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Assessment of the Probiotic Potential of a Dairy Product Fermented by *Propionibacterium freudenreichii* in Piglets

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Supporting Information

ABSTRACT: Dairy propionibacteria, including *Propionibacterium freudenreichii*, display promising probiotic properties, including immunomodulation. These properties are highly strain-dependent and rarely studied in a fermented dairy product. We screened 10 strains, grown in a newly developed fermented milk ultrafiltrate, for immunomodulatory properties in vitro. The most anti-inflammatory strain, *P. freudenreichii* BIA129, was further tested on piglets. *P. freudenreichii*-fermented product improved food intake and growth of piglets. Colonic mucosa explants of treated pigs secreted less interleukin 8 (−25%, $P < 0.05$) and tumor necrosis factor α (−20%, $P < 0.05$), either in basal conditions or after a lipopolysaccharide challenge. By contrast, the gut structure, barrier function (measured ex vivo in Ussing chambers), microbial diversity (assessed by 16S rRNA pyrosequencing), and colonic short-chain fatty acid content were unchanged, assuming maintenance of normal intestinal physiology. In conclusion, this work confirms in vivo probiotic properties of dairy propionibacteria-fermented products, which are promising for the prevention or healing of inflammatory bowel diseases.

KEYWORDS: *probiotic, dairy propionibacteria, immunomodulation, pig, intestinal barrier*

INTRODUCTION

Dairy propionibacteria, especially *Propionibacterium freudenreichii* (PF), are considered as potential probiotics for both animal and human use. This relies on different potential benefits to health, recently reviewed.[1] Consumed in high amounts within Emmental and other types of Swiss cheese, PF is a GRAS bacterium known to stimulate growth of bifidobacteria,[2] which has been shown to be beneficial for human health. Tablets containing supernatants or live freeze-dried cultures of PF are already commercialized to improve intestinal transit.

PF is also a promising immunomodulatory probiotic. It induces high levels of the regulatory anti-inflammatory cytokine interleukin 10 (IL-10) in human peripheral blood mononuclear cells (PBMCs) in a strain-dependent manner.[3] Propionibacterial variable surface compounds are involved; strains displaying key surface proteins induce cytokines, while strains covered with surface β-glucan do not.[4] The most stress-tolerant strains of PF produce short-chain fatty acids (SCFAs) in the gut,[5,6] which are generally recognized as beneficial metabolites.[7] In particular, they display trophic effects on healthy colonocytes but cytotoxic effects on human colon cancer cells.[8] Accordingly, PF consumption enhances apoptotic depletion of intestinal cells damaged by 1,2-dimethylhydrazine (DMH).[9] This is consistent with the ability of some probiotic bacteria to prevent the development of 1,2-dimethylhydrazine (DMH)-induced colonic tumorigenesis.[10] Interestingly, propionic acid was also shown to play a beneficial role in the modulation of gut inflammation.[11] Other beneficial propionibacterial metabolites include 1.4-dihydroxy-2-naphthoic acid (DHNA), known as a bifidogenic compound,[12] which also attenuates experimental colitis in mice.[13,14], and conjugated linoleic acid,[15] known to modulate inflammation. Accordingly, several dairy propionibacteria protect against different experimental colitis models in rodents.[3,16] Moreover, consumption of the JS strain of PF was reported to result in reduction of the serum basal level of C-reactive protein, a biomarker of inflammation, in a human trial.[17] Altogether, these data strongly suggest a beneficial potential of selected strains of dairy propionibacteria and of their metabolites in different disorders, including dysbiosis and gut inflammatory diseases.

Health-beneficial properties were shown to be highly strain-dependent in PF,[3,6] as in other probiotic bacteria.[18] Surprisingly, the strain dependence of these effects and the corresponding molecular mechanisms, responsible for such variability, were poorly explored. Probiotic efficacy also widely

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Table 1. Bacterial Strains and Their Origins

Strain number [a]	Identification	Origin
MG1363 [b]	*Lactococcus lactis*	Cheese starter
NCFM	*Lactobacillus acidophilus*	Commercial strain/Danisco
Ls33	*Lactobacillus salivarius*	Commercial strain/Danisco
IPL A12-1	*Pediococcus acidilactici*	IPL collection
BB536	*Bifidobacterium longum*	Morinaga Milk Industry Ltd
BIA1	*Propionibacterium freudenreichii*	Cheese
BIA118	*Propionibacterium freudenreichii*	Gruyere cheese
BIA127	*Propionibacterium freudenreichii*	Cheese
BIA129	*Propionibacterium freudenreichii*	Cheese
BIA131	*Propionibacterium freudenreichii*	Cheese
BIA136	*Propionibacterium freudenreichii*	Human feces
BIA455	*Propionibacterium jensenii*	Buttermilk
BIA456	*Propionibacterium freudenreichii*	Raclette cheese
BIA457	*Propionibacterium freudenreichii*	Human feces
BIA458	*Propionibacterium freudenreichii*	Cheese

[a]CIRM-BIA = Centre International de Ressources Microbiennes—Bactéries d'Intérêt Alimentaire, INRA, Rennes, France; IPL = Institut Pasteur de Lille, Lille, France. [b]Some bacterial strains (gray shading) were used as reference strains for immune cell stimulation as previously described.

depends on the delivery vehicle used. First, this vehicle determines the amount of live propionibacteria reaching the colon, and this amount remains a major bottleneck.[19] Second, it determines the growth conditions, including the substrate used for bacterial growth. This may drastically affect the bacterial cell composition and the functional properties of the probiotic.[20] Thus, it is obvious that relevant screening of propionibacteria strains and of delivery vehicles constitutes a prerequisite before preclinical and clinical studies.

In this work, we screened a set of 10 dairy propionibacteria strains for their immunomodulatory potential on a well-established PBMC model shown to be predictive of anti-inflammatory properties in probiotic bacteria.[18] To take into account the impact of growth in a dairy product, we compared the yeast extract lactate (YEL) laboratory medium and a food-grade dairy product, exclusively fermented by dairy propionibacteria that we recently designed (based on either milk or milk ultrafiltrate).[21] As milk proteins or derived peptides are known to modulate the immune system,[22] we used fermented milk ultrafiltrate (UF; milk aqueous fraction, depleted in proteins), rather than fermented milk, to limit this impact. Using this delivery vehicle, we investigated the impact of the most promising immunomodulatory PF strain on a piglet model. We verified the absence of an adverse effect on the piglets' health and growth and sought a preventive effect toward pro-inflammatory signals at the gut level. Indeed, safety of the treatment and prevention of inflammation offset are key issues in this context before any further trial.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The strains originated from the collections of the Centre International de Ressources Microbiennes—Bactéries d'Intérêt Alimentaire (CIRM-BIA) and of the Institut Pasteur de Lille (Table 1). Reference strains for immune cell stimulation were cultivated as previously described.[18] Dairy propionibacteria were routinely cultivated at 30 °C without shaking either in YEL medium[23] or in UF supplemented with 50 mM food-grade sodium L(+) lactate (galaflow SL60, purity ≥97%, Société Arnaud, Paris, France) and 5 g/L casein hydrolysate (Organotechnie, La Courneuve, France), sterilized by 0.2 μm filtration (Nalgene, Roskilde, Denmark). Growth kinetics are presented in Supplemental Figure 1 (Supporting Information).

UF was obtained by an ultrafiltration process.[24] Briefly, raw milk was skimmed and ultrafiltered using UF pilot equipment (TIA, Bollene, France) equipped with organic spiral membrane with a molecular weight cutoff of 5000 (Koch International, Lyon, France). The ultrafiltrate collected was then sterilized by 0.2 μm filtration (Nalgene) and stored at 4 °C.

PBMC Isolation and Induction of Cytokine Release. PBMCs were isolated from the blood of four healthy donors, and reference bacterial strains were prepared as previously described.[18] Propionibacteria were harvested from fermented UF or YEL and treated the same way. Bacteria were resuspended in PBS containing 20% glycerol and added to PBMCs. This resulted in a bacteria-to-cell ratio of approximately 10:1. PBS containing 20% glycerol was used as a negative (nonstimulated) control. After 24 h of stimulation, culture supernatants were collected, clarified by centrifugation, and stored at −20 °C until cytokine analysis. These were quantified by enzyme-linked immunosorbent assay (ELISA) using BD Pharmingen antibody pairs (BD Biosciences, San Jose, CA) for IL-10, interferon γ (IFN-γ),

and IL-12p70 and R&D systems (Minneapolis, MN) for human tumor necrosis factor α (TNF-α), as previously described.[18] The IL-10:IL-12 ratio, previously shown to be predictive of in vivo anti-inflammatory potential,[18] was calculated.

Food Fermentation for Animal Consumption. The strain used in the in vivo trial was *P. freudenreichii* ssp. *shermanii* CIRM-BIA129. UF supplemented with 50 mM sodium lactate and 5 g/L casein peptone[21] was inoculated (1%) with PF and incubated for 3 days at 30 °C (early stationary phase). The dairy propionibacteria concentration was determined in each culture to calculate the daily dose. Physicochemical analyses of the dairy products, fermented or not, were performed according to the procedure described by Thierry et al.[25] and are presented in Table 2.

Table 2. Composition of the Fermented Product[a]

measured param	sterile milk UF	fermented milk UF	aqueous phase of fermented milk UF[b]
pH	6.94	5.12	5.12
total dry matter concn	63.97	58.55	56.77
total nitrogen concn	5.67	5.75	4.94
nonprotein nitrogen concn	5.5	4.94	4.85
total ash concn	7.3	7.26	7.36
lactose concn	50.90	–	41.34
lactate concn	5.39	–	0.07
acetate concn	0	–	1.31
propionate concn	0	–	4.64
pyruvate concn	0	–	0.90
citrate concn	1.75	–	1.79
succinate concn	0	–	0.57

[a]The results are the means of two independent experiments. All parameters except pH are expressed as grams per kilogram. A dash means not determined. [b]The aqueous phase was separated by centrifugation followed by sterile filtration.

Animal Procedure. The experimental protocol was designed in compliance with recommendations of the French law (2001-464 29/05/01) and EEC (86/609/CEE) for the care and use of laboratory animals under the certificate of authorization to experiment on living animals no. 3569. Sixteen ((Pietrain × Landrace) × Large White) piglets from the experimental herd of INRA St-Gilles (France) were used. Eight pairs of sex- and weight-matched 7 week old littermates were housed individually in stainless cages in a temperature-controlled (23 °C) and 12 h/12 h dark/light cycle room. Initial body weights were similar between the two groups (19.7 ± 0.5 kg versus 19.4 ± 0.4 kg for control and PF-treated piglets, respectively, $P > 0.05$). Piglets were weighed twice a week. They were fed ad libitum a weaning diet (net energy 10.6 MJ/kg and 195 g of crude protein/kg of dry matter, Cooperl-Hunaudaye, Lamballe, France). Food intake was measured daily. Pigs had free access to water.

PF-treated piglets were gavaged every morning for 14 days with fermented UF providing daily 2×10^{10} CFU/mL PF. This corresponds to the amount found in approximately 10 g of Emmental cheese. Control pigs were gavaged daily with 10 mL of an unfermented UF for 14 days as well. At the end of the treatment period, pigs were sacrificed 3 h after their last meal by electronarcosis and exsanguination. Blood was collected in heparinized tubes, centrifuged, and plasma stored at −20 °C for later haptoglobin analysis. The proximal colon was dissected, and digesta were collected. A fraction was immediately analyzed for its propionibacteria population, and another fraction was immediately frozen in liquid nitrogen and stored at −80 °C until SCFA quantification and microbiota sequencing. Segments of mucosa were rinsed with cold sterile saline and then placed either into Ringer buffer at 4 °C for immediate Ussing chamber analysis or into RNA later (cross-section of tissue) at 4 °C for 24 h and then maintained at −80 °C for later reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. An adjacent segment was used for immediate explant cultures as previously described.[26] Five centimeter adjacent segments were also rinsed and fixed in 4% formaldehyde buffer for 24 h, dehydrated in ethanol, and stored at 4 °C before being embedded in paraffin for further histological analyses.

Counting of Propionibacteria in Fecal and Colonic Samples. Feces were collected at day 0 (before treatment) and at day 14 (end of the treatment). Colon contents were collected at sacrifice in the same way. Feces (or colon contents) freshly collected were immediately frozen and stored at −80 °C until analysis. Their propionibacteria concentrations were measured by qPCR as described previously.[5] Results are expressed as log [bacteria] per gram of sample.

Short-Chain Fatty Acid Analysis in Colonic Samples. SCFAs were extracted in a cold Tris buffer (50 mM, pH 7.5) and stored at −20 °C until gas-phase chromatography analysis. Proteins were precipitated by incubation for 1 h at 4 °C in the presence of oxalic acid (0.03 M final concentration). SCFAs were separated on a BP21 column and quantified by a flame ionization detector as previously described.[27] Isocaproic acid was used as an internal standard because it was absent from piglet colonic samples (data not shown). Samples were analyzed in duplicate, and the results are expressed as millimolar in wet matter.

16s rRNA Pyrosequencing in Colon Content Samples. This was performed as previously described[28] and is detailed in the Supporting Information.

Histology. Histological sections (5 μm) were stained with hematoxylin and eosin and examined under a light microscope (Nikon ECLIPSE E400, Nikon Instruments, France), and image analysis was performed (NIS-Elements AR3.0 software, Nikon Instruments). The crypt depth and surface were measured in 15−20 well-oriented crypts per pig. Histological sections were also examined for inflammation or lesion signs. All measurements were conducted by one investigator, who was unaware of the pig group from which the section was prepared.

Ussing Chambers. Colonic tissues were stripped of longitudinal muscle, opened along the antimesenteric border, and then mounted in an Ussing chamber (World Precision Instrument, Stevenage, U.K.). The paracellular and transcellular passages were determined as already described.[26]

Quantitative RT-PCR Analysis. Total RNA was extracted from tissues with Trizol reagent and chloroform and precipitated by 2-propanol, and the pellets were resuspended in 100 μL of water. Extracted RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE) and treated with the DNA-free kit (Ambion, Austin, TX). RNA quality was assessed by fluorimetry with the RNA 6000 nano LabChipH kit in a 2100 Bioanalyser (Agilent Biotechnologies, Santa Clara, CA). Reverse transcription and real-time quantitative PCR for GAPDH, TLR4, IL-1β, IL-6, TNF-α, IL-10, TGF-β, IL-4, and IL-13 genes were performed as already described.[26]

Explant Culture. The explant culture protocol was performed as already described.[26] Briefly, small pieces of colonic mucosa were incubated with 0, 50, 100, or 200 μg/mL lipopolysaccharides (LPSs; Sigma-Aldrich). Then IL-8 and TNF-α concentrations were determined by ELISA (R&D Systems Europe, Lille, France) in supernatants.

Plasma Haptoglobin Analysis. Haptoglobin concentrations were determined in the plasma by a pig-dedicated colorimetric kit (Tridelta Ltd., Maynooth, Ireland).

Statistical Analysis. Statistical analyses were performed using the general linear model procedure of Statistical Analysis Systems software (SAS Institute, Cary, NC), testing the piglet pair and treatment effect with the t test as subsequent multiple comparisons when appropriate. For cytokine secretion data, the model also included the LPS dose and LPS dose × treatment interaction. All results are presented as means ± SEM. Differences between groups were declared significant at $P < 0.05$. For 16s rRNA analysis, the statistical analysis at the taxonomic level was performed using DESeq (http://www.bioconductor.org/packages/2.6/bioc/html/DESeq.html). Briefly, P values obtained by the t test were corrected according to Benjamini−Hochberg for multiple factors. For diversity and richness indexes, a classical Welsh

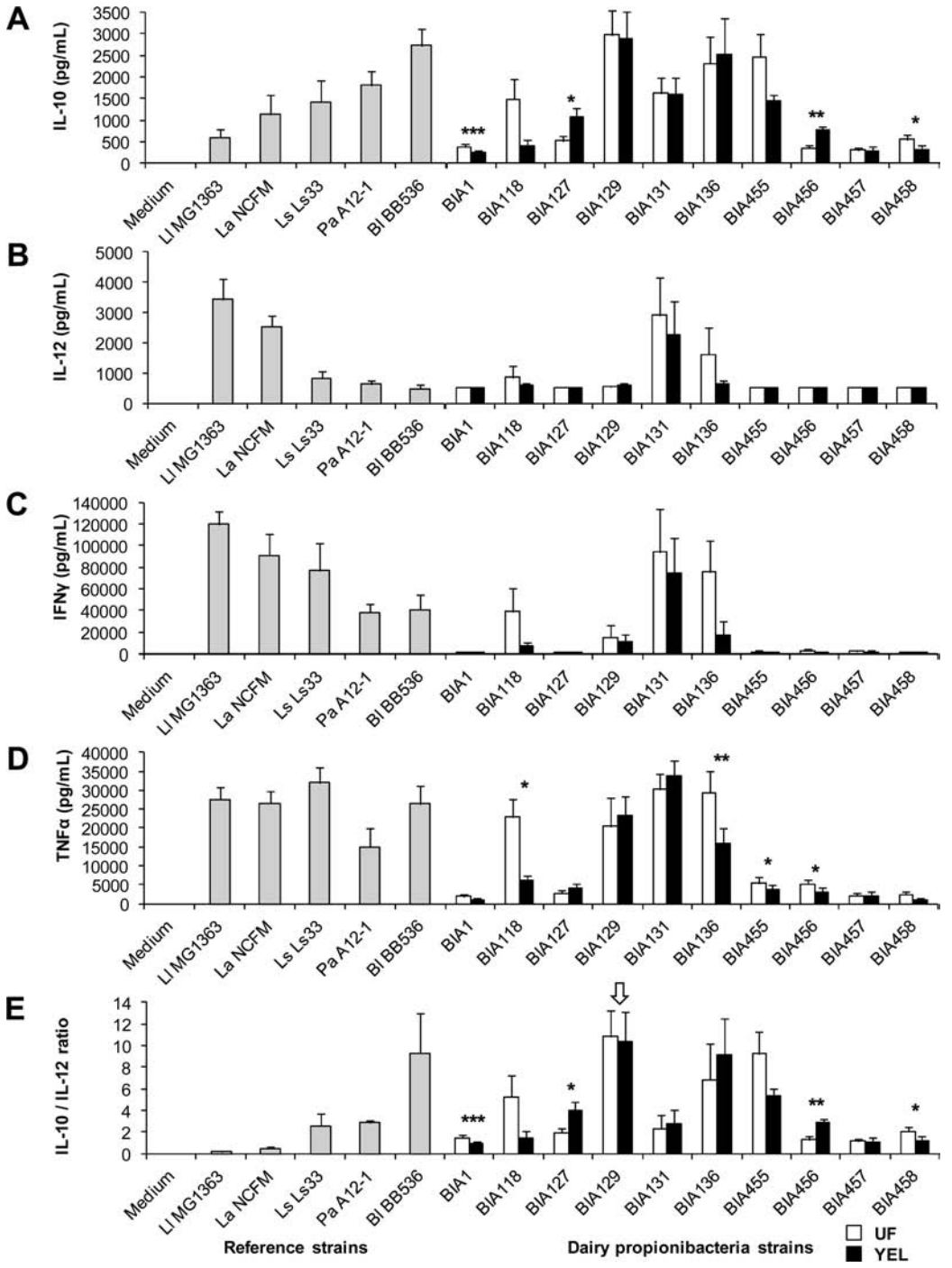

Figure 1. Production of cytokines by peripheral blood mononuclear cells in response to bacteria. IL-10 (A), IL-12 (B), IFN-γ (C), TNF-α (D), and the IL-10:IL-12 ratio (E) were analyzed by ELISA in the supernatants collected from 24 h cultures of human PBMCs with reference bacteria (gray bars) and dairy propionibacteria cultivated in supplemented UF (white bars) or in YEL (black bars). Data are expressed as the mean ± SEM (n = 4 healthy donors). Reference bacteria: Ll, *Lactococcus lactis*; La, *Lactobacillus acidophilus*; Ls, *Lactobacillus salivarius*; Bl, *Bifidobacterium longum*; Pa, *Pediococcus acidilactici*. The strain selected for the in vivo trial is indicated by an arrow. Key: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (UF vs YEL).

two-sample t test was carried out, and the Bonferroni correction was applied as noted for multiple comparison correction.

RESULTS AND DISCUSSION

Development of a *P. freudenreichii*-Fermented Milk Ultrafiltrate. Food-grade sodium lactate and casein hydrolysate were added to milk ultrafiltrate, the aqueous phase of milk, devoid of protein, to allow growth of propionibacteria. Indeed, these are the optimal sources of carbon and of nitrogen that dairy propionibacteria use for growth. As shown in Supplemental Figure 1 (Supporting Information), PF growth parameters were similar in this experimental dairy product and in the laboratory reference YEL medium; however, the initial lag phase was longer. Maximal propionibacterial concentration was reached within 72 h in both media. This was 6.00×10^9 and 3.93×10^9 CFU/mL in YEL and in supplemented UF, respectively. Composition analysis of supplemented UF, before and after fermentation, shown in Table 2, revealed

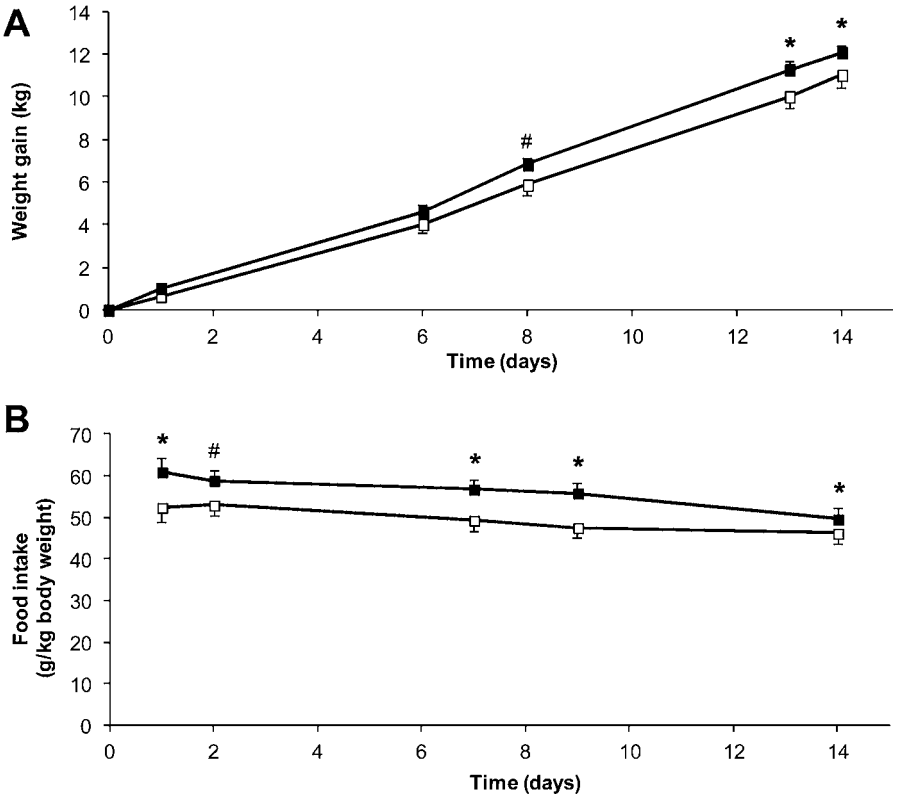

Figure 2. Weight gain and food intake of control (□) and PF-treated (■) pigs. Weight gain (A) was measured in the control and pigs gavaged daily with 2×10^{10} CFU of *P. freudenreichii* CIRM-BIA129 for 2 weeks. Food intake (B) was also measured during the same period and is expressed as grams of food per kilogram of body weight. PF-treated pigs exhibited a greater body weight gain and greater food consumption than control pigs. Key: *, $P < 0.05$ compared to control; #, $P < 0.07$ compared to control ($n = 8$).

consumption of 5.32 g of lactate (almost the totality of provided lactate) and 9.36 g of lactose (only 18% of provided lactose) by PF. Fermentation led to acidification to pH 5.12 and production of organic acids, mainly propionate (4.64 g/kg) and acetate (1.31 g/kg), in fermented UF. Propionibacteria were harvested from both types of cultures at the same physiological stage (early stationary phase, 72 h) for further screening based on immunomodulatory properties.

In Vitro Screening of Dairy Propionibacteria Strains Revealed Strain-Dependent Immunomodulation. This work extended the PBMC-based screening[3] to 10 strains and gave further insight into the immunomodulatory property variability within the *P. freudenreichii* species. Cytokine induction patterns, determined on human PBMCs, were indeed variable (Figure 1), as demonstrated by the wide range of cytokine secretion for the four measured cytokines: 250−2500 pg/mL for IL-10 (Figure 1A), undetectable level to 1140 pg/mL for IL-12 (Figure 1B), undetectable level to 120 000 pg/mL for IFN-γ (Figure 1C), and 1350−24780 pg/mL for TNF-α (Figure 1D). No pro-inflammatory pattern was observed among dairy propionibacteria, as the release of pro-inflammatory mediators was very low, compared to that of *Lactococcus lactis*. This was the case for IL-12 (Figure 1B) and to a lesser extent for TNF-α and IFN-γ (Figure 1C,D). This investigation confirmed the great diversity of anti-inflammatory profiles among dairy propionibacteria, which can be estimated by the IL-10:IL-12 ratio (Figure 1E).

The molecular bases of such variability are currently under investigation. Strains BIA1, BIA456, BIA457, and BIA458 induced little or no cytokine release. These strains, by contrast with the others, possess a surface β-glucan polysaccharide layer, which was recently shown to coincide with the absence of an immunomodulatory property, this layer being thought to hide anti-inflammatory moieties in *P. freudenreichii*.[4,29] A great variability was also reported within this species regarding genomic profiles[30] and probiotic properties, including stress tolerance,[31] metabolic activity in the gut,[6] and B_{12} vitamin production.[32] This deserves attention to develop new and efficient probiotic products containing dairy propionibacteria with specific health benefits.

Immunomodulation by Propionibacteria Depended on the Growth Medium. For the first time this work evidenced immunomodulatory properties of dairy propionibacteria grown and ingested in a fermented dairy product. Indeed, promising immunomodulatory properties were recently reported for these bacteria when grown in the YEL laboratory culture medium.[3] However, the majority of food-borne beneficial bacteria are consumed within dairy products, and fermented dairy products are responsible for immunomodulatory effects.[33,34] In this respect, the proteome and the metabolic activity of *Lactobacillus rhamnosus* GG have recently been shown to vary greatly, whether this probiotic bacterium is grown on MRS laboratory medium or on an industrial-type whey-based medium, indicating different functionalities and characteristics.[20] Cellular protein expression was indeed shown to vary depending on the growth medium in lactic acid bacteria[20,35] and bifidobacteria.[36] Furthermore, the growth media and conditions were shown to determine immunomodulatory properties of lactic acid bacteria.[37,38] In our work, the medium that allowed growth of propionibacteria modulated

their immunomodulatory pattern. As shown in Figure 1, cytokine induction can vary, whether the strain is grown on the YEL laboratory culture medium or on the milk UF dairy product. Indeed, the pro-inflammatory cytokine TNF-α was higher when strains BIA118, BIA136, BIA455, and BIA456 were grown on UF. Similar variations were observed for IFN-γ and for IL-12. By contrast, all the strains induced IL-10, whatever the growth medium. The impact of the growth medium on functional properties, including immunomodulation, should thus be taken into account in studies aimed at establishing the effects of a candidate bacterium and/or probiotic product. This screening allowed the selection of the most promising anti-inflammatory *P. freudenreichii* strain, namely, the CIRM-BIA129 strain, for further preclinical studies.

Propionibacteria Survived in the Gastrointestinal Tract. Piglets were gavaged daily for 14 days either with UF fermented by *P. freudenreichii* CIRM-BIA129, providing 2×10^{10} CFU of propionibacteria daily, or with sterile UF used as a control. Piglets remained healthy throughout the experiment. Moreover, the PF treatment was well-tolerated by the animals with evidence of neither discomfort nor distress. The fecal propionibacteria population levels were undetectable before treatment (day 0); however, propionibacteria reached 6.75 ± 0.22 log/g in feces 1 day before slaughtering (day 14) and 6.26 ± 0.17 log/g in colon contents at slaughtering (day 15) in the PF-treated group. Propionibacteria remained undetectable in the control group throughout the experiment.

***P. freudenreichii* CIRM-BIA129 Improved Piglet Growth and Food Intake.** No adverse effect on piglet general health and growth was observed. On the contrary, consumption of *P. freudenreichii*-fermented UF led to a 10% higher weight increase during the overall 2 week period compared to control piglets ($P < 0.01$, Figure 2A). This greater weight correlated with a greater food consumption (+13% over the whole period, $P < 0.05$, Figure 2B), starting on the first day of dairy consumption. Several studies have investigated the effect of different probiotics on piglet performance. They led to different results, depending on the probiotic used, the timing of administration, or the dose of probiotic administered. However, they often described increased food intake, body weight gain, and/or food conversion ratio in piglets.[39−45] Although reaching statistical significance, these changes were always within the range of 5−10% compared to control piglets. This is similar to what we observed in piglets as a result of *P. freudenreichii* CIRM-BIA129 consumption. Promotion of growth was already reported in calves and piglets consuming probiotic combinations containing lactic acid bacteria, bifidobacteria, and dairy propionibacteria or pure cultures of *P. freudenreichii*, leading to 9−15% higher weight gain.[46] Although hypothetical, the growth-promoting effect of propionibacteria could be attributed to production of vitamins by propionibacteria, by the modulation of the intestinal microbiota, or by anti-inflammatory properties. Consequently, probiotics, including dairy propionibacteria, can be regarded as a safer alternative to antibiotics as growth promoters.[47] Finally, probiotic consumption was recently shown to modulate fatty acid composition in pig meat, with increased concentrations of conjugated linoleic acid.[48] Propionibacteria being known to produce such lipids in fermented dairy products,[15] their impact on meat quality also deserves attention.

***P. freudenreichii* CIRM-BIA129 Exerted an Immunomodulatory Effect on the Pig Proximal Colon.** Quantitative RT-PCR analysis of cytokines and TLR4 expression in tissues and IL-8 and TNF-α secretion by explant cultures were used to assess the impact of PF treatment on the immune function of the piglet's proximal colon. Treatment with PF had no effect on TLR4 and cytokine mRNA levels in the piglet proximal colonic mucosa (Table 3). There was no increase of TNF-α or IL-8 secretion in response to LPSs in the colon explants from control pigs (LPS treatment, $P = 0.37$ and 0.27 for TNF-α and IL-8, respectively, Figure 3). PF treatment did not modify this unresponsiveness to LPSs of the proximal colon (Figure 3). However, basal pro-inflammatory cytokine secretion was reduced by an average of 25% for TNF-α for all the LPS doses tested ($P < 0.0001$, Figure 3A) and by 26% and 15% for IL-8 at LPS doses of 50 and 100 μg/mL, respectively ($P < 0.0001$, Figure 3B) in PF-treated pigs compared to control pigs. In addition, although not significant, plasmatic haptoglobin was reduced by 33% in the PF-treated group (1.04 ± 0.2 and 0.70 ± 0.18 mg/mL, for control and PF-treated pigs, respectively, $P = 0.23$).

Table 3. mRNA Levels of Targeted Genes in the Colon of Piglets[a]

targeted gene	control	PF-treated
TLR-4	0.260 ± 0.044	0.302 ± 0.054
IL-1β	0.081 ± 0.019	0.054 ± 0.011
TNF-α	0.016 ± 0.004	0.015 ± 0.002
IL-6	0.006 ± 0.001	0.005 ± 0.001
IL-10	0.029 ± 0.004	0.024 ± 0.003
TGF-β1	0.596 ± 0.083	0.571 ± 0.079
IL-13	0.014 ± 0.004	0.022 ± 0.008

[a]Targeted gene expression was expressed relative to the GAPDH transcript level. Values are means ± SEM ($n = 8$).

Several studies have already reported an anti-inflammatory effect of probiotic bacteria or yeast against LPS-induced inflammatory challenge, such as *Saccharomyces boulardii*,[49] diverse species of bifidobacteria,[50,51] or of lactobacilli.[52−54] A role of inhibition of NF-κB activation by the probiotics has been suggested.[49,50] Accordingly, *Lactobacillus paracasei* was shown to inhibit inflammation triggered by the pathogen *Escherichia coli* O157:H7 in mice, with lower cumulative morbidity.[55] In the present study, we expand this anti-inflammatory effect of probiotics toward LPSs to *P. freudenreichii*, provided under the form of fermented milk.

***P. freudenreichii* CIRM-BIA129 Caused No Major Modification of the Gut Microbiota and Physiology.** A 16S rRNA-based analysis was carried out on the microbial diversity of the colonic content of both PF-treated and control piglets. We generated a data set consisting of 155 095 filtered high-quality, classifiable 16S rRNA gene sequences with a mean average (±SD) of 9693 ± 3866 sequences per sample. The taxonomic assignation at the family level for all the samples is shown in Figure 4A. No significant statistical difference was observed between the two groups of samples at the phylum, family, and genus levels. Moreover, the microbial richness, estimated by the Chao1 index, and the biodiversity, assessed by a nonparametric Shannon index, showed no statistical differences between groups at operational taxonomy unit (OTU) cutoffs of 0.03, 0.05, and 0.10. At an OTU cutoff of 0.03, an average (±SD) of 1087 ± 398 clusters was obtained.

So far, very few studies have investigated the bacterial composition of the digestive tract in piglets by pyrosequencing. Importantly, and in contrast with other studies,[56] the samples

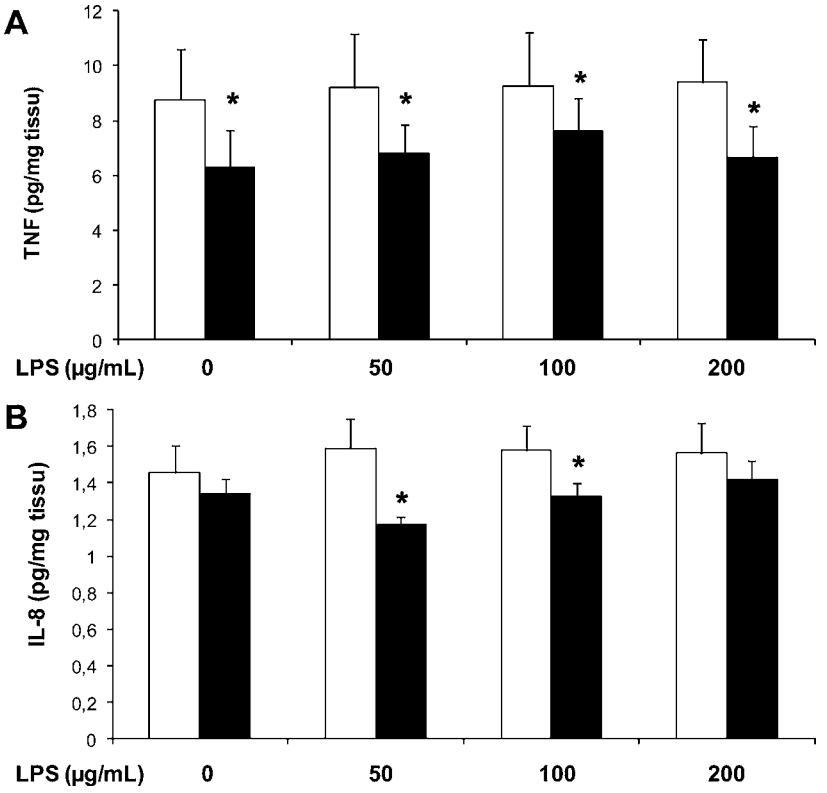

Figure 3. Pro-inflammatory cytokine secretion of proximal colon explants from control (□) and PF-treated (■) pigs in response to different doses of LPSs. TNF-α (A) and IL-8 (B) secretions of colonic explants were measured after 20 h of exposure to different doses of LPSs. PF-treated pigs exhibited lower TNF-α and IL-8 secretion than control pigs. As expected, the colon of control pigs did not respond to LPSs, irrespective of the dose. PF treatment did not modify this tolerance to LPSs. An asterisk indicates $P < 0.05$ compared to control pigs ($n = 8$).

were not pooled and eight biological replicates were studied for each group. Interestingly, our results, indicating *Lactobacillus* sp. and *Clostridium* sp. as two ubiquitous genera of the colonic microbiota, confirmed the results obtained in ileum samples.[57] The microbiota was highly dominated by firmicutes with a mean average (±SD) of 95.9 ± 2.3%. At the family level, the majority of assigned reads belonged to the Lactobacillaceae family with an average (±SD) of 59.9 ± 15.9%. Interestingly, similarly high proportions of firmicutes and Lactobacillaceae were observed in the ileum of piglets.[56] We estimated the maximum number of unique species level (3% dissimilarity) operational taxonomic units in the colon of these pigs. These predictions indicated that there may be as many as 821 different species in the colon in pigs. The OTU-clustering at 0.03% is generally recognized as the clustering level representing the taxonomic level of species. The average (±SD) number of OTUs (0.03%) was 1087 ± 398. This number was similar to the 821 OTUs identified on average by Dowd et al.[57] Interestingly, the diversity and richness of the samples for the OTU-clustering levels of 0.03, 0.05, and 0.10 were not statistically modified by the treatment. The absence of a statistically significant difference, when considering dominant families, above 1% of the whole microbiota, indicates no drastic microbiota perturbation as a result of dairy propionibacteria consumption. We nevertheless observed that three minor families, known to include potential pathogenic bacteria, tended to be lower in the PF-treated group. This was statistically significant for the Porphyromonadaceae family (Figure 4B). Such a modulation of nondominant but opportunistic pathogens was already described as a result of dairy propionibacteria consumption[58−60] and may depend on the production of bacteriocins and SCFAs by propionibacteria.[1] Thus, some changes at the species level or in rare species cannot be ruled out.

No major modification of colonic fermentation variables was observed. The total SCFA concentration was not affected in the PF-treated group (149.9 ± 4.0 mM) compared to the control group (150.6 ± 8.0 mM). The concentrations of each major SCFA in the colon also remained unchanged whatever the treatment group (Figure 4C). The three major SCFAs, acetic, propionic, and butyric acids, were at the same level in the two groups at concentrations of 85, 40, and 17 mM, respectively.

The proximal colon crypt depth and surface were evaluated on hematoxylin and eosin-stained sections. No difference was observed between PF-treated and control pigs for those two parameters (crypt depth, PF 472 ± 16 vs control 463 ± 18 μm, $P > 0.05$; crypt surface, PF 32490 ± 1283 μm^2 vs control 33999 ± 1864 μm^2, $P > 0.05$). Moreover, the examination of histological sections did not reveal any sign of inflammation or lesion in PF-treated pigs (data not shown). The epithelial barrier function of the proximal colon was evaluated by measuring the flux of small molecules (FD-4, 4 kDa) or large molecules (HRP, 40 kDa) across the tissue mounted in Ussing chambers. There was no difference of paracellular (PF 703 ± 140 (ng/cm^2)/h vs control 698 ± 85 (ng/cm^2)/h, $P > 0.05$) nor of transcellular (PF 123 ± 17 (ng/cm^2)/h vs control 140 ± 32 (ng/cm^2)/h, $P > 0.05$) permeability for these two functional probes between control and PF-treated pigs. Several studies demonstrated that probiotic bacteria affect the intestinal morphology by increasing the villus and/or crypt length in different animal models.[61,62] However, most of the time only the jejunum and less often the ileum were influenced by

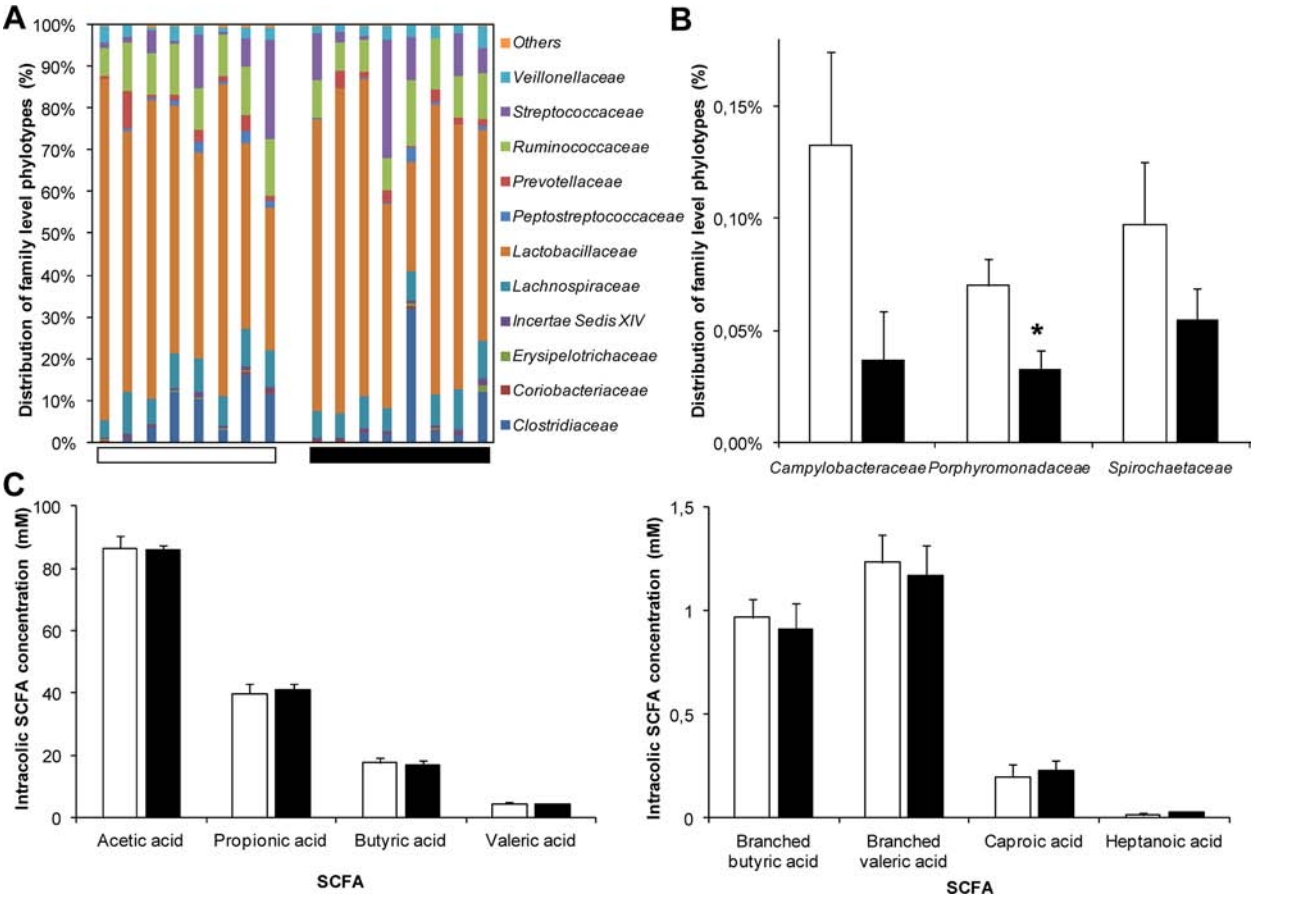

Figure 4. Microbiota and SCFA profiles of control (□) and PF-treated (■) pigs. (A ,B) 16S rRNA-based analyses of colonic contents expressed as percentages of sequences per bacterial family. (A) Dominant families, above 1%, are shown. (B) Three minor families, below 1%, are shown. An asterisk indicates $P < 0.05$ compared to control pigs ($n = 8$). (C) Colonic concentrations of SCFAs were determined by gas-phase chromatography. Isocaproic acid was added as an internal standard to check the repeatability of analysis. The repeatability coefficient of the isocaproic acid amount was 1.46%.

probiotic bacteria administration, with little or no effect on the colon, suggesting a trophic effect of probiotic bacteria administration in the proximal rather than distal gastrointestinal tract. Similarly, a recent review summarized the effect of probiotic bacteria on intestinal barrier function, focusing on the mucus, IgA secretion, production of antibacterial peptides, bacteria adherence, and tight junction protein expression.[63] Regarding tight junction proteins which directly regulate permeability of small probes, the general scheme is that probiotic bacteria enhance barrier function by increasing the expression of the key protein of the tight junctions. However, these results were obtained mainly on cell lines (T84, Caco-2, HT-29) or in diseased conditions.[63] The only report on healthy animals did not show any effect of a combination of *Lactobacillus helveticus* and *L. rhamnosus* upon ileal and colonic barrier function in rats.[64] A study also investigated the effect of a 6 h infusion of *Lactobacillus plantarum* in the duodenum of healthy volunteers on the tight junction protein arrangement in biopsy samples. They observed translocation of the scaffold protein zonula occludens ZO-1 to the tight junction region after a short-term treatment with *L. plantarum*.[65] However, functional consequences on permeability were not evaluated. Taken together, these data suggest that the effect of probiotics cannot be extrapolated so easily from in vitro to in vivo conditions and that probiotics do not seem to alter basal physiology in normal conditions as illustrated by our results with *P. freudenreichii* CIRM-BIA129.

The present study constituted a first preclinical step in the exploitation of the great diversity of strains of *P. freudenreichii* in terms of probiotic potential and development of an adapted fermented dairy delivery vehicle. No side effect was observed, while dairy propionibacteria survived the passage through the piglet digestive tract. Growth promotion and anti-inflammatory immunomodulation confirmed the potential of selected strains of dairy propionibacteria as animal probiotics. These results were promising and open new perspectives for further investigations on the mechanisms of *P. freudenreichii* probiotic properties, taking into account their strain specificity and the impact of the delivery vehicle.

■ ASSOCIATED CONTENT

Supporting Information

A comparative growth curve of *P. freudenreichii* grown on rich YEL medium versus milk ultrafiltrate and description of the method used for 16s rRNA pyrosequencing in colonic samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

[▽]These authors contributed equally to this work

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ABBREVIATIONS

CIRM-BIA, Center International de Ressources Microbiennes—Bactéries d'Intérêt Alimentaire; DSS, dextran sodium sulfate; LPSs, lipopolysaccharides; PBMCs, peripheral blood mononuclear cells; PF, *Propionibacterium freudenreichii*; UF, milk ultrafiltrate; YEL, yeast extract lactate

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