Promising immunomodulatory effects of selected strains of dairy propionibacteria evidenced in vitro and in vivo

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Running title: Anti-inflammatory properties of dairy propionibacteria
Abstract

Immunomodulatory properties of dairy propionibacteria, analyzed on human PBMCs, revealed a highly strain-dependent induction of anti-inflammatory cytokine interleukin IL-10. Two selected strains of *Propionibacterium freudenreichii* showed a protective effect against models of colitis in mice, suggesting a probiotic potential predicted by immune-based selection criteria for these cheese starter bacteria.

Selected immunomodulatory probiotic bacteria can counteract inflammation of the intestine through multiple regulatory activities and may be either complementary or an alternative to conventional treatments towards Inflammatory Bowel Disease (IBD), a growing health concern in developed countries. Probiotic strains such as lactobacilli and bifidobacteria are able to induce anti-inflammatory cytokines in human peripheral blood mononuclear cells (PBMCs) *in vitro* and were shown to exert efficient anti-inflammatory effects on colitis *in vivo* (9, 26). However, little is known about the immunomodulatory potential of highly consumed starter bacteria such as dairy propionibacteria. Consumption of fermented products has an impact on immune system function (25) and the bacterial content of these products is responsible for immunomodulation (6, 10). Dairy propionibacteria display various probiotic properties either similar or distinct from those reported for probiotic bifidobacteria and lactic acid bacteria (3). Although an anti-inflammatory potential of a few dairy propionibacteria strains was occasionally suggested *in vitro* (15) or in animals (22, 24, 31), no reliable observation was established in terms of strain variability and of specific mechanisms involved. In addition, supplementation with dairy propionibacteria in human randomized, placebo-controlled, double-blind trials has mainly concerned mixtures comprising probiotic bacteria assigned to other genera than *Propionibacterium*, but rarely with propionibacteria...
alone (16). Thus, because of synergistic effects, it is not possible to attribute observed health benefits to a specific bacterium *per se* within the mixtures.

While some criteria such as adhesion and related immunomodulation through epithelial cells may impact on further performance *in vivo*, depending on the expected probiotic effect (13, 21, 27), each probiotic strain is preferably characterized for its immune activity on immunocompetent cells (2, 19, 21, 33) or in cell co-culture models before being proposed for clinical application (23). Results obtained may help to evaluate the specific potential for the strain(s) to induce immune responses of the Th1 or the anti-inflammatory type. Here we questioned whether this *in vitro* approach is applicable to propionibacteria and whether results match with *in vivo* pathological animal models which mimic human gastrointestinal diseases and immune disorders.

**In vitro immunomodulatory-based screening.** By using a previously described *in vitro* assay of cytokine release by human PBMCs (8, 9), we evaluated the cytokines induction pattern for a set of 10 dairy propionibacteria. This included *P. freudenreichii* (*Pf*) CIRM-BIA1, CIRM-BIA118, CIRM-BIA456, ITGP18, ITGP20, SI48, SI41, LSIP11, LSIP23 and *P. jensenii* (*Pj*) CIRM-BIA455. These strains were provided by the culture collections CIRM-BIA (INRA STLO), ITG (Actilait, Rennes, France) or Laboratoires STANDA (Caen, France) and grown at 30°C in YEL medium until early stationary phase in microaerophilic conditions (18). A first screening was performed on these 10 strains, using PBMCs from four distinct donors. Tumor necrosis factor alpha (TNFα), interleukin (IL)-10, interferon γ (IFNγ) and IL-12p70 cytokines were measured by ELISA (9). It revealed a highly strain-dependent induction of IL-10, covering the range (150 to 2500 pg/ml), generally obtained with reference strains of lactobacilli (*L. acidophilus* NCFM and *L. salivarius* Ls33, kindly provided by Danisco), lactococci (*Lc. lactis* MG1363, Institute of Food Research, Norwich, UK) and
bifidobacteria (*B. longum* 5336, from Morinaga Milk Industry Ltd) cultivated and analyzed as recently described (8). Briefly, we observed strong, moderate and weak inducers of IL-10 for propionibacteria at a bacteria:host cell ratio of 10:1 (MOI of 10, data not shown). We then selected 5 strains to verify the dose dependency of the IL-10 induction (MOI of 5, 10 and 50), confirming that the MOI of 10 dose was the most appropriate for screening purpose (Fig. 1A and 1E). *Pf* ITGP20 and SI48 were found to be the most anti-inflammatory strains, while *Pj* BIA455 and *Pf* BIA118 were less anti-inflammatory, although still more than the *Pf* BIA1 type strain (Fig. 1A). Interestingly, for all the *Pf* strains tested, release of pro-inflammatory mediators was very low: weak for TNFα (Fig. 1B) and almost undetectable for IL-12 and IFNγ (Fig. 1C and 1D). The latter cytokine levels were even well below the levels described for strains that offer little or no protection in *in vivo* inflammation models (9), see cutoff levels in Fig. 1B-1D, while IL-10 levels were clearly at or above the cut-off level that generally allows protection in a TNBS induced colitis model (9), Fig. 1A. This interesting absence of pro-inflammatory cytokines was confirmed at the transcriptional level for a high, intermediary and lower anti-inflammatory strain. Quantitative RT-PCR was performed on RNA from PBMCs, isolated 4.5 h after induction (supplemental Fig. S1A, S1B, S1C) and matched with ELISA quantifications of proteins of the corresponding donors in 24h supernatants (Fig. S1D, S1E, S1F). This investigation confirmed the large diversity in the immunomodulatory profiles amongst propionibacteria, quantified directly by the IL-10 cytokine, rather than the traditional IL-10/IL-12 ratio, which obviously, is inappropriate here.

Efficacy of selected candidates in animal models. The strain *Pf* SI48, showing a particularly high anti-inflammatory profile, was further tested in a 5-days prophylactic oral treatment (5×10⁸ CFU per mouse and per day or the corresponding vehicle) in the acute trinitrobenzene sulfonic acid (TNBS)-induced acute colitis model in mice (8). This “gold standard” model
was used as described previously (female Balb/c mice, from Charles River, France, aged 7 weeks, n=10 per group). *Pf* SI48 significantly lowered the colitis-associated weight loss at day 2 (10.5 % versus 16 %, p < 0.05, Fig. 2A), confirmed by significant changes of the macroscopic (Fig. 2B and 2C, corresponding to 45 % of protection, p < 0.05), and histological scores (Fig. 2C and 2F). Consistently, inflammatory markers such as colon length and colonic myeloperoxidase (MPO) activity were also significantly reduced in the *Pf* group (Fig. 2D and 2E, respectively).

In addition, we determined whether preventive treatment with *P. freudenreichii* ssp could attenuate the severity of colonic injury and inflammation of mice infected with *Citrobacter rodentium*. We used a non-lethal model of resistant Balb/c mice sustaining a discrete and moderate colitis, but with quantifiable parameters (12, 32). Groups of 8 mice received either a 5-days prophylactic oral treatment with *Pf* BIA118 or *Pf* SI48 (5×10⁸ CFU per day and per mouse) or the corresponding vehicle before a single oral inoculum (1x10⁹ CFU) of *C. rodentium* strain DBS120 was administered (29). The counts of the fecal pathogens increased progressively in infected-control mice (Fig. 3A) together with a slight but significant decrease in whole body weight (Fig. 3B) at 10-days post infection. The colitis-associated markers were also characterized by splenomegaly (assessed by spleen weight) and increased blood Serum Amyloid A levels (Fig. 3C and 3D). Colons exhibited increased MPO activity (see above) and crypt hyperplasia (assessed by crypts length measurements on histological colon sections (Fig. 3E and 3F, respectively). Although both *Pf* strains reduced fecal pathogen counts only until day 5, strain *Pf* BIA118 significantly, and strain *Pf* SI48 to a lesser extent, lowered most of the parameters measured at the end of the experiment (Fig. 3). It could be argued that such lowering rather evokes a delay of the onset of colitis than a direct anti-inflammatory effect. However, based on previous kinetic studies at day 6 and 12 post infection (PI) (Breton J. *et al*, unpublished data), we know that (i) the markers investigated are
highly correlated with fecal (and caecal) pathogen counts and time; (ii) Moreover, blood SAA is an early marker that reaches a maximum at day 6 PI before it decreases at day 12 PI. Consequently, beside an anti-infectious effect, such a delay would not reduced SAA levels in probiotic-treated animals. Preventive effects of probiotics against *C. rodentium* were already described for lactobacilli and yeasts (14, 34) but, to our knowledge, never for dairy propionibacteria. Alvarez *et al.* reported that feeding a *Propionibacterium acidipropionici* supplement to mice prior to *Salmonella typhimurium* administration afforded a partial protection against colonization by the pathogen, measured by a decrease in tissue colonization by *S. typhimurium* and an increase in the mice survival rate (1). Here, *Pf* strains did not affect colonization of *C. rodentium* on the longer term, but mainly alleviated inflammatory symptoms linked to the infection.

**A key role for surface compounds?**

We suspected some surface compounds to be involved in the observed immunomodulatory effects of dairy propionibacteria. Therefore, in a preliminary attempt to estimate the importance of surface proteins, we removed the surface layer protein(s) by guanidium chloride treatment as described previously (20) and as depicted by scanning electron microscopy (Fig. 1F). This treatment indeed decreased the *in vitro* cytokine induction, turning the strains to display a more pro-inflammatory profile on human PBMCs (Fig. 1G). Although the importance of surface layer proteins was already observed for a probiotic *Lactobacillus* ssp. this result needs to be extensively confirmed *in vivo* for different propionibacteria *Pf*. Similarly, we can hypothesize that other components such as exopolysaccharides (EPS) may also play an important role in the bacteria-host interaction as reported for other probiotic genera, using both *in vitro* and *in vivo* models (4, 28, 30). Presence of EPS is strain-dependent in *Pf* (5) and preliminary data based on stimulation of PBMCs indicate a role of *Pf* EPS in
such interaction (data not shown). Finally, as established for bifidobacteria (11), specific cell-wall-associated proteins, which are known to be highly strain-dependent in \textit{P}. \textit{freudenreichii} \textit{ssp} \textit{shermanii}, may also contribute to a certain extent to the distinct immunomodulatory behavior of these bacteria.

The now available genome sequence of \textit{P. freudenreichii} \textit{ssp} \textit{shermanii} CIRM-BIA1 (strain included in this study) (7) will offer molecular approaches to further address this question.

Conclusions:

In the past, selection of probiotic strains was mainly empiric and based on technological criteria. As probiotic intervention has strain-specific immunomodulatory effects, it is essential to reliably select the most promising candidates for desired health applications. We described before that the differential activation of human PBMCs by bacterial strains can be considered as a predictive tool to identify gram positive probiotic strains with a potential anti-inflammatory effect \textit{in vivo} (9). Here we compared the immune–based biodiversity of a set of first 10, then 5 dairy propionibacteria together with established probiotic reference strains and found an overall characteristic pattern with high levels of IL-10 and very low induction of IL-12, TNF\(\alpha\) and IFN\(\gamma\). In agreement with these profiles we obtained significant \textit{in vivo} protection against colitis in mice, suggesting that selected strains of dairy propionibacteria and/or fermented dairy products containing these bacteria can possibly play a role in a diet designed to prevent and/or limit the severity of IBD in humans. Moreover, experiments with \textit{C. rodentium} infection in mice, proved similar efficacy in the context of infectious disease.

Further mechanistic studies based on the \textit{Propionibacterium} envelope are required in order to substantiate and select such strains for clinical investigations.

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REFERENCES


LEGEND TO THE FIGURES

Figure 1. *In vitro* immunomodulatory-based screening. Comparative anti-inflammatory IL-10 (A) and pro-inflammatory TNFα, IL-12 and IFNγ cytokine responses of human PBMCs (B, C, D) for 4 reference and 5 propionibacteria strains at a bacterial density corresponding to a multiplicity of infection (MOI) of 10. (E): IL-10 response at distinct dosing (MOI of 5, 10 and 50). Anti-inflammatory role of surface layer protein(s). (F): Transmission electron microscopy appearance of untreated (left panel) and guanidium chloride-treated *P. freudenreichii* BIA118 and SI48, showing removal of specific surface layer. (G): IL-10 and IL-12 response of *P. freudenreichii* BIA118 and SI48 and the corresponding extracted bacteria (MOI of 10 for all strains). Immuno-competent cells were stimulated by bacteria for 24 h and collected supernatants analyzed by ELISA. Data are expressed in pg/ml as means from 4 distinct healthy blood donors and standard deviation, (the dashed lines indicate the cut-off of *in vitro* threshold of corresponding strain previously shown to be protective *in vivo* (9); dashed window for 1D corresponds to a 25 times magnification).

Figure 2. Protective effect of 5-days of oral treatment with *P. freudenreichii* SI48 (5x10⁸ CFU per day) on TNBS colitis. (A): 2-days body-weight loss (% of initial) for healthy control mice (empty circles), vehicle-TNBS-treated animal (black circles) and SI48-fed mice (grey circles). (B): Individual macroscopic disease scores (Wallace score), (C): Macroscopic and histological damage scores (Ameho score), (D): Changes in colon length (cm), and (E): Colonic MPO activities, all recorded 48 hours after induction of colitis. Results are expressed as mean ± SEM (n=10 per group), *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle, Mann–Whitney U-test or the Student-t test where appropriate. (F): Representative histological sections from an untreated mouse (upper panel) and from mice after induction of TNBS.
colitis following treatment with either vehicle (mid panel) or with *P. freudenreichii* SI48 (lower panel); May-Grünwald and Giemsa-stained 5 µm paraffin sections, x40.

Figure 3. Protective effect of 5-days of oral treatment with *P. freudenreichii* BIA118 and SI48 (5x10^8 CFU per day) on 10-days *C. rodentium* (strain DBS120) induced colitis. (A): Time course of pathogens enumeration in pooled feces following the distinct treatments. (B): Weight changes (% of initial) for healthy control mice (empty circles) and infected control (full circles), BIA118- (squares) and SI48-fed mice (triangles), (C): Spleen weight, (D): Blood Serum Amyloid A protein (mg/ml), (E): Colonic MPO activities and (F): crypt length (µm) measured on at least 10 well orientated sections per animal per group. Results are expressed as mean ± SEM (n=8 mice per group), excepted for weight changes % on 3B for clarity. nd: not detected. (*) 0.05 < P < 0.1; *P < 0.05; **P < 0.01; ***P < 0.001 versus infected animals, Mann–Whitney *U*-test or the Student-t test where appropriate.
Figure 2

A) Weight change (%)

B) Individual Wallace Score

C) Colitis severity

D) Colon length (cm)

E) Colon MPO (arbitrary units)

F) Histological (Ameho score)
Figure 3

A. Viable C. rodentium counts (CFU x 10^6/g of stool)

B. Weight change (% of initial)

C. Spleen weight (mg)

D. Blood SAA (µg/mL)

E. Colon MPO (arbitrary units)

F. Crypt length (µm)