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# Cheese matrix protects the immunomodulatory surface protein SlpB of *Propionibacterium freudenreichii* during *in vitro* digestion

Houem Rabah[a,b], Olivia Ménard[a], Floriane Gaucher[a,c], Fillipe Luiz Rosa do Carmo[a,d], Didier Dupont[a], Gwénaël Jan[a,*]

[a] STLO, INRA, Agrocampus Ouest, 35 000, Rennes, France
[b] Pôle Agronomique Ouest, Régions Bretagne et Pays de la Loire, F-35042 Rennes, France
[c] Bioprox, 6 rue Barbès, 92532 Levallois-Perret, France
[d] Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (ICB/UFMG), 31270-901 Belo Horizonte, Brazil

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ABSTRACT

*Propionibacterium freudenreichii is* a traditional Swiss-type cheeses starter and constitutes an emergent probiotic, exerting several beneficial effects, including anti-inflammatory modulation of gut inflammation. This feature relies on several metabolites and on surface proteins, with a prominent role of the surface protein SlpB. In this study, we firstly investigated the relevance to avoid SlpB digestive proteolysis, by comparing the effect of i) *P. freudenreichii* CIRM-BIA 129, ii) its native Slps, or iii) peptides resulting from Slps digestive proteolysis, with respect to modulation of HT-29 cells response to a lipopolysaccharide (LPS) challenge. The anti-inflammatory effect exerted by *P. freudenreichii* CIRM-BIA 129 and by its native surface proteins (Slps) on HT-29 cells was abolished by digestive proteolysis. This result confirmed the importance to protect immunomodulatory surface proteins from digestive proteolysis in order to allow gut immune system modulation. Thus, we examined the effect of dairy matrices on *P. freudenreichii* viability and on SlpB integrity during digestion. In comparison with liquid matrices, the cheese matrix provided an enhanced tolerance towards digestive stresses and protection of SlpB towards proteolysis, during two *in vitro* digestion models: static and dynamic. Taken together, these results show that cheese is an adequate delivery vehicle for *P. freudenreichii* immunomodulatory proteins. This opens perspectives for the development of fermented dairy functional foods aimed at target populations at high risk for diet-related diseases with an inflammatory component.

## 1. Introduction

*Propionibacterium freudenreichii* is an emergent probiotic bacterium, belonging to dairy propionibacteria, within the group of Actinomycetales. It possesses the GRAS (Generally Recognized As Safe, USA, FDA) and QPS (Qualified Presumption of Safety, EFSA, European Union) status (EFSA Panel on Biological Hazards (BIOHAZ), 2013). Dairy propionibacteria, including *P. freudenreichii*, are typically exploited in the food industry for the production of vitamins and for the manufacture of Swiss-type cheeses, including Emmental (Rabah, Rosa do Carmo, and Jan, 2017; Thierry et al., 2011). Recently, several beneficial effects exerted by *P. freudenreichii* were reported, including anti-inflammatory modulation in the context of gut inflammation (Cousin, Mater, Foligné, and Jan, 2010; Rabah et al., 2017). Dairy products fermented by *P. freudenreichii*, alone or in combination with the probiotic *Lactobacillus delbrueckii*, were efficient to attenuate a chemically-induced colitis in mice (Benoît Foligné et al., 2016; Plé et al., 2015; Plé et al., 2015). Accordingly, a pilot clinical study indicates healing of ulcerative colitis in patients, as a result of consumption of dried *P. freudenreichii* whey culture (Mitsuyama et al., 2007; Suzuki et al., 2006). These data highlight the probiotic potential of *P. freudenreichii* to promote remission from IBD. The anti-inflammatory properties of *P. freudenreichii* are strain-dependent (Foligné, Breton, Mater, and Jan, 2013) and mediated by several metabolites, such as 1, 4-dihydroxy-2-naphtoic acid (DHNA), short-chain fatty acids (SCFAs) (Rabah et al., 2017) and by immunomodulatory surface proteins which present structural characteristics of S-layer proteins (Slps) (Deutsch et al., 2017; Le Maréchal et al., 2015). S-layer proteins belong to the outermost S-layer lattice, non-covalently anchored to the cell wall, and alternately present in Gram-positive and Gram-negative bacteria (Gerbino, Carasi, Mobili, Serradell, and Gómez-Zavaglia, 2015). They display a variety of functions such as adhesion, sieving of molecules, and protection against environmental stresses (Gerbino et al., 2015; Lortal, Rouault, Cesselin, and Sleytr, 1993). In the

* Corresponding author at: INRA UMR1253 STLO, 65 rue de Saint Brieuc, 35042 Rennes cedex, France.
*E-mail address:* gwenael.jan@inra.fr (G. Jan).

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reference *P. freudenreichii* CIRM-BIA 1 strain, nine genes encoding putative S-layer proteins were identified within the genome (Falentin et al., 2010). In contrast with SlpA from the strain *P. freudenreichii* CIRM-BIA 118, which is confirmed as a true S-layer protein (de sa Peixoto et al., 2015; Lortal et al., 1993), the protein designated previously as S-layer protein B (SlpB) from *P. freudenreichii* CIRM-BIA 129 was not shown to form a true surface layer. However, it was detected by surface proteomics (Le Maréchal et al., 2015) and our studies pointed out the key role of SlpB in *P. freudenreichii's* anti-inflammatory properties and in interaction with immune cells (Deutsch et al., 2017; Le Maréchal et al., 2015). SlpB is also involved in the adhesion to human intestinal epithelial cells (Carmo et al., 2017). These results indicate a prominent role of the surface protein SlpB as a Microbe-Associated Molecular Pattern (MAMP) in this probiotic/host cross-talk, with promising anti-inflammatory applications. This is consistent with previous studies indicating that Lactobacilli S-layer proteins interact with immune cells, such as dendritic cells, *via* Pathogen-Recognition Receptors (PRRs), within the gut, inducing tolerance response leading to attenuated colonic inflammation (Konstantinov et al., 2008; Lightfoot et al., 2015). During *P. freudenreichii* consumption, susceptibility of its bioactive surface proteins to proteolysis within the digestive tract thus becomes a crucial question, not yet addressed. The delivery vehicle may determine the transit of intact surface proteins through the host intestinal tract. We investigated the efficacy of the “Swiss-type” cheese matrix as a protective delivery vehicle of anti-inflammatory potential of *P. freudenreichii* to target intact surface proteins to the colon. Indeed, cheese environment enhances propionibacteria tolerance towards acid and bile salts stresses (Gagnaire, Jardin, Rabah, Briard-Bion, and Jan, 2015). Furthermore, the presence of a proteinaceous matrix may exert a protective effect towards enzymatic proteolysis, allowing undamaged bacteria and surface proteins to reach the digestive tract. In the present study, we first assessed the impact of SlpB proteolytic degradation on its immunomodulatory properties, using on colon epithelial HT-29 cells. We then investigated the potential of cheese matrix, as a vehicle, to deliver intact anti-inflammatory *P. freudenreichii* MAMP to the colon. In that aim, we compared efficacy of dairy liquid matrices and of a solid Swiss-type cheese matrix, using two *in vitro* digestion models.

## 2. Material and method

### 2.1. Bacterial strains and dairy matrices preparation

The *Propionibacterium freudenreichii* strain CIRM-BIA 129 was provided as certified pure cultures by the CIRM-BIA International Biological Resource Center (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, INRA, Rennes, France) and routinely cultivated in YEL (Yeast Extract Lactate medium). Milk ultrafiltrate and milk media were prepared by adding 5 g/L of casein peptone (Organotechnie, France) and 100 mM of sodium DL-lactate (Sigma), as described previously (Cousin et al., 2012). *P. freudenreichii* was grown at 30 °C, without agitation, in microaerophilic conditions until stationary phase (72 h). Single-strain cheese was prepared with *P. freudenreichii* CIRM-BIA 129 using a process close to ‘Swiss-type’ cheeses as described previously (Plé, Richoux, et al., 2015). Briefly, *P. freudenreichii* was cultivated in sterile whole milk, supplemented with cream, casein powder, casein peptone and sodium lactate, until stationary phase. After coagulation, (chy-max®Extra, Chr. Hansen),the curd was cut, heated (40 °C, 10 min), molded, pressed, dried under laminar flow, wrapped and storage at 4 °C. The biochemical composition of the cheese was: dry matters 58 g/100 g, lipids 28 g/100 g, proteins 29 g/100 g, carbohydrates 0 g/100 g, and calcium 840 mg/100 g (Plé, Richoux, et al., 2015). The propionibacteria amounts reached $3.10^9$ CFU/mL in milk ultrafiltrate, $7.10^9$ CFU/mL in milk and $1.10^{10}$ CFU/g in single-strain cheese.

### 2.2. Surface proteins extraction and peptides preparation

Surface proteins (Slps) extraction was performed as described previously (Le Maréchal et al., 2015). Briefly, *P. freudenreichii* CIRM-BIA 129 cultures in milk ultrafiltrate (40 mL) were centrifuged (7000 × *g*, 10 min) to remove the supernatants and the pellets were washed twice with cold PBS buffer. The pellets were then suspended in guanidine chloride-5 M (4 mL, Sigma), and incubated at 50 °C during 15 min. These suspensions were centrifuged (20,000 × *g*, 20 min) to recover the supernatants. The proteins extracts were dialyzed against water during 24 h and then against PBS. Slps-derived peptides were prepared by treating 50 µg of Slps with 2.5 µg of trypsin corresponding to an activity of 17.8 U/µg (Promega). The samples were incubated for 8 h at 37 °C. The complete digestion of Slps was checked by SDS-PAGE (data not shown). Resulting peptides were recovered by filtration through, successively, 20 kDa and 10 kDa-CutOff filters (Vivaspin, Sartorius stedim biotech) without dilution, nor concentration.

### 2.3. HT-29 cells challenging

HT-29 cells were routinely cultivated in T-25 flasks in complete medium DMEMc (10% (*v*/v) fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin sulphate) at 37 °C with 5% $CO_2$. Trypsin/EDTA (Gibco, Saint Aubin, France) was used to release adherent cells for subculturing. For the experiment, cells were seeded in 12-well plates and the growth medium was changed every 2 days. HT-29 cells were cultured until complete confluence, $1.10^6$ cell/mL-well. Prior to challenging cells, complete medium was replaced with antibiotic-free medium and HT-29 cells were then subjected to the different treatments according to (Duary, Batish, and Grover, 2014). Briefly, cells were stimulated 3 h with 100 ng/mL lipopolysaccharide (LPS from *E. coli*, Sigma), or 7 h with probiotic preparations. These last were: *P. freudenreichii* CIRM-BIA 129 ($1.10^7$ CFU/mL-well, corresponding to an MOI of 10, as determined previously (Benoît Foligné et al., 2010), or Surface proteins (Slps) (50 µg/mL-well), or peptides (50 µg/mL-well). After different stimulation conditions, the HT-29 cells was monitored and the viability percentage are listed in Table S1.

### 2.4. Total RNA isolation and gene expression analysis by qRT-PCR

Cellular RNA was isolated with Trizol reagent (Invitrogen Ambion), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Table 1 lists interleukine and chemokine genes analyzed (Duary et al., 2014). Amplification was performed on a CFX96 real-time system (Bio-Rad, Marne la Coquette, France). Real-time PCR reactions were set up in CFX96 real-time system (Bio-Rad, Marne la Coquette, France). Each PCR reaction was performed in a 16 µL reaction mixture containing 5 µL SYBR Green PCR Master Mix (Biorad), 5 µL of properly diluted cDNA (280 ng of cDNA for all genes), 3 µL mixture of each primer at 300 nM. The negative controls (with no DNA template, only primer pair, water and SYBR Green PCR Master Mix) for each primer set were included in each run. Amplification was carried out on a CFX96 Real-Time System (Bio-Rad) for 3 min at 95 °C and 40 cycles of 2 steps consisting of 5 s at 95 °C and 30 s at 60 °C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager. The transcript level was normalized to the transcript level of GAPDH and actin genes (housekeeping gene, see Table S1). Finally, the results are presented as fold change using $2^{-\Delta\Delta CT}$ method for an unknown sample *versus* the control (untreated HT-29 cells).

### 2.5. Static digestion model

Digestion of three fermented dairy matrices was carried out simultaneously using the consensus static *in vitro* digestion protocol as described previously (Minekus et al., 2014), see Table 2. In detail, simulated salivary (SSF) simulated gastric (SGF) and simulated duodenal