats were aged 12 weeks at the start of the experiment (mean weight 291 (SEM 4) g). They were randomly separated into four groups of six animals housed in four sterile Plexiglas isolators (Ingénia, Vitry-sur-Seine, France). Within each isolator, rats were kept in pairs in standard macrolon cages containing a bed of wood shavings. They were given free access to autoclaved tap water and a pelleted semi-synthetic diet (Scientific Animal Food and Engineering, Augy, France) sterilised by γ irradiation at 45 kGy (IBA Mediris, Fleurus, Belgium). To reproduce the diversity of a human-type diet, the food contained lipids and proteins of animal and plant origins, sucrose and cooked starch (Table 3). Analytical compounds of DM were: crude proteins, 18 %; crude fat, 8 %; ash, 6 %; carbohydrates 68 % (energy 19·33 MJ/kg DM; Eurofins Scientific Analytics, Nantes, France). Throughout the study, isolators were maintained in controlled conditions of light (07.00–19.00 hours), temperature (20–22°C) and humidity (45–55 %).

Experimental design

All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals. On day 1, all rats were orally administered with 1 ml of a whole fresh faecal suspension made from the stools of a healthy

Table 3. Composition of the diet

Ingredient	Content (g/kg DM)
Mashed potato	290
Maize starch	289·85
Sucrose	50
Casein	50
Soya isolate*	120
Maize oil	30
Lard	30
Cholesterol	0·15
Cellulose	60
Mineral additive†	70
Vitamin additive‡	10

* Nurish 1500 (DuPont Protein Technologies, St Louis, MO, USA).

† The mineral additive provided (g/kg diet): Ca, 2·11; P, 5·46; Na, 2·74; K, 3·67; Mg, 1·02; Fe, 0·10; Cu, 0·09; Mn, 0·55; Zn, 0·31; I, 0·0043; Co, 0·0007.

‡ The vitamin additive provided (per kg diet): vitamin A acetate, 6·88 mg; vitamin D_3, 62·5 μg; DL-α-tocopheryl acetate, 175 mg; menadione sodium bisulfite, 35·2 mg; thiamin hydrochloride, 22·4 mg; riboflavin, 15 mg; nicotinic acid, 100 mg; calcium DL-pantothenate, 7·5 mg; pyridoxine hydrochloride, 12·15 mg; folic acid, 5 mg; D-biotin, 0·3 mg; cyanocobalamin, 0·05 mg; L-ascorbic acid, 0·8 mg; choline chloride, 1·56 g; myoinositol, 150 mg.

adult human subject. This subject had followed a normal diet over 3 weeks but with the exclusion of fermented products containing propionibacteria (no consumption of Swiss-type and other pressed cheeses) in order to avoid the presence of propionibacteria in faecal content. Fresh stools (2 g) were transferred in an anaerobic glove box and dispersed in 200 ml Brain Heart Infusion broth (Difco, Becton Dickinson, Le Pont de Claix, France); the suspension was subsequently transferred in the isolators and given to rats using a sterile stainless-steel stomach tube. The rats were given 3 weeks to allow the microbiota to settle in the digestive tract and the rat physiology to adapt to the new bacterial status. On day 21, the human microbiota-associated rats were randomly allocated to four treatments, namely control, TL3, TL133 and TL1348 strains. Each rat was orally administered daily for 3 weeks either with 1·0 ml of physiological saline solution (control group) or with 2×10^{10} CFU of the corresponding *P. freudenreichii* strain (TL3, TL133 or TL1348) suspended in 1·0 ml of physiological saline solution. The bacterial suspensions were prepared every day from early stationary-phase propionibacteria cultures.

On day 21 (initial time), day 31 (medium time) and day 41 (final time), faecal pellets were freshly collected from each rat and divided into two samples. One was stored at −80°C until analysis of bacterial enzymic activities. The second was immediately 10-fold diluted with a dilution medium (casein enzymic hydrolysate (2 g/l), yeast extract (2 g/l), NaCl (5 g/l), KH_2PO_4 (1 g/l); pH 7.0). Of this dilution, 1 ml was used for enumeration of propionibacteria and one volume of this dilution was added to three volumes of 4 % paraformaldehyde for overnight fixation, then stored at −80°C until analysis of the microbiota by fluorescent *in situ* hybridisation (FISH) (Rigottier-Gois *et al*. 2003*a*).

On day 42, rats were weighed and killed by CO_2 asphyxiation. The caecum was removed, the caecal pH was measured and the content was weighed. Two samples were used immediately for enumeration of propionibacteria and for RNA extraction followed by RT-PCR. The remaining part was distributed into several vials stored at −80°C for SCFA analysis.

Enumeration of propionibacteria in faecal and caecal samples

Freshly collected faeces and caecal contents were immediately dispersed in the dilution medium. Samples from serial 10-fold dilutions were poured into the Pal-Propiobac® selective agar (Laboratoires Standa, Caen, France) added with metronidazole (4 mg/l) for propionibacteria enumeration in samples of intestinal origin, as described previously (Jan *et al*. 2002*b*). Plates were then incubated anaerobically at 30°C for 1 week before colony counting. Results were expressed as log CFU/g faeces or caecal content.

Analysis of bacterial enzymic activities in faecal samples

β-Glucuronidase and β-galactosidase activities were measured spectrophotometrically (λ 400 nm) by the rate of release of *p*-nitrophenol from the *p*-nitrophenylglycoside as described by Andrieux *et al*. (1998). Analyses were performed in duplicate. Enzymic activity was expressed as μmol product formed/min per g wet faeces.

Ribonucleic acid extraction from caecal samples and cDNA synthesis

In order to detect *P. freudenreichii* transcarboxylase 5S mRNA (Hervé *et al*. 2006), total caecal microbial RNA were isolated using the Nucleospin RNA II kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions but with modifications and partially adapted steps as described below. Into a 2 ml microtube (VWR, Nogent sur Marne, France), 60 mg of caecal sample were mixed with 300 to 500 mg of Zr beads (100 μm; VWR), 100 μl of cold TE 1x, 350 μl of buffer RA1 (supplied with the kit) and 3·5 μl of β-mercaptoethanol. After homogenisation with vortex at maximal speed, the tubes containing the Zr beads were shaken twice at 2000 **g** for 40 s in a Fast Prep instrument (FP 120, Bio101; Savant, Holbrook, NY, USA) with rapid cooling on ice between steps. After shaking, the tubes were centrifuged at 7000 **g** for 2 min. The supernatant fractions were transferred to new tubes, and total RNA were rapidly subjected to chloroform–phenol extraction and ethanol precipitation. Pellets were washed with 70 % ethanol and re-suspended in 120 μl of RNAse-free water. To remove contaminating DNA, a DNAse treatment was performed on 20 μg of total nucleic acid with deoxyribonuclease I, amplification grade (Invitrogen, Cergy Pontoise, France). Following incubation at 30°C for 30 min, RNA were concentrated to 20 μl with the RNeasy® MinElute Cleanup (Qiagen, Hilden, Germany). RNA integrity was determined after electrophoresis on agarose gel containing ethidium bromide. RNA concentration and purity were estimated by the Ribogreen® RNA Quantification Kit (Molecular Probes, Eugene, OR, USA) and by spectrofluorometry (Kontron Instruments, Rungis, France), respectively. One portion of 10 μl (approximately 10 μg) of concentrated RNA was reverse-transcribed to cDNA using Superscript II RT (Invitrogen, Cergy-Pontoise, France) with 100 pmol of the reverse primer (Hervé *et al*. 2006). In parallel, the remaining 10 μl (10 μg) of RNA were treated in the same way, except for the absence of RT, and were considered as a negative control. After the reaction, the synthesised cDNA was purified using Qiaquick® PCR Purification kit (Qiagen).

The reaction mixture of the TaqMan™ real-time PCR amplification (in a final reaction volume of 25 μl) contained 1 × of Quantitect Probe PCR Master Mix (Qiagen), 200 nM of the probe and 300 nM of each of the primers. All the primers and probe used in the present study were synthesised by QIAGEN Operon Europe (Cologne, Germany). To detect the transcarboxylase mRNA, 10 μl of total cDNA were used. To verify the purity of the amplicon, samples of 10 μl were analysed by electrophoresis on a 4 % (w/v) agarose 3:1 (Amresco, Solon, OH, USA) gel containing ethidium bromide.

Short-chain fatty acid analysis in caecal samples

Samples were water extracted and proteins were precipitated with phosphotungstic acid. A volume of 0·1 μl supernatant fraction was analysed for SCFA on a gas–liquid chromatograph (Autosystem XL; Perkin Elmer, Saint-Quentin-en-Yvelines, France) equipped with a split-splitless injector, a flame-ionisation detector and a capillary column (15 m × 0·53 mm, 0·5 μm) impregnated with SP 1000 (FSCAP

Nukol; Supelco, Saint-Quentin-Fallavier, France). Carrier gas (He) flow rate was 10 ml/min and inlet, column and detector temperatures were 175°C, 100°C and 280°C, respectively. 2-Ethylbutyrate was used as the internal standard (Rabot *et al*. 2000). Samples were analysed in duplicate. Data were collected and peaks integrated using the Turbochrom v. 6 software (Perkin Elmer, Courtaboeuf, France).

Faecal microbiota analysis by fluorescent in situ hybridisation combined with flow cytometry

FISH analysis was carried out as described previously (Rigottier-Gois *et al*. 2003*a*), using the following probes to detect bacterial groups in the faecal samples (Table 4): Eub338 (total bacteria); Ato291 (*Atopobium*, *Eggerthella* and *Collinsella*); Bac303 (*Bacteroides* and *Prevotella*); Bif164 (*Bifidobacterium*); Clep866 (*Clostridium leptum*); Erec482 (*Eubacterium rectale–C. coccoides*); Enter1432 (enterobacteria); Lab158 (*Lactobacillus* and *Enterococcus*). Oligonucleotidic probes were 5′-labelled with indodicarbocyanine (Cy5) or fluorescein isothiocyanate and purified by HPLC (Integrated DNA Technologies, Coralville, IA, USA). The fixed faecal samples were washed in TE buffer (100 mM-tri(hydroxymethyl)-aminomethane (Tris)–HCl, 50 mM-EDTA) and permeabilised with lysozyme (1 mg/ml) in TE buffer for 10 min. After centrifugation, the pellet was washed in PBS and re-suspended in hybridisation buffer (0·9 M-NaCl, 20 mM-Tris–HCl, pH 8·0, 0·01 % SDS, and 30 % formamide). Of this suspension, 40 μl were mixed with 10 μl of a mixture containing the fluorescein isothiocyanate-labelled Eub338 probe (20 ng/μl) and one of the group-specific Cy5-labelled probe (20 ng/μl). Hybridisation was performed at 35°C in the dark for 16 h; then, a volume of 150 μl of hybridisation solution was added to the reaction mixture and cells were pelleted at 4000 ***g*** for 15 min. Non-specific binding of the probes was removed by incubating pelleted cells at 37°C for 20 min in washing buffer (0·065 M-NaCl, 20 mM-Tris–HCl, 5 mM-EDTA, pH 8·0, and 0·01 % SDS). Cells were pelleted again and suspended in 200 μl PBS. Samples of 100 μl were added with 0·5 ml of FACS Flow (Becton Dickinson, Franklin Lakes, NJ, USA) for data acquisition by flow cytometry, using a FacsCalibur flow cytometer (Becton Dickinson). An air-cooled Ar ion laser (488 nm) and a red diode laser (635 nm) were used for excitation, and the green and red signals of the bacteria were collected in the FL1 (515–545 nm) and FL4 (653–669 nm) detectors, respectively. The acquisition threshold was set on side scatter channel and 10^5 fluorescent events were stored in list mode files. Subsequent analysis was performed with the Cell Quest software (Becton Dickinson). An FL1 histogram was built to determine the total number of bacteria present in the sample and hybridising with Eub338. A gate was created in the FL1 histogram and a FL4 histogram was subsequently used to determine the proportion of cells hybridising with the group-specific Cy5 probe. This proportion was corrected by eliminating the background of red fluorescence, determined by using the Non-Eub338–Cy5 probe as a negative control. Results were expressed as the proportion of cells hybridising with the group-specific Cy5 probe in relation to the total bacteria hybridising with the Eub338–fluorescein isothiocyanate probe.

Statistical analysis

The effect of propionibacteria consumption on rats' body and caecal weight, on caecal pH, SCFA concentration and enzymic activities, and on the number of culturable propionibacteria in faeces and caecal contents was analysed using a one-way ANOVA. When ANOVA indicated significant differences, groups were compared in pairs with the Student-Newman-Keuls multiple comparison test. Inter-group similarity of the faecal microbiota at the start of the experiment was checked using a one-way ANOVA. Effect of propionibacteria consumption on this parameter was assessed by comparing each group at initial *v*. final time using a paired Student's *t* test, and by comparing groups in pairs at the end of the experiment using a one-way ANOVA followed by a Student–Newman–Keuls multiple comparison test. Statistical significance was set at $P<0{\cdot}05$. Calculations were performed using the Statview® software (version 5.0; SAS Institute, Cary, NC, USA). All data were expressed as mean values with their standard errors (n 6).

Table 4. Oligonucleotidic probes used in fluorescent *in situ* hybridisation analysis

Probes	Sequences from 5′ to 3′*	OPD code	Reference
Eub338	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18	Amann *et al.* (1995)
Non-Eub338	ACATCCTACGGGAGGC	NA	Wallner *et al.* (1993)
Ato291	GGTCGGTCTCTCAACCC	S--Ato-0291-a-A-17	Harmsen *et al.* (2000)
Bac303	CCAATGTGGGGGACCTT	S--Bacto-0303-a-A-17	Manz *et al.* (1996)
Bif164	CATCCGGCATTACCACCC	S-G-Bif-0164-a-A-18	Langendijk *et al.* (1995)
Clep866	GGTGGATWACTTATTGTG	S--Clep-0866-a-A-18	Lay *et al.* (2005*b*)
Clep866 competitor 1†	GGTGGA*A*WACTTATTGTG		
Clep866 competitor 2†	GGTGGATWACTTATTG*C*G		
Erec482	GCTTCTTAGTCARGTACCG	S--Erec-0482-a-A-19	Franks *et al.* (1998)
Enter1432	CTTTTGCAACCCACT	S--Ent-1432-a-A-15	Sghir *et al.* (2000)
Lab158	GGTATTAGCAYCTGTTTCCA	S--Lab-0158-a-A-20	Harmsen *et al.* (1999)

OPD, Oligonucleotide Probe Database.
* R is an A or G, Y is a C or T and W is an A or T.
† Targets of the Clep866 oligonucleotide competitors 1 and 2 are Sb. termiti and AF001743 respectively; the names correspond to RDP Short-ID (Lay *et al.* 2005*b*).

Results

Tolerance of dairy propionibacteria towards digestive stresses and abilities for short-chain fatty acid production in vitro

With the goal to select propionibacteria candidates for *in vivo* assays, twenty-four strains of *P. freudenreichii* and one of *P. acidipropionici*, isolated either from dairy products or from human faecal microbiota, were tested *in vitro*, on the one hand according to their resistance to digestive stresses and, on the other hand, according to propionate production ability in a medium mimicking the human colonic content. The strain survival was measured following sequential exposures to acidic pH and ox bile at 37°C. Results are represented in Fig. 1 (A), strains being classified by decreased order of survival. Digestive stress tolerance was highly variable, depending on the strain. The strains showing the best rate of survival during digestive stress challenge, that is to say the strains that underwent a viability loss below 1-log value, are four *P. freudenreichii shermanii* strains, TL133, TL1348, TL213 and TL1123, and one *P. freudenreichii freudenreichii* strain, TL3. Most of the strains showed a moderate loss of viability between 1- and 2-log values, whereas four out of the twenty-five strains (TL33, TL166, TL165 and TL2) were shown to be the less tolerant strains and underwent a drop in viability greater than 2-log values.

With the aim to select the best SCFA-producing strains, propionibacteria were cultured in Gibson modified medium at 37°C and propionate production was quantified after 24 h incubation (Fig. 1 (B)). A set of strains produced an amount of propionate close to the 1·32 g/l value predicted for the lactate provided (3 g/l) to be completely metabolised. Twelve strains showed thus a propionate production above 1 g/l, including TL213, TL133, TL3 and TL1348. By contrast, a set of strains produced lower amounts of propionate. All the tested strains led to comparable concentrations of propionate after 3 d of incubation, showing that the rate, but not the yield, of propionic fermentation varied (data not shown).

It is noteworthy that both digestive stress tolerance and propionate production rate are strain-dependent. These screenings allowed us to select three strains for *in vivo* assays, two *P. freudenreichii* subsp. *shermanii* (TL133 and TL1348) and one *P. freudenreichii* subsp. *freudenreichii* (TL3), which fulfilled the two selection criteria.

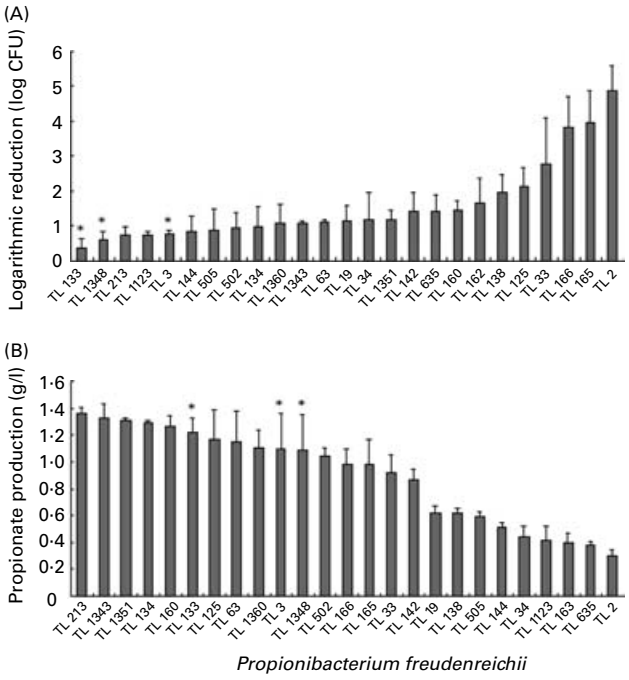

Fig. 1. *In vitro* screening of *Propionibacterium* strains: resistance to digestive stress (A) and production of propionate (B). (A) Viability reduction of twenty-five strains after acid challenge followed by bile challenge. (B) Production of propionate after 24 h of culture in a medium mimicking the content of the human colon. Data are means with their standard errors represented by vertical bars. * The three best candidates selected for *in vivo* experiments.

Survival of selected strains of propionibacteria in the gastrointestinal tract of rats

As shown in Table 5, no dairy propionibacteria could be detected in rats' faeces at the beginning of the experiment. This remained true in control animals throughout the experiment. In groups consuming propionibacteria, recovery varied according to the strain and the time of analysis. Indeed, after 10 d of propionibacteria administration (medium time), faecal concentration did not exceed 6-log values per g for the TL3 strain whereas it reached 8-log values for the TL133 and TL1348 strains. This 2·5-log difference in terms of survival was minimised during further propionibacteria feeding. Hence, after another 10 d, about 1-log difference was observed between the TL3 group and the other TL groups. Indeed, the dairy propionibacteria level in the faeces increased to a 7-log value per g in the TL3 group, whereas it remained constant until the end of the experiment (final time) in the TL133 and TL1348 groups. Surprisingly, at the end of the experiment, caecal concentrations of the three strains were very similar. Indeed, close to 8-log of propionibacteria per g were enumerated in the caecal contents of rats that had consumed propionibacteria, regardless of the strain (Table 5).

Metabolic activity of selected strains of propionibacteria in the gastrointestinal tract of rats

With the aim of examining if strains were metabolically active in the rat digestive tract, expression of the gene encoding methylmalonyl-CoA carboxyltransferase, a specific and key enzyme of *P. freudenreichii* metabolism, was analysed using reverse transcription PCR. Transcarboxylase mRNA was detected in the caecal contents of all rats consuming propionibacteria (threshold cycles 27·2 (SEM 0·2); 26·8 (SEM 0·5) and 26·2 (SEM 0·2) for the TL3 (*n* 4), TL133 (*n* 5) and TL1348 (*n* 4) groups respectively), regardless of the strain, whereas no expression of this transcript was detected in the control group (*n* 5).

Effect of propionibacteria consumption on caecal pH, short-chain fatty acids and enzymic activities

First, it is noticeable that rats' health remained good throughout the experiment, whether the animals belonged to the control or TL groups. Consumption of propionibacteria had no significant effect on weight gain (25 (SEM 2) g/rat); consequently, rats' body weight at the end of the experiment was

Table 5. Enumeration of dairy propionibacteria in faeces and caecal contents of rats fed with *Propionibacterium freudenreichii* TL3, TL133 or TL1348 strains for 20 d†

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

	Control (log CFU/g)	TL3 (log CFU/g)		TL133 (log CFU/g)		TL1348 (log CFU/g)	
	Mean	Mean	SEM	Mean	SEM	Mean	SEM
Faeces							
Initial time (day 21)	ND	ND		ND		ND	
Medium time (day 31)	ND	5·78*	0·49	8·22	0·42	8·51	0·78
Final time (day 41)	ND	6·69*	0·60	7·92	0·29	8·37	0·54
Caecal contents (day 42)	ND	7·81	0·42	7·75	0·29	8·04	0·28

CFU, colony-forming units; ND, no propionibacteria were detected.

* At each time point, mean value was significantly different from those of the other groups (ANOVA and Student–Newman–Keuls test; $P<0·05$).

† Data are log values of bacterial counts. No propionibacteria were detected either in the control group, or at the start of the experiment, regardless of the group.

the same among groups (316 (SEM 4) g). Food consumption was also similar in all groups, ranging from 2·6 kg (*n* 6; 20 d) in the TL3 group to 3·2 kg (*n* 6; 20 d) in the control group. No significant differences in caecal weight and pH were observed between groups (Table 6).

By contrast, the total SCFA concentration was significantly enhanced in the caecal content of rats receiving the TL133 *P. freudenreichii* strain compared with the three other groups with a significant increase of acetate, propionate and butyrate (Table 6). Nevertheless, the relative proportions of acetate, propionate and butyrate did not differ between groups; they were 77·8 (SEM 0·3), 13·2 (SEM 0·3) and 9·0 (SEM 0·2) %, respectively. With regard to minor SCFA, consumption of propionibacteria significantly decreased the concentration of branched-chain fatty acids, regardless of the strain.

β-Galactosidase and β-glucuronidase activities were measured in the faecal contents at the end of the experiment. No significant difference was observed between groups, either for β-galactosidase activity (6·6 (SEM 0·4) μmol/min per g), or for β-glucuronidase activity (1·5 (SEM 0·1) μmol/min per g).

Effect of propionibacteria consumption on the faecal microbiota diversity

The impact of dairy propionibacteria consumption on the faecal microbiota composition was assessed by FISH combined with flow cytometry at the start of the experiment, i.e. 3 weeks after all initially germ-free rats had been inoculated with the human faecal microbiota. No significant difference was observed between groups, indicating that the microbiota composition was basically the same in all rats before propionibacteria administration (Fig. 2 (A)). The most abundant clusters were *C. coccoides–E. rectale*, detected with the Erec482 probe (Table 4), and *C. leptum*, detected with the Clep866 probe; they represented 35·3 (SEM 0·6) and 30·2 (SEM 0·8) % of the total eubacteria, respectively. The *Bacteroides* group, detected with the Bac303 probe, accounted for 25·5 (SEM 1·0) % of the bacterial population and the *Bifidobacterium* group, detected with the Bif164 probe, was the fourth major group (6·2 (SEM 0·2) %). The four other phylogenetic groups, i.e. *Atopobium*, enterobacteria and *Lactobacillus—Enterococcus*, accounted together for less than 5 % of the total. After 3 weeks of propionibacteria administration, analysis of the faecal microbiota was performed again (Fig. 2 (B)). The proportion of the *Bacteroides* cluster had significantly increased in the TL133 group (27·2 (SEM 1·1) % at initial time *v.* 35·6 (SEM 2·8) % at final time), whereas it remained stable in the other groups. Similarly, rats from the TL1348 group harboured a slightly higher proportion of enterobacteria at the end of the experiment (1·8 (SEM 0·2) % at initial time *v.* 3·2 (SEM 0·4) % at final time), though it remained in the same low order of magnitude as the other

Table 6. Effect of TL3, TL133 and TL1348 supplementation on the weight, pH and short-chain fatty acid concentrations of the caecal contents

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

	Control		TL3		TL133		TL1348	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Weight (g)	5·80	0·43	3·86	0·60	5·72	0·24	5·56	0·39
PH	6·54	0·07	6·59	0·06	6·58	0·11	6·49	0·05
Acetate (μmol/g)	30·14	4·19	29·85	1·30	39·61*	1·49	25·03	3·23
Propionate (μmol/g)	5·23	0·67	4·70	0·31	6·80*	0·19	4·22	0·46
Butyrate (μmol/g)	3·34	0·56	3·54	0·25	4·80*	0·30	2·87	0·37
Valerate and caproate (μmol/g)	1·03	0·10	0·71	0·06	0·73	0·11	0·74	0·13
Branched-chain fatty acids (μmol/g)	0·38*	0·06	0·19	0·03	0·23	0·01	0·24	0·03
Total SCFA (μmol/g)	40·12	5·41	38·99	1·79	52·17*	1·67	33·10	4·12

* Mean value was significantly different from those of the other groups (ANOVA and Student–Newman–Keuls test; $P<0·05$).

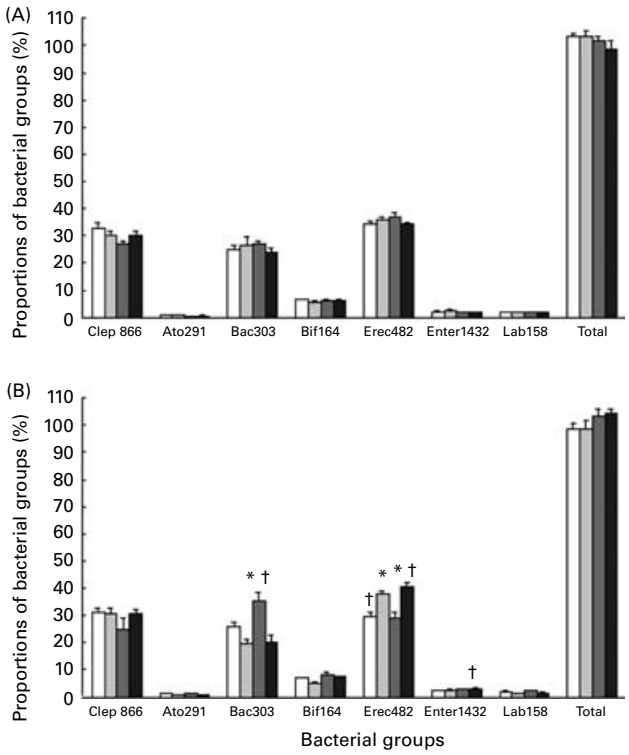

Fig. 2. Analysis of faecal microbiota of rats before (A) and after (B) a 20 d consumption period of *Propionibacterium freudenreichii* TL133 (■), TL1348 (■) or TL3 (■) strain, and for control rats (□). Proportions of *Clostridium leptum* subgroup (Clep866), *Atopobium* (Ato291), *Bacteroides* (Bac303), *Bifidobacterium* (Bif 164), *C. coccoides–Eubacterium rectale* (Erec482), *Enterics* (Enter1432) and *Lactobacilli–Enterococci* (Lab158) groups in control rats and propionibacteria-supplemented rats were assessed in the faeces using fluorescent *in situ* hybridisation combined with flow cytometry. Data are means (*n* 6) with their standard errors represented by vertical bars and are the proportions of cells hybridising with a specific probe among cells hybridising with the universal Eub338 probe. * Mean value was significantly different from that of the control group at the same time (ANOVA and Student–Newman–Keuls test; $P<0{\cdot}05$). † Mean value was significantly different from that of the same group at initial time (paired Student's *t* test; $P<0{\cdot}05$).

groups. Finally, the proportion of the *C. coccoides–E. rectale* cluster spontaneously decreased with time in the control group (34·1 (SEM 1·4) % at initial time *v.* 29·8 (SEM 1·2) % at final time) whereas it did not change in the TL3 and TL133 groups and increased in the TL1348 group (34·1 (SEM 0·6) % at initial time *v.* 40·3 (SEM 1·8) % at final time). On the whole, at the end of the experiment, the proportion of the *C. coccoides–E. rectale* cluster appeared to be higher in the TL3 and TL1348 groups than in the control and TL133 groups.

Discussion

Probiotic potential has been described for a limited number of dairy *Propionibacterium* strains and mainly depends on the ability (1) to survive and reach substantial populations within the digestive tract and (2) to maintain a metabolic activity in the colon. The selection of strains of *P. freudenreichii* best adapted for survival in the gut is a prerequisite to the development of an efficient probiotic product.

The present study showed a tremendous variability in the probiotic abilities, as revealed *in vitro*. The criteria of screening chosen were tolerance towards digestive stresses and rate of SCFA synthesis in conditions mimicking the intestinal environment (37°C, artificial intestinal content medium). The first criterion, acid and bile stress, is supposed to reflect the robustness of the strain in the adverse environment of the human digestive tract and is frequently considered. Variability among strains of propionibacteria was already observed during acid stress (Jan *et al.* 2000) and bile stress (Warminska-Radyko *et al.* 2002). However, while acid adaptation was shown to trigger bile salts sensitivity (Leverrier *et al.* 2004), the succession of both stresses, which is more stringent, was not taken into account for propionibacteria strain selection. The second criterion would reflect the ability to maintain a metabolic activity, SCFA propionate being one of the endproducts of propionibacteria obligatory fermentative metabolism. Tremendous differences in performance were observed, suggesting that not all the isolated strains have a probiotic potential. On one hand, the *in vitro* method can be applied to a large number of strains in order to expand screening. On the other hand, screenings have to be confirmed by *in vivo* investigations, as *in vitro* challenges are far from the conditions prevailing in the digestive tract.

In the present study, survival of the three strains of *P. freudenreichii* selected *in vitro* was demonstrated *in vivo*, and high-level populations were detected in the intestine of human microbiota-associated rats. These animals constitute a now well-validated model for experimental studies aimed at evaluating the effects of functional foods, including probiotics, in gastrointestinal physiology (Djouzi *et al.* 1997; Kleessen *et al.* 2001; Humblot *et al.* 2004; Imaoka *et al.* 2004). Indeed, human microbiota inoculated to germ-free rats colonise the foreign gastrointestinal tract while retaining their major characteristics in terms of bacterial populations, enzyme activities and metabolite profiles (Andrieux *et al.* 1991; Rumney & Rowland, 1992). Furthermore this model facilitates investigations on the interactions between food compounds and the human gut microbiota, since it is easier to control animal diets than those of human subjects. In addition, it enables several repetitions of the same microbiota for one dietary condition, thus avoiding the fluctuations observed by several authors in human experiments (Tannock, 2001; Zoetendal *et al.* 2001). Finally, this model allows using invasive investigations in order to evaluate propionibacteria metabolic activity *in situ*. Since SCFA are absorbed by the colonic mucosa, their quantification within the intestine is essential, while their concentration in faeces may not reflect their production rate.

Despite the possible competition with the human microbiota, ingested propionibacteria reached populations between 10^7 and 10^8 CFU/g in faeces after several days of administration. As a comparison, another dairy propionibacterium, *P. jensenii*, reached a concentration of 10^8 to 10^9 CFU/g in faeces of rats following administration at a daily dose of 10^{10} CFU (Huang *et al.* 2003), in agreement with the present results. While similar populations of *P. freudenreichii* (close to 10^8 CFU/g) were found for each TL strain in the rats' caecal contents, up to 1-log value less of viable cells were detected in the faeces than in the caeca of rats consuming the TL3 strain. A possible explanation for TL3 viability drop could be a particular sensitivity of this strain to hydric stress due to massive water absorption in the colon. Indeed,

the efficacy of osmoadaptation is highly strain-dependent within the *P. freudenreichii* species (Boyaval *et al.* 1999; Cardoso *et al.* 2004). TL3 may also be more sensitive to the barrier effect of the human microbiota. The minimal level allowing a micro-organism to influence the environment is generally recognised as 10^6 to 10^8 CFU/g intestinal content (Marteau *et al.* 1993). The caecal and faecal propionibacteria populations reached in the present study by TL133 and TL1348 thus suggest that they may have had an impact on the colon ecosystem and/or metabolism.

The metabolic activity of propionibacteria during transit in the digestive tract remains a key question. To evaluate it, a molecular method based on the specific detection of the mRNA encoding the propionibacteria transcarboxylase within the complex intestinal microbiota was used (Hervé *et al.* 2006). This protein, which contains sequences strictly specific to dairy propionibacteria, is a key enzyme of the transcarboxylase cycle involved in propionic fermentation. Transcription of transcarboxylase occurs during fermentation and stops in late stationary phase when starvation occurs. The corresponding mRNA is thus considered as a biomarker of metabolic activity (Hervé *et al.* 2006). The results obtained in the present study demonstrate that the three *P. freudenreichii* strains initiate transcarboxylase gene transcription during transit as the mRNA is detected in the caecal content of propionibacteria-supplemented rats, yet undetected in that of control rats. It can thus be hypothesised that these bacteria encountered conditions allowing active metabolism during transit in the digestive tract. Propionic fermentation leads to the production of the SCFA acetate and propionate. However, a very high proportion of SCFA is taken up by the colonic mucosa, which is why SCFA are preferentially quantified in the caecum rather than in the faeces. This was investigated in propionibacteria-supplemented and control human microbiota-associated rats. The overall SCFA caecal concentration slightly increased as a result of TL133 ingestion, while TL3 and TL1348 failed to exert such an effect. Acetate, propionate and butyrate concentrations were enhanced, while branched-chain fatty acids were lowered. A modification in both the amount and the composition of caecal SCFA was reported as a result of ingestion of milk containing *P. acidipropionici* or *P. freudenreichii* in mice (Perez Chaia & Zarate, 2005). Furthermore, in a human study using a commercially available *P. freudenreichii* probiotic preparation, the recovery of live propionibacteria seemed to be related to an enhanced content in SCFA and a lower branched-chain fatty acid ratio, in faecal samples (Jan *et al.* 2002*b*). Moreover, a lower branched-chain fatty acid ratio was also observed as a result of yoghurt consumption by infants (Guerin-Danan *et al.* 1998). As suggested by the authors, the decrease in these fatty acids, characteristic of bacterial proteolytic reactions which lead to the production of toxic metabolites, may provide a healthful influence on the host. In the present study, the *P. freudenreichii* TL133 strain may thus have exerted a beneficial influence on the intestinal metabolism.

Other effects on the gut ecosystem were further sought during the present study. No significant variations in the colon β-galactosidase and β-glucuronidase enzymic activities occurred during propionibacteria ingestion. In contrast, it has been reported that ingestion of *P. acidipropionici* increases β-galactosidase and decreases β-glucuronidase activities in the caecum of mice (Perez Chaia *et al.* 1999; Perez Chaia & Zarate, 2005). However, such an effect was not observed in the caecal contents of rats neither during the present study, nor with *P. jensenii* in rat faeces (Huang *et al.* 2003). These results seem to confirm that modulation of this bacterial enzyme activity is species-dependent. No major modification of the gut microbial ecology occurred in rats consuming the propionibacteria. Using cluster-targeting probes, the microbial composition of faecal samples was analysed by FISH combined with flow cytometry; this method estimates the relative proportion of a bacterial group within the total number of bacteria. The *Bacteroides* group, which is predominant in the human faecal microbiota, was increased in rats fed with the TL133 strain. However, this population increase remained within the proportion range from 0·0 to 47·9 % of *Bacteroides* cells obtained in a human study involving twenty subjects (Rigottier-Gois *et al.* 2003*b*). By contrast, the *P. freudenreichii* TL1348 strain increased the faecal population of *C. coccoides–E. rectale* to a proportion (40·3 %) slightly exceeding the maximum value (38·5 %) observed by Rigottier-Gois *et al.* (2003*a*) among twenty-five human subjects; nevertheless, these values are fully compatible with the proportions observed by Lay *et al.* (2005*b*)in a group of twenty-one subjects or in the 100 subjects they analysed for a large-scale molecular analysis of the normal colonic microbiota in healthy human subjects from Northern Europe (Lay *et al.* 2005*a*). No modification of the bifidobacteria population was detected, in contrast with other reports. Indeed, a bifidogenic effect was observed *in vitro* with cell-free extracts of *P. freudenreichii* (Kaneko *et al.* 1994), due to the presence of bifidogenic compounds (Mori *et al.* 1997; Isawa *et al.* 2002). Similarly, ingestion of *P. freudenreichii*, under the form of whey cultures, either heat-inactivated (Satomi *et al.* 1999) or not (Hojo *et al.* 2002), or as freeze-dried live bacteria (Bougle *et al.* 1999), resulted in a higher faecal bifidobacteria concentration in human subjects. It must be stressed that the bifidobacteria proportion in the human microbiota used in the present study was high, about 6 %, compared with the 2 % average values obtained in the human studies performed (Lay *et al.* 2005*a*,*b*). Furthermore, the bifidogenic property would depend on the strain within the *P. freudenreichii* species (N Roland, personal communication). On the whole, the present study shows that modification of the colon ecosystem by propionibacteria is limited and strain-specific.

To summarise, three strains of *P. freudenreichii*, firstly selected *in vitro* on the basis of stress challenges and metabolic assays, are able to survive at high population levels and to be metabolically active in the gastrointestinal tract of human microbiota-associated rats. Furthermore, while no drastic effect on the rats' physiology was observed as a result of propionibacteria ingestion, strains modulated differently the intestinal microbiota composition and SCFA content. The present study thus confirms the need for large-scale screenings of dairy propionibacteria, involving simple *in vitro* assays followed by *in vivo* validation experiments, to reveal the probiotic abilities of specific strains. Here, one particular strain, namely TL133, displayed an interesting probiotic potential by increasing the caecal concentration of major SCFA while decreasing branched-chain fatty acids. Colonic SCFA, produced by bacterial fermentation of dietary fibre and starch in the large bowel, are known for their beneficial impact on colonic health, including development of normal colonic

epithelial cells and protection against cancer and ulcerative colitis (Hague *et al.* 1997). Furthermore, propionibacterial SCFA are potent inducers of apoptosis in colon cancer cells *in vitro* (Jan *et al*. 2002*a*; Lan *et al*. in press). The effects of a selected strain of *P. freudenreichii* on the intestinal microbial ecosystem and metabolism, which would lead to an enhanced production of major SCFA *in vivo*, opens further perspectives for the probiotic application of dairy propionibacteria. The same *in vivo* model should allow studying the impact of selected strains of dairy propionibacteria on experimental colon carcinogenesis.

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