

Survival and beneficial effects of propionibacteria in the human gut: in vivo and in vitro investigations

Gwénaél JAN^{a*}, Pauline LEVERRIER^{a,b}, Isabelle PROUDY^b,
Nathalie ROLAND^b

^a Laboratoire de Recherches de Technologie Laitière, INRA, 65 rue de St Briec,
35042 Rennes Cedex, France

^b Standa Industrie, 68 rue R. Kaskoreff, 14050 Caen Cedex 4, France

Abstract — Propionibacteria, which are largely used as Swiss-type cheese starters, also constitute promising human probiotics because of their beneficial effects on intestinal bifidobacteria and bacterial enzyme activities. To be efficient, they must survive acid stress imposed within the stomach and the surfactant effects of bile salts. We thus investigated the ability of *Propionibacterium freudenreichii* (strain *Propiofidus*[®] SI41) to adapt to, and survive these stresses. In an in vivo study, 7 volunteers received *Propiofidus*[®] SI41 in 3 different modes. The probiotic was supplied during 3 periods (i) in classical capsules at a low dose (5×10^9 CFU·d⁻¹), (ii) in the same capsules at a higher dose (5×10^{10} CFU·d⁻¹), (iii) in acid-resistant capsules at the low dose. In the first case, propionibacteria were recovered above 10^5 CFU·g⁻¹ in only 8 out of 14 faecal samples. In the two following cases, they were enumerated in all faecal samples between 10^5 and 10^7 CFU·g⁻¹. The recovery of live propionibacteria in faecal samples seemed to be related to a higher content in short chain fatty acids. This study, which clearly showed the role of gastric protection in the survival of propionibacteria, led us to investigate stress adaptation in this strain. Acid tolerance response was evidenced: transient exposure to pH 5 afforded protection towards acid challenge at pH 2. Moreover, we showed that pre-exposure to a moderate bile salts pre-treatment at $0.2 \text{ g}\cdot\text{L}^{-1}$ sharply increased its survival against a subsequent lethal challenge ($1 \text{ g}\cdot\text{L}^{-1}$). Important changes in cellular protein synthesis were observed during adaptation using two-dimensional electrophoresis. Global protein synthesis decreased during adaptation treatments. In contrast, specific proteins were overexpressed during acid adaptation and others during bile salts adaptation. Among the up-regulated polypeptides, a carboxyl carrier protein (BCCP) and the recombinase RecR were identified during both stress responses while the universal chaperonins GroEL and GroES, as well as the replicase RepB, were only up-regulated during acid adaptation. These results bring new insights on the tolerance of *P. freudenreichii* to acid and bile salts, which should be taken into consideration for the development of probiotic preparations.

Propionibacterium / probiotic / stress / adaptation / proteome

Oral communication at the 3rd International Symposium on Propionibacteria, Zurich, Switzerland, July 8–11, 2001.

* Correspondence and reprints

Tel.: (33) 2 23 48 57 41; fax: (33) 2 23 48 53 50; e-mail: gjan@labtechno.roazhon.inra.fr

Résumé — Survie et effets bénéfiques des bactéries propioniques dans l'intestin humain : études in vivo et in vitro. Les propionibactéries, qui sont largement utilisées comme levain d'affinage des fromages à pâte pressée cuite, constituent également des probiotiques humains prometteurs du fait de leurs effets bénéfiques sur la flore bifide et les activités enzymatiques bactériennes, au niveau de l'intestin. Pour être efficaces, elles doivent survivre au stress acide subi dans l'estomac et à l'action détergente des sels biliaires. Nous avons donc étudié la capacité de *Propionibacterium freudenreichii* (souche *Propiofidus*[®] SI41) à s'adapter et à survivre à ces stress. Dans une étude in vivo, 7 volontaires ont reçu *Propiofidus*[®] SI41 selon 3 modalités. Le probiotique était fourni pendant 3 périodes (i) dans des gélules classiques à une faible dose (5×10^9 UFC·j⁻¹), (ii) dans les mêmes gélules à une dose plus élevée (5×10^{10} UFC·j⁻¹), ou (iii) dans des gélules acido-résistantes à la plus faible dose. Dans le premier cas, les propionibactéries ont été retrouvées à plus de 10^5 CFU·g⁻¹ dans seulement 8 échantillons fécaux sur 14. Dans les deux cas suivants, elles ont été détectées dans tous les échantillons à une concentration comprise entre 10^5 and 10^7 UFC·g⁻¹. La présence de propionibactéries viables dans les échantillons fécaux semblait reliée à une concentration plus élevée d'acides gras à courte chaîne. Cette étude, qui montre clairement le rôle de la gastro-protection vis-à-vis de la survie des bactéries, nous a amenés à rechercher l'adaptation au stress chez cette souche. L'acquisition de tolérance en réponse au stress acide a été mise en évidence : l'exposition transitoire à pH 5 protège d'une épreuve acide à pH 2. De plus, la pré-exposition à une dose modérée de sels biliaires ($0,2 \text{ g} \cdot \text{L}^{-1}$) augmente nettement sa survie lors d'une épreuve létale ($1 \text{ g} \cdot \text{L}^{-1}$). Des modifications importantes de la synthèse des protéines cellulaires ont été observées par électrophorèse bidimensionnelle durant l'adaptation. La synthèse protéique globale diminue pendant les traitements d'adaptation. En revanche, des protéines spécifiques sont sur-exprimées pendant l'adaptation à l'acidité et d'autres pendant l'adaptation aux sels biliaires. Parmi les polypeptides sur-exprimés, une carboxyl carrier protein (BCCP) et la recombinase RecR ont été identifiées dans les deux réponses alors que les chaperonines universelles GroEL et GroES ainsi que la réplicase RepB sont seulement induites par l'adaptation acide. Ces résultats apportent de nouvelles données sur la tolérance de *P. freudenreichii* aux stress digestifs, qui doivent être prises en considération pour la mise au point de préparations probiotiques.

***Propionibacterium* / probiotique / stress / adaptation / protéome**

1. INTRODUCTION

Probiotics are defined as live microbial food supplements that beneficially affect the host health when ingested. These microorganisms are thought to help prevent and/or lessen various disorders such as diarrhoea, lactose intolerance, inflammatory bowel disease, irritable bowel syndrome, hypercholesterolemia and food allergy [15, 19]. They can also improve local immunity and exert a positive effect on carcinogenesis, although the corresponding mechanisms of action remain to be fully elucidated [25, 31]. Claimed bacterial probiotics belong to the *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Bacteroides*, *Bacillus* and *Propionibacterium* geni.

Propionibacteria display several promising characteristics, including the produc-

tion of vitamin B12, which led to consideration of their use as human probiotics [18]. Pérez Chaia et al. showed that they positively modulate intestinal microflora in mice by modifying the number of anaerobes and coliforms. Furthermore, they were able to down-regulate the carcinogenic β -glucuronidase, azoreductase and nitroreductase activities in these animals [23]. Propionibacteria were also shown to be a source of β -galactosidase, which would be liberated as a result of bile salts permeabilisation within the gut [32]. They could thus be useful to prevent lactose intolerance. It has also been demonstrated that these bacteria favour the growth of bifidobacteria, both in vitro [20] and in the human intestine [3]. It is noteworthy that some strains of dairy propionibacteria produce bacteriocins. *P. thoenii*, in particular, produces PLG-1, a bacteriocin able to inhibit growth and survival of *Listeria*

monocytogenes and *Yersinia enterocolitica* in fermented milk [16, 17]. This reinforces their potentialities as probiotics. The characterisation of a cytoplasmic protein, which exhibits reactivating effects on both prokaryotic and eukaryotic organisms [29, 30], might further highlight the beneficial role of these bacteria in human alimentation [27].

Efforts have thus been concentrated on the effect of digestive stresses on propionibacteria. The study performed by Bouglé et al. on healthy human volunteers revealed that some strains of dairy propionibacteria survived the transit through the human digestive tract [3]. In vitro, we have shown a great diversity among strains in the ability to adapt and survive acid stress [11]. However, for most of the strains tested, no lethal effect was detected for pH values above 2.5. This was confirmed in an artificial gastric juice by Zarate et al. [32]. An adaptive response was evidenced in strain SI41 and was shown to require the synthesis of a set of acid stress proteins [12].

In this study, the survival rate of SI41 was followed in healthy human volunteers with regard to the impact of ingested dose and acid-resistant capsules. The impact of propionibacteria ingestion on the faecal concentration of SCFAs was also investigated. In vitro, we were able to show a promising adaptation of this *P. freudenreichii* strain to the complex and stressful ecosystem of the human digestive tract. The mechanisms underlying this adaptive response were investigated using a proteomic approach. Stress-activated proteins were identified which respond to acidic pH or to the surfactant bile salts. This study thus constitutes a first insight into the molecular mechanisms of digestive stress adaptation in *P. freudenreichii*. Understanding and controlling this response is likely to determine the efficacy of propionibacterial probiotic products.

2. MATERIALS AND METHODS

2.1. Human subject and probiotic administration

Seven healthy volunteers (3 males and 4 females) between 25 and 35 years old participated in this study. It was divided into 3 periods of 4 weeks (d 1 to d 28), each period separated by a 2-week interval. Each period began with a one-week control time during which volunteers consumed neither *P. freudenreichii* SI41, nor Swiss-type cheese. In each period, the volunteers received a daily treatment from d 8 to d 21, consisting of the ingestion of one capsule of freeze-dried bacteria. In the first and second periods, the volunteers received 5×10^9 and 5×10^{10} CFU per day respectively of *P. freudenreichii* SI41 (Propiofidus[®], Standa-Industrie, Caen, France) provided in classical capsules. In the third period, the volunteers received 5×10^9 CFU per day of SI41 provided in acid-resistant capsules. Swiss-type cheese was withdrawn from the volunteers' alimentation throughout the 3 periods.

2.2. Counting of propionibacteria in faecal samples

Faeces were collected 4 times in each period: at d 7 (just before beginning the treatment), at d 14 (after the first week of treatment), at d 21 (after 2 weeks of treatment) and at d 28 (one week after the end of the treatment). Freshly collected faeces were immediately placed in anaerobic conditions at 4 °C. One half of each sample was frozen and kept at -20 °C for SCFA analysis. The other half was 1/10 diluted in physiological peptone water and homogenised in stomacher bags. Further dilutions (from 1/10 000) were poured into Pal-propiozac[®] selective agar added to an antibiotic cocktail (Standa-Industrie, Caen, France) and metronidazole (4 mg·L⁻¹). Plates were incubated anaerobically at 30 °C for one week

prior to colony counting. Results are expressed as log CFU·g⁻¹ of faeces. For samples with no detectable propionibacterium, the indicated result is less than 10⁴ CFU·g⁻¹, this being the detection limit in our conditions.

2.3. Pulse-field gel electrophoresis (PFGE) analysis of faecal propionibacteria

Typical colonies of propionibacteria were isolated from Pal-propionibac[®] selective agar plates (3 or 5 clones per plate) and cultured in yeast extract-lactate (YEL) medium, as well as SI41. DNA samples were prepared according to Gautier et al. [7], and digested for 4 h at 37 °C using the restriction enzyme *Xba*I (Eurogentec, Liège, Belgium). Electrophoresis was run for 20 h at 14 °C on 1% agarose gels submitted to 200 V, with pulses at 2 and 20 s, using a Chef DRII system (Bio-rad, Richmond, UK).

2.4. Faecal SCFA analysis

Five grams of each faecal sample was thawed and was 1/2 diluted in mercuric chloride solution (1/50 dilution of a saturated solution) prior to vortex homogenisation. The resulting mixture was centrifuged (45 000 g, 25 min, 4 °C). 0.1 mL of phosphotungstic acid (1/5 dilution of a saturated solution) was added to 1 mL of supernatant. Deproteinisation was conducted overnight at 4 °C prior to centrifugation (14 000 g, 10 min, 4 °C). Supernatants were immediately frozen for further analysis. SCFA composition was determined using a gas liquid chromatograph (Varian 3400, Varian France, S.A.) on a column (1.5 m long × 2 mm I.D.) packed with 80/100 mesh Chromosorb 101 and nitrogen as carrier gas, according to Jouany [13]. Results are expressed as µmol of SCFA per gram of faeces.

2.5. Bacterial growth for in vitro studies

Strain SI41 was routinely cultivated at 30 °C, without shaking, on YEL broth and stored at -80 °C in the same medium, supplemented with 15% glycerol. All the cultures used were less than 5 passages from an original stock. Exponential phase cells were obtained as follows: an overnight preculture was used to inoculate fresh YEL medium (1/100) and cells were harvested as optical density (650 nm) reached 0.5, corresponding to a population of 5 × 10⁸ CFU·mL⁻¹.

2.6. Stress adaptation and extreme challenge

Exponential phase cells were acid-adapted as follows: bacteria were centrifuged and resuspended in acidified YEL at pH 5 prior to incubation for 30 min. For bile salts adaptation, an equimolar mixture of sodium cholate and sodium deoxycholate was added to a sub-lethal concentration (0.2 g·L⁻¹) prior to incubation for 4 h at 30 °C. An extreme acid challenge consisted of exposing propionibacteria for 60 min to pH 2 and extreme bile salt challenge by exposing them for 60 s to 1 g·L⁻¹ bile salts. All experiments were conducted at 30 °C. At the end of the treatments, surviving bacteria were enumerated. Therefore, samples were diluted in peptone water (0.1% peptic digest of meat, Biokar Diagnostics, Beauvais, France), pH 7.0, containing 0.9% NaCl and poured into YEL medium containing 1.5% agar for maximal recovery. CFU were determined after 6 d of anaerobic incubation at 30 °C.

2.7. Radioactive labelling, whole-cell protein extraction and two-dimensional electrophoresis

Control and adapted cells were labelled essentially as described previously using

500 μCi of [^{35}S]methionine/cysteine protein labelling mix (ICN Pharmaceuticals, Orsay, France) per 1 mL subsample of bacterial culture [12]. Protein extracts were subjected to high-resolution two-dimensional electrophoresis according to the method described by O'Farrel [22] and modified by Görg et al. [8]. Equal amounts of radioactivity were loaded onto first dimension gels (1 000 000 dpm) and isoelectric focusing was performed using immobilised pH gradient (IPG) strips (18 cm pH 4 to 7 Dry Strips, Amersham Pharmacia Biotech, Uppsala, Sweden) in a focusing solution containing 7 mol·L⁻¹ urea, 2 mol·L⁻¹ thiourea, 25 mmol·L⁻¹ DTT, 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) and 2% IPG-Buffer (Amersham Pharmacia Biotech) according to Rabilloud [24]. Isoelectric focusing was conducted on a Multiphor II unit using a gradient mode yielding 60 000 Vh. The strips were equilibrated in the presence of DTT and then iodoacetamide [2] and placed on top of a uniform SDS-14% polyacrylamide gel for the second dimension using an Isodalt two-dimensional electrophoresis system (Amersham Pharmacia Biotech). Gels were dried and electronic autoradiograms acquired on a Storm Phosphorimager (Amersham Pharmacia Biotech). The quantitative analysis of spots was performed using the two-dimensional gel computer analysis software Melanie II (Bio-Rad, Hercules, USA).

3. RESULTS

3.1. Recovery of viable propionibacteria from faeces during the different periods

First period (n = 7). At the beginning of the experiment, faecal propionibacteria were not detected in the faeces of 3 volunteers or detected at a small level for the 4 others (<4.90 log CFU per gram). During

the period of daily ingestion of 10⁹ CFU of SI41 in classical capsules, propionibacteria were recovered in the faeces of 6 volunteers after the first week and in all volunteers after 2 weeks, with a mean value of 5.50 \pm 1.11 log CFU·g⁻¹ (Tab. I). During this first treatment, results were highly variable between individuals. For volunteer 2, faecal propionibacteria remained very low, whereas for volunteer 5, faecal propionibacteria rose to high levels from the first week. One week after the end of the treatment, faecal propionibacteria became undetectable, except for 3 volunteers with a level of 5.00 to 6.48 log CFU·g⁻¹.

Second period (n = 5). Surprisingly, at d 7, faecal propionibacteria were still counted at a high level in 3 volunteers (about 6.40 log CFU·g⁻¹). During the treatment consisting of ingestion of 10¹⁰ CFU per day of SI41 in classical capsules, propionibacteria were recovered in the faeces of all volunteers, ranging from 5.00 to 7.20 log CFU·g⁻¹, with a mean level of 6.25 \pm 0.66 log CFU·g⁻¹ (Tab. I). After one week of washout, propionibacteria were still numbered in 2 volunteers.

Third period (n = 6). During the time of ingestion of 10⁹ CFU per day of SI41 in gastro-protected capsules, faecal propionibacteria were found in all volunteers, from 4.95 to 6.81 log CFU·g⁻¹, with a mean level of 6.12 \pm 0.59 log CFU·g⁻¹ (Tab. I). Again, high levels of faecal propionibacteria were found in some volunteers before and after the treatment.

3.2. Composition of the faecal propionibacteria population as determined by molecular typing

Propionibacteria strains are easily differentiated on the basis of restriction fragment length profiles as revealed by PFGE [7]. Thus, 94 faecal clones were identified using PFGE and were chosen as follows: 9 clones at d 7 of period 2 (3 different

Table I. Recovery of propionibacteria from human faecal samples before and during ingestion of *Propiofidus*[®] SI41.

Volunteers	Treatment 1 ^a				Treatment 2 ^b				Treatment 3 ^c			
	d 7	d 14 ^d	d 21	d 28	d 7	d 14	d 21	d 28	d 7	d 14	d 21	d 28
Vol 1	4,00 ^e	4,00	5,88	< 4	6,46	5,83	6,36	5,11	< 4	6,51	6,56	5,26
Vol 2	< 4	< 4 ^f	4,00	< 4	< 4	5,40	6,46	< 4	< 4	5,43	5,77	< 4
Vol 3	< 4	4,70	6,85	< 4	6,43	6,69	6,83	< 4	6,15	4,95	6,70	< 4
Vol 4	4,00	6,00	6,08	6,11	6,30	6,32	6,36	< 4	< 4	6,79	6,23	6,80
Vol 5	< 4	7,15	6,30	6,48	< 4	7,20	5,00	6,11	6,38	6,81	6,11	< 4
Vol 6	4,85	4,85	4,70	< 4	N.D.	N.D.	N.D.	N.D.	< 4	5,68	5,95	5,89
Vol 7	4,30	5,68	6,76	5,00	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
mean ± s	N.D.	5,50 ± 1.11	N.D.	N.D.	N.D.	6,25 ± 0.66	N.D.	N.D.	N.D.	6,12 ± 0.59	N.D.	N.D.

^a Patients received 5×10^9 CFU of freeze-dried *Propiofidus*[®] SI41 in classical capsules.

^b Patients received 5×10^{10} CFU of freeze-dried *Propiofidus*[®] SI41 in classical capsules.

^c Patients received 5×10^9 CFU of freeze-dried *Propiofidus*[®] SI41 in acid resistant capsules.

^d Shaded columns indicate the treatment period, d 14: one week of treatment, d 21: two weeks of treatment.

^e Results are expressed as log CFU·g⁻¹ of faeces.

^f Value assimilated to 4 for the mean calculation.

N.D. not determined.

volunteers); 10 clones at d 7 of period 3 (2 different volunteers); 60 clones during the third treatment, at d 14 and d 21 (in all volunteers) and 15 clones at d 28 of period 3 (3 volunteers). Among the 60 clones isolated during supplementation time, 93% were identified as SI41. The profiles of the 34 other clones, all isolated outside the supplementation periods, were highly different from SI41 but were all typical of the species *P. freudenreichii*. They thus evidenced 25 different propionibacteria strains of human intestinal origin.

3.3. Faecal level of SCFA

First, and due to the small number of volunteers and the high variability among the results, obtained data were pooled for the 3 periods and divided into 2 groups, in order to investigate the influence of ingestion of SI41 on faecal SCFA; (1): results obtained from faeces collected during supplementation of SI41 (at d 14 and d 21

of the 3 periods, n = 36); (2): results obtained from faeces collected outside ingestion time (at d 7 and d 28 of the 3 periods, n = 38). With this analysis, no significant difference could be observed between the 2 groups of data, for all measured SCFA, either regarding their concentration, or their profile (data not shown).

Due to the frequent apparitions of faecal propionibacteria outside ingestion times, it appeared interesting to investigate the relationship between the presence of these bacteria (SI41 or endogenous strain) and the level of SCFA in the faeces. For this, results were divided into 2 new subgroups; "PAB +": faeces containing more than 4 log CFU·g⁻¹ of propionibacteria, collected or not during ingestion of SI41 (n = 53) and "PAB -": faeces without detectable propionibacteria, essentially outside the time of ingestion of SI41 (n = 21). Results are indicated in Table II and were statistically compared with the Student test. It appeared that, in the

Table II. Concentration and composition of SCFAs in the two subgroups of faecal samples.

	“faecal PAB –” subgroup ¹ (n = 21)	“faecal PAB +” subgroup ¹ (n = 53)
Concentration of SCFA ($\mu\text{mol}\cdot\text{g}^{-1}$):		
total	32.88 (16.57) ²	50.02 (27.68) ² **
acetate	18.35 (10.01)	28.55 (17.18)**
propionate	4.88 (2.41)	7.46 (3.84)**
butyrate	5.21 (3.20)	8.81 (5.82)**
valerate + caproate	1.40 (0.79)	1.96 (1.27)
isobutyrate + isovalerate	3.04 (1.34)	3.25 (1.54)
% of total SCFA:		
acetate	55.6 (6.1)	56.1 (6.4)
propionate	14.9 (2.3)	15.3 (2.4)
butyrate	15.4 (4.5)	17.2 (4.1)
valerate + caproate	4.3 (0.9)	3.9 (0.9)
isobutyrate + isovalerate	9.8 (2.6)	7.5 (3.1)**

¹ “Faecal PAB –”: all faecal samples containing no detectable propionibacteria and “faecal PAB +”: all faecal samples containing more than 4 log (CFU·g⁻¹) of propionibacteria.

² Results are expressed as means (s).

** Results significantly different between the 2 subgroups, $p < 0.01$ (Student test).

presence of propionibacteria in the faeces, the faecal concentrations of acetate, propionate and butyrate were clearly higher, compared with the subgroup without faecal propionibacteria ($p < 0.01$). Regarding the fermentative profile, expressed in percent of total SCFA, no difference was observed between the 2 subgroups, except for isobutyrate and isovalerate, whose percentage significantly decreased in the presence of faecal propionibacteria ($p < 0.01$).

3.4. Adaptation to acid and bile salts stresses

Results obtained in vivo have shown that the SI41 strain was, at least partially, able to adapt to digestive stresses, while other tested strains were never recovered in a cul-

tivable state from human volunteers (data not shown). We thus looked for acid and bile salts tolerance acquisition in SI41 in vitro. The viability of SI41 dropped rapidly when bacteria were shifted from optimal to lethal conditions. Indeed, a 4 log decrease in viable cell counts was recorded upon exposure to pH 2 or 1 g·L⁻¹ bile salts (Fig. 1). However, pre-treatment at sublethal doses of these stresses restored viability during a subsequent lethal challenge. Indeed, acid-adapted cells reached 87% survival at pH 2 and bile salts-adapted ones 22.5% during a challenge with 1 g·L⁻¹ bile salts. In order to investigate overlaps between stress regulons, the influence of a mild thermal pre-treatment was also checked. Heat adaptation protected cells from extreme acid challenge and 3% survival was achieved versus 0.0025% for unadapted cells

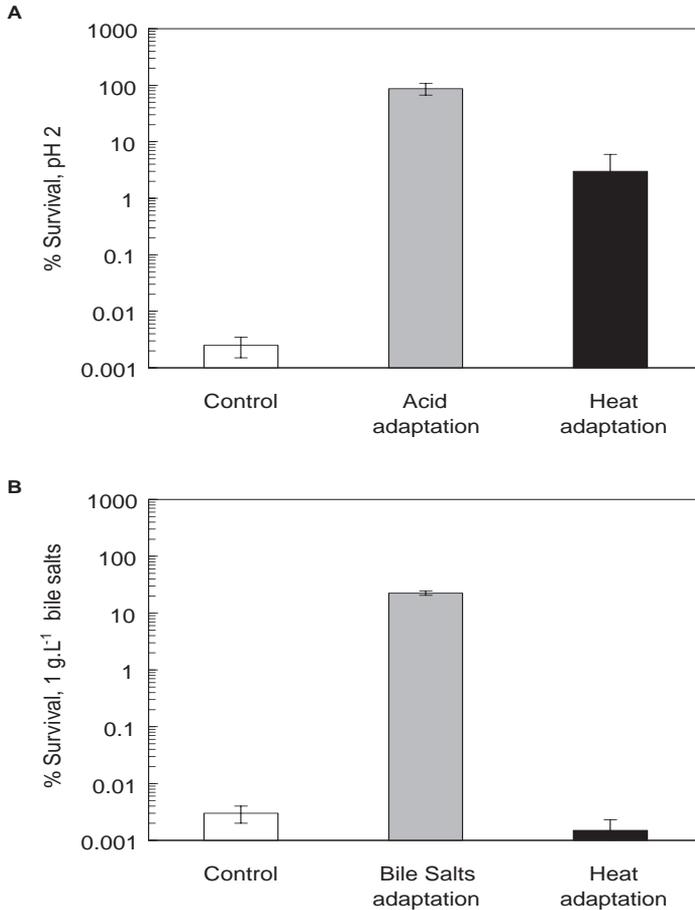


Figure 1. Effect of homologous and heterologous pre-treatments on *P. freudenreichii* tolerance to acid and bile salts challenge. Exponential cells were submitted to lethal acid (pH 2, **A**) or lethal bile salts (1 g·L⁻¹, **B**) challenge without pretreatment (control), after homologous pre-treatments (pH 5, acid adaptation; 0.2 g·L⁻¹ bile salts, bile salts adaptation) or after a heterologous heat pretreatment (52 °C). Each point is the average of at least 4 independent experiments and standard deviations are shown by bars.

(Fig. 1A). By contrast, heat adaptation had no protective effect towards an extreme bile salts challenge and a lower survival rate (0.0015%) was obtained than without pre-treatment (0.003%, Fig. 1B).

3.5. Analysis of acid and bile salts adaptation proteins

To elucidate the molecular mechanisms leading to stress tolerance, we looked for

stress protein over-expression during acid and bile salts protecting pre-treatments. Pulse labelling and two-dimensional gel electrophoresis were used to resolve the maximum of cellular ³⁵S-labelled proteins of *P. freudenreichii* cells undergoing adaptation either at pH 5 or in the presence of 0.2 g·L⁻¹ bile salts. Both stresses led to dramatic changes in the protein pattern (Fig. 2). The synthesis of the majority of the approximately 900 proteins expressed in

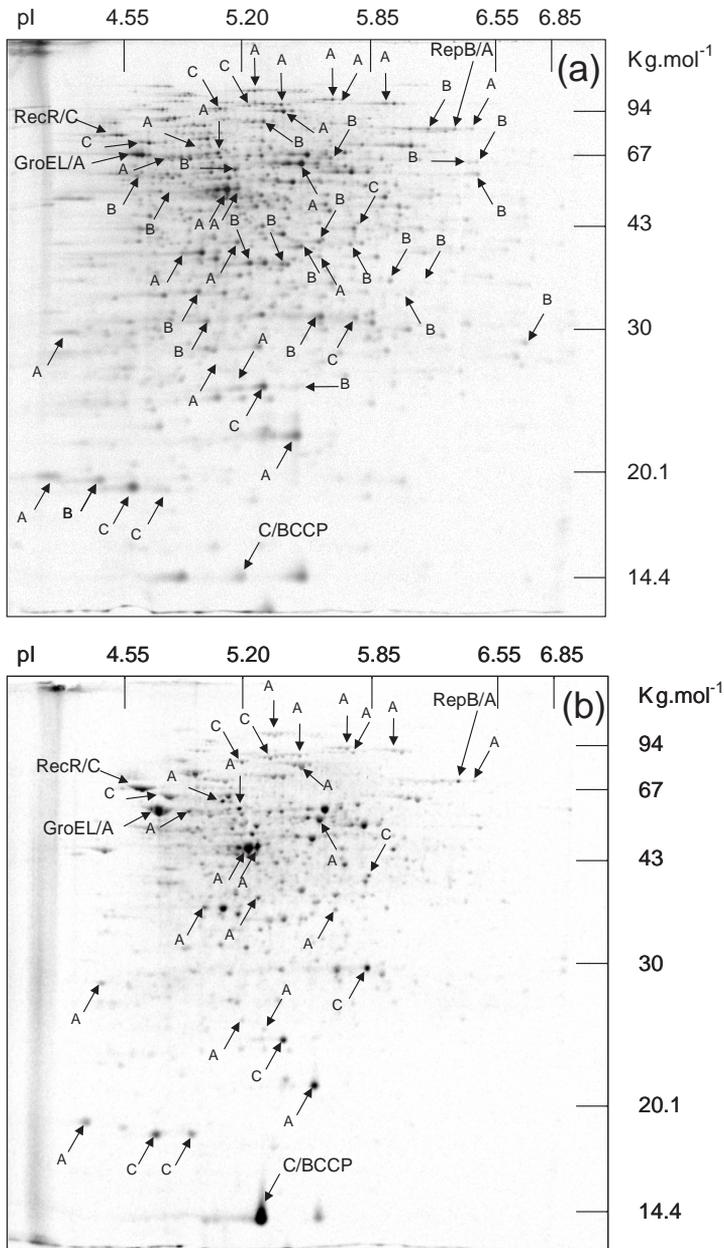


Figure 2. Analysis of *P. freudenreichii* cellular protein synthesis rate during exponential growth and during stress adaptation. Proteins were labelled in untreated (a), acid-adapted (b) or bile-salts-adapted (c) cells. The different groups of stress proteins are marked with capital letters: A, specific to acid, B, specific to bile salts and C, common to both stresses. The RepB, RecR, GroEL and BCCP proteins identified previously are indicated. Growth, labelling and two-dimensional analysis were performed in at least 3 independent experiments, showing less than 10% variation in relative spot intensities.

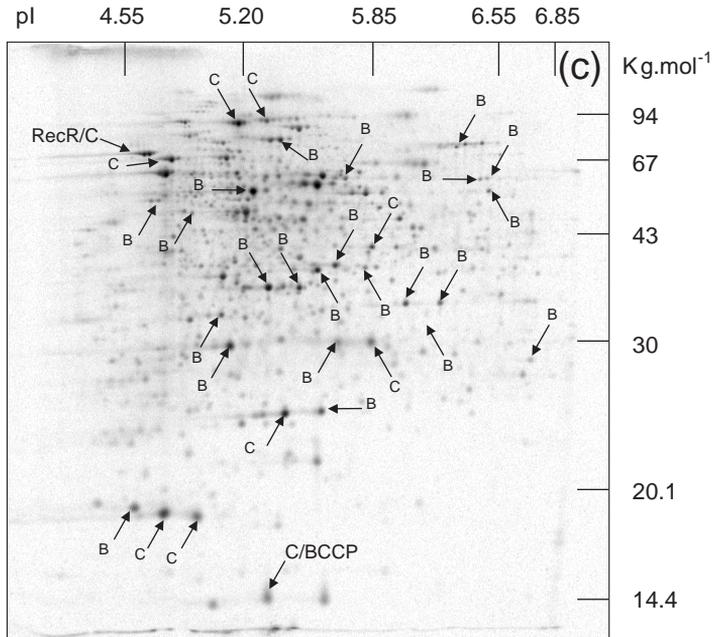


Figure 2 (continued).

control cells showed reduction or even complete inhibition in the adapted cells. On the other hand, at least 56 proteins appeared to be overexpressed in stressful conditions. Among these, 23 were specifically enhanced by acid treatment (Fig. 2B), 23 by bile salt treatment (Fig. 2C), while 10 stress proteins were common to both stimuli (Fig. 2A). The subset of 10 common stress proteins contains two spots previously identified as BCCP, the biotin-containing carboxyl carrier sub-unit of the methylmalonyl- CoA transcarboxylase (TC) from *P. freudenreichii* subsp. *shermanii*, as well as a protein highly homologous to *Bacillus subtilis* recombinase RecR [12]. The subset of 20 acid stress proteins, on the other hand, contained the universal chaperonin GroEL previously identified [12].

4. DISCUSSION

4.1. Human study

The aim of this study was to confirm the adaptation and survival of SI41 in the human digestive tract and to study the influence of the ingested dose as well as that of gastroprotection. As shown previously, this strain survived and was recovered at a concentration exceeding 10^6 viable propionibacteria (identified by genomic fingerprinting as SI41) per gram of faeces. It is generally recognised that this bacterial concentration is sufficient for a probiotic to exert a beneficial effect on colon ecology and host physiology [5]. However, the selective Pal-propionibac[®] medium used in this study to inhibit the growth of enteric

bacteria underestimates propionibacteria counts, compared to the rich YEL medium, especially if cells are stressed or damaged (Jan, unpublished data). It is thus likely that the viability determined using this medium is underestimated. Except for volunteer 5, who displayed high and similar amounts of propionibacteria, whatever the treatment, this study stressed the importance of the ingested dose and of gastro-protection. Propionibacteria were recovered above 10^6 CFU·g⁻¹ from all the faecal samples only when the volunteers ingested the highest dose or acid-resistant capsules. These capsules restored a propionibacteria viability comparable to that afforded by the highest dose. It is thus tempting to speculate that, in these individuals, the drastic acidic conditions of the stomach were responsible for the poor survival of SI41. Acid stress being the bottleneck of probiotic survival, it should thus be bypassed either by using these acid-resistant capsules, or by triggering acid tolerance in SI41. However, even in the case of gastroprotection, survival of propionibacteria was evaluated at only around 2% due to the other stresses encountered. In the best cases, propionibacteria thus represented only 0.001% of the total faecal flora. As a comparison, a similar study performed with bifidobacteria on 8 human volunteers allowed the numeration of 8.8 ± 0.1 log CFU·g⁻¹ of faeces [4]. This probiotic was ingested in the form of 375 g of fermented milk per day containing a daily intake of 11.8 log CFU·d⁻¹, which is considerably higher than the doses used in our study. By contrast, *Lactobacillus rhamnosus* GG was given to human volunteers in classical capsules with daily doses (8 and 10 log CFU·d⁻¹) comparable to our work [26]. This probiotic was not recovered from the faeces at the lowest dose and was numerated between 3 and 5 log CFU·g⁻¹ using the highest dose. In another investigation using *Lactobacillus rhamnosus* GG (6×10^{10} CFU per day in fermented whey), the probiotic was detected at concentrations varying from 4 to 9 log CFU·g⁻¹ of

faeces, depending on the volunteer [1]. Interestingly, *L. rhamnosus* was still detected in substantial amounts in colonic biopsies one week after supplementation while no viable *L. rhamnosus* was detected at that time in the faeces. The authors concluded that the study of faecal samples alone was not sufficient for evaluating colonisation by a probiotic. Nevertheless, further investigations are needed to improve *P. freudenreichii* survival in the human gut. Special attention should therefore be focussed on stress tolerance acquisition and on the way of administration (cheese, fermented milk or whey, freeze-dried bacteria, encapsulation). As for other probiotics, a survey of the volunteers after the test period revealed that SI41 did not persist in the human gut, as it was no longer detected one week after the treatment.

To our knowledge, the presence of dairy propionibacteria in the human gut has never been reported, albeit sought [3, 28]. In this study, some of the human volunteers were shown to harbour *P. freudenreichii* strains distinct from SI41, although Swiss-type cheese was withdrawn from their alimentation. The ingestion of SI41 might have favoured the emergence of endogenous strains, which were undetectable at the beginning of the experiment. These human-derived strains should be considered for future developments and might well display physiological characteristics adapted to the gut ecosystem.

This work did not evidence a marked increase in propionate or other SCFA in the faeces, as an effect of SI41 ingestion. However, the amount of the major SCFAs (acetate, propionate and butyrate) seemed to be higher in faecal samples rich in propionibacteria, either SI41 or human strains. By contrast, the proportion of the isoacids isobutyrate and isovalerate seemed lower in these samples. Nevertheless, the high variability between volunteers, in terms of faecal counts of propionibacteria as well as in terms of SCFAs

concentrations, does not allow us to draw clear conclusions. Thus, further investigations on the influence of ingesting human-derived propionibacteria are needed to establish their impact on volatile fatty acid production, which was demonstrated to exert beneficial effects on human health [21].

4.2. In vitro study

In a previous report, we demonstrated in vitro the ability of dairy propionibacteria to sense, adapt to and survive acidic environments. The acid stress susceptibility was shown to be highly variable among strains of a same species [11]. Experiments conducted in human volunteers confirmed this high variability. Indeed, some strains, including SI41, were recovered after transit through the human digestive tract, while others succumbed to this challenge ([3], this work). It thus seemed essential to investigate the stress tolerance response in this promising strain. In a first report, we have shown that the acid tolerance response was surprisingly efficient in this strain, leading to 100% survival at pH 2 [12]. Here, we extended this investigation to adaptation to the surfactant bile salts. An adaptive response was also demonstrated for this second stress and sharply increased survival in the presence of lethal concentrations of these toxic compounds. With the aim of discriminating between general and specific stress responses, we looked for cross-protection between digestive stresses and heat stress, generally recognised as the most general stimulus. Surprisingly, heat stimulation afforded protection against an acid challenge, but no cross-protection towards bile salts stress could be detected, whatever the temperature tested. These data underline the overlap of acid and heat regulons, as previously observed in other bacteria, whether they are Gram-positive [9], or negative [10]. However, heat stress, which was shown to confer cross-protection against bile salts stress in the Gram-positive *Enterococcus*

faecalis [6], had on the contrary adverse effects on bile salts susceptibility in *P. freudenreichii* SI41. Considering that in *E. faecalis*, heat adaptation had no positive effect on acid tolerance, it becomes obvious that interaction between stress regulons is not only complex but also specific to the organism studied. Physiological data were confirmed by the proteomic approach. Indeed, among the 54 located stress proteins, only 10 were common to acid and bile salts. Acid tolerance response was shown to involve over-expression of the universal chaperonin GroEL. It is thus clear that acid adaptation triggers the heat shock response. This is indeed consistent with the cross-protection observed between acid and heat stress. On the other hand, heat shock response does not seem to be induced by bile salts, in accordance with the lack of cross-protection. Furthermore, only 10 out of the 34 bile salts stress proteins were also induced by acid stress. This is quite the opposite to what was observed in *E. faecalis*. In this last, both responses are closely related and 18 out of 21 bile salts stress polypeptides are heat shock proteins [6]. These data show that bile salt stress triggers a rather specific response in *P. freudenreichii*.

The efficacy of most of the recognised human probiotics is mainly limited by their ability to adapt to and survive abiotic stresses. While some of them are killed by the successive stresses of the human digestive tract (mainly acid and bile salts), others don't reach this last because they fail to survive the technological process and storage of the product [14]. Understanding and mastering the cellular processes leading to efficient tolerance towards these multiple environmental constraints thus constitutes a major prerequisite for the development of a propionibacterial probiotic preparation. We have demonstrated that, in contrast to other strains, *P. freudenreichii* subsp. *shermanii* SI41 showed promising characteristics in this respect. The use of proteomic tools is currently giving clues

about the mechanisms leading to the efficient response of SI41 to unfavourable conditions. A more complete analysis of molecular events occurring during homologous and heterologous protective pre-treatments will allow the optimisation of probiotic preparations in a pragmatic and experienced way.

ACKNOWLEDGMENTS

The authors thank A. Rouault for expert technical assistance and P. Boyaval for his constant interest in this work. The participation of the volunteers is gratefully acknowledged.

REFERENCES

- [1] Alander M., Satokari R., Korpela R., Saxelin M., Vilpponen-Salmela T., Mattila-Sandholm T., von Wright A., Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption, *Appl. Environ. Microbiol.* 65 (1999) 351–354.
- [2] Beis A., Lazou A., Removal of artifactual bands associated with the presence of 2-mercaptoethanol in two-dimensional polyacrylamide gel electrophoresis, *Anal. Biochem.* 190 (1990) 57–59.
- [3] Bougle D., Roland N., Lebeurrer F., Arhan P., Effect of propionibacteria supplementation on fecal bifidobacteria and segmental colonic transit time in healthy human subjects, *Scand. J. Gastroenterol.* 34 (1999) 144–148.
- [4] Bouhnik Y., Pochart P., Marteau P., Arlet G., Godere I., Rambaud J.C., Fecal recovery in humans of viable *Bifidobacterium* sp. ingested in fermented milk, *Gastroenterology* 102 (1992) 875–878.
- [5] Collins M.D., Gibson G.R., Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut, *Amer. J. Clin. Nutr.* 69 (1999) 1052S–1057S.
- [6] Flahaut S., Hartke A., Giard J.C., Benachour A., Boutibonnes P., Auffray Y., Relationship between stress response toward bile salts, acid and heat treatment in *Enterococcus faecalis*, *FEMS Microbiol. Lett.* 138 (1996) 49–54.
- [7] Gautier M., de Carvalho A.F., Rouault A., DNA fingerprinting of dairy propionibacteria strains by pulsed-field gel electrophoresis, *Curr. Microbiol.* 32 (1996) 17–24.
- [8] Görg A., Postel W., Gunther S., The current state of two-dimensional electrophoresis with immobilized pH gradients, *Electrophoresis* 9 (1988) 531–546.
- [9] Hartke A., Bouché S., Giard J.C., Benachour A., Boutibonnes P., Auffray Y., The lactic acid stress response of *Lactococcus lactis* subsp. *lactis*, *Curr. Microbiol.* 33 (1996) 194–199.
- [10] Heyde M., Portalier R., Acid shock proteins of *Escherichia coli*, *FEMS Microbiol. Lett.* 57 (1990) 19–26.
- [11] Jan G., Rouault A., Maubois J.L., Acid stress susceptibility and acid adaptation of *Propionibacterium freudenreichii* subsp. *shermanii*, *Lait* 80 (2000) 325–336.
- [12] Jan G., Leverrier P., Pichereau V., Boyaval P., Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*, *Appl. Environ. Microbiol.* 67 (2001) 2029–2036.
- [13] Jouany J.P., Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentator contents, *Sci. Aliments* 2 (1982) 131–144.
- [14] Kailasapathy K., Chin J., Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp., *Immunol. Cell Biol.* 78 (2000) 80–88.
- [15] Kopp-Hoolihan L., Prophylactic and therapeutic uses of probiotics: a review, *J. Amer. Diet. Assoc.* 101 (2001) 229–238.
- [16] Lyon W.J., Glatz B.A., Isolation and purification of propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*, *Appl. Environ. Microbiol.* 59 (1993) 83–88.
- [17] Lyon W.J., Sethi J.K., Glatz B.A., Inhibition of psychrotrophic organisms by propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii*, *J. Dairy Sci.* 76 (1993) 1506–1513.
- [18] Mantere-Alhonen S., Propionibacteria used as probiotics – A review, *Lait* 75 (1995) 447–452.
- [19] Mombelli B., Gismondo M.R., The use of probiotics in medical practice, *Int. J. Antimicrob. Agents* 16 (2000) 531–536.
- [20] Mori H., Sato Y., Taketomo N., Kamiyama T., Yoshiyama Y., Meguro S., Sato H., Kaneko T., Isolation and structural identification of bifidogenic growth stimulator produced by *Propionibacterium freudenreichii*, *J. Dairy Sci.* 80 (1997) 1959–1964.
- [21] Mortensen P.B., Clausen M.R., Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease, *Scand. J. Gastroenterol. Suppl.* 216 (1996) 132–148.
- [22] O'Farrell P.H., High resolution two-dimensional electrophoresis of proteins, *J. Biol. Chem.* 250 (1975) 4007–4021.

- [23] Pérez Chaia A., Zarate G., Oliver G., The probiotic properties of propionibacteria, *Lait* 79 (1999) 175–185.
- [24] Rabilloud T., Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis, *Electrophoresis* 19 (1998) 758–760.
- [25] Rolfe R.D., The role of probiotic cultures in the control of gastrointestinal health, *J. Nutr.* 130 (2000) 396S–402S.
- [26] Saxelin M., Pessi T., Salminen S., Fecal recovery following oral administration of *Lactobacillus* strain GG (ATCC 53103) in gelatine capsules to healthy volunteers, *Int. J. Food Microbiol.* 25 (1995) 199–203.
- [27] Vorobjeva L., Physiological peculiarities of propionibacteria – present facts and prospective applications, *Sci. Prog.* 83 (2000) 277–301.
- [28] Vorobjeva L.I., Ol'sinskaia N.L., Naumova E.S., Dubinin A.V., Shemchuk L.F., Search for propionic acid bacteria in the human intestine, *Zh. Mikrobiol. Epidemiol. Immunobiol.* (1987) 7–12.
- [29] Vorobjeva L.I., Khodjaev E., Cherdinceva T.A., The study of induced antimutagenesis of propionic acid bacteria, *J. Microbiol. Meth.* 24 (1996) 249–258.
- [30] Vorobjeva L., Zinchenko A., Khodjaev E., Ponomareva G., Gordeeva E., Isolation and some properties of the nucleoprotein complex of *Propionibacterium freudenreichii* subsp. *shermanii*, *Lait* 79 (1999) 187–196.
- [31] Wollowski I., Rechkemmer G., Pool-Zobel B.L., Protective role of probiotics and prebiotics in colon cancer, *Amer. J. Clin. Nutr.* 73 (2001) 451S–455S.
- [32] Zarate G., Chaia A.P., Gonzalez S., Oliver G., Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids, *J. Food Protect.* 63 (2000) 1214–1221.