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Chapter

Propionibacterium freudenreichii: General Characteristics and Probiotic Traits

Vinícius de Rezende Rodovalho, Diego Lucas Neres Rodrigues, Gwénaël Jan, Yves Le Loir, Vasco Ariston de Carvalho Azevedo and Eric Guédon

Abstract

Propionibacterium freudenreichii is a Gram-positive dairy probiotic bacterial species that has been used as a ripening starter in the production of Swiss-type cheese for a long time. It has been exploited for the optimization of cheese production, including ripening capacities and aroma compounds production, but also for the production of vitamin B12 and organic acids. Furthermore, it has emerged in the probiotics landscape owing to several beneficial traits, including tolerance to stress in the gastrointestinal tract, adhesion to host cells, anti-pathogenic activity, anticancer potential and immunomodulatory properties. These beneficial properties have been confirmed with *in vitro* and *in vivo* investigations, using several omics approaches that allowed the identification of important molecular actors, such as surface proteins, short-chain fatty acids and bifidogenic factors. The diversity within the species was shown to be an important aspect to take into consideration, since many of these properties were strain-dependent. New studies should dive further into the molecular mechanisms related to the beneficial properties of this species and of its products, while considering the complexities of strain diversity and the interactions with the host and its microbiota. This chapter reviews current knowledge on the possible impact of *P. freudenreichii* on human health.

Keywords: *Propionibacterium freudenreichii*, propionibacteria, probiotics, immunomodulation, food microbiology

1. Introduction

The denomination "probiotics" comprises living microorganisms, including bacteria and yeasts, with health-promoting properties and suitable for safe consumption, as confirmed by their dietary uses for thousands of years of human history [1–3]. Lactic acid bacteria and bifidobacteria comprise traditional probiotic bacteria species, widely documented and commercialized [3, 4]. However, different species have emerged in the probiotics landscape, such as the dairy species *Propionibacterium freudenreichii* [4, 5], which is phylogenetically related to bifidobacteria (**Figure 1**) [4].

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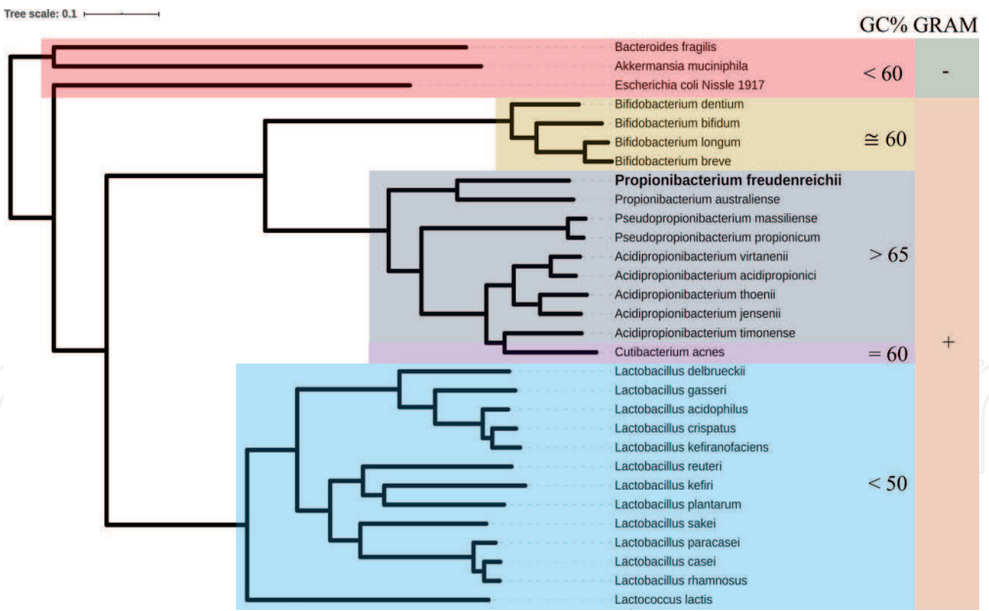


Figure 1.
Phylogenetic tree showing genomic similarity between health-promoting P. freudenreichii *species, other probiotic or closely-related species.*

The former *Propionibacterium* genus encompassed a group of microorganisms with importance in industry and health, due to the production of valuable metabolites, food, cosmetic and pharmacological products [6]. Previously, this genus included classic dairy propionibacteria species and skin-associated pathogenic propionibacteria [7]. However, a genome-based taxonomy reevaluation suggested the reclassification of cutaneous bacteria into the *Cutibacterium* genus, together with the inclusion of two other new genera for formerly classic propionibacteria, *Acidipropionibacterium* and *Pseudopropionibacterium* [7]. *P. freudenreichii*, which is one of the most notable dairy propionibacteria species, kept its former taxonomic classification [4, 7].

P. freudenreichii is a Gram-positive, high GC-content, mesophilic, aerotolerant, non-motile, non-spore forming bacterium, that shows low nutritional requirements and survives in harsh environments [5, 8, 9]. Regarding morphology, it is a pleomorphic rod microorganism, with aggregation tendency, forming clusters that resemble Chinese characters [5] (**Figure 2**). This bacterium, isolated from samples of Emmental cheese, was first described by Orla Jensen and von Freudenreich in 1906 [10]. Recently, *P. freudenreichii* strains have been identified in fecal samples from a discrete cohort of human preterm breast-fed infants, suggesting that it could be a component of the healthy human gut microbiota [11].

P. freudenreichii is able to use several carbon sources (e.g., glycerol, erythriol, L-arabinose, adonitol, galactose, D-glucose, D-fructose, D-manose, inositol, arbutine, esculine, lactose, lactate and gluconate) in the fermentation process to produce propionate, together with acetate, succinate and carbon dioxide (CO_2) [9, 12, 13]. Unlike other species, *P. freudenreichii* is able to reduce pyruvate into propionate via the transcarboxylase cycle (also referred to as Wood–Werkman cycle), which is a cyclic process coupled to oxidative phosphorylation, that allows a higher ATP yield than in other propionate-producing bacteria [9]. In its turn, pyruvate is a metabolic node molecule, which may be used either for the NADH-generating synthesis of acetate, or for the NADH-consuming synthesis of propionate [14]. In a strain-dependent manner, the bacterium modulates the proportions of pyruvate that are reduced into propionate or oxidized into acetate and CO_2, thus maintaining the

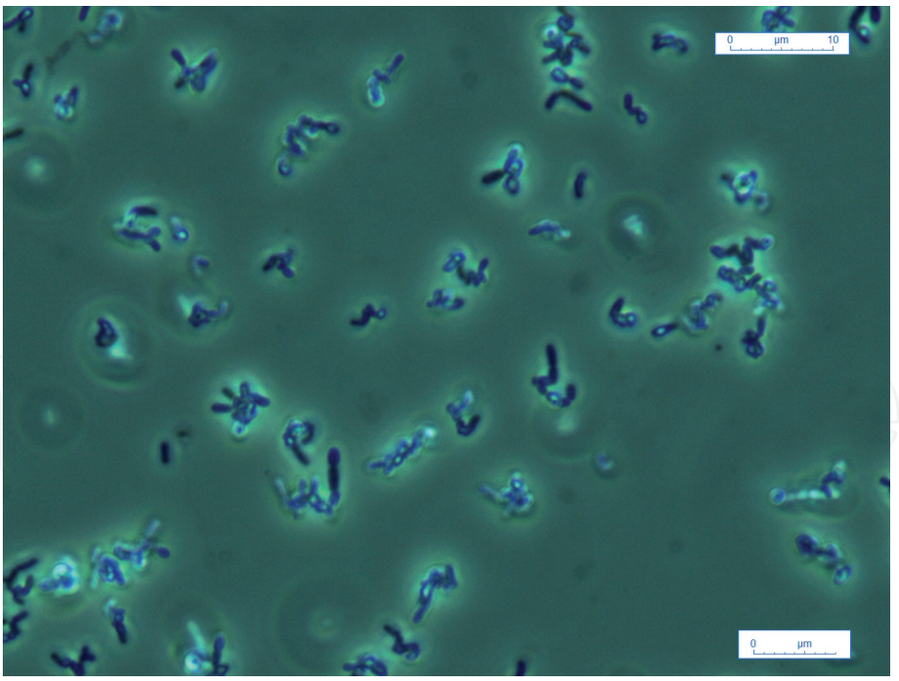


Figure 2.
Optical microscopy image showing the morphological aspect of a P. freudenreichii *CIRM-BIA129 culture, with typical aggregates resembling Chinese characters.*

redox equilibrium [9]. Therefore, this species encompasses biochemically versatile strains, that find different applications in several contexts [5].

2. Technological importance

P. freudenreichii is widely used for the production of Swiss-type cheeses, such as Emmental [5, 15] (**Figure 3**). In such dairy matrices, the CO_2 gas that is produced during fermentation forms bubbles that diffuse slowly, creating characteristic holes, or "eyes", in cheese architecture [9, 12]. Cheese flavor is related to propionate and acetate, as well as the products of amino acids catabolism and fat hydrolysis by propionibacteria [16, 17]. Importantly, these dairy products containing *P. freudenreichii* displayed anti-inflammatory properties *in vivo* [18–20], increasing the recognition of this bacterium and of its products as health-promoting. Therefore, dairy propionibacteria are considered 2-in-1 bacteria, with both fermentative and probiotic properties, which makes them ideal for the development of health-promoting fermented food [5, 18].

This bacterium is also well recognized to encompass a pathway for vitamin B12 (cobalamin) synthesis [8, 9]. Vitamin B12 is a water-soluble vitamin, which plays a key role in the functioning of the brain, of the nervous system and in the



Figure 3.
Emmental cheese produced using P. freudenreichii *CIRM-BIA129, in conjunction with* Streptococcus thermophilus *and* Lactobacillus delbrueckii.

production of blood [21]. It is also a co-factor of methylmalonyl-CoA mutase, which catalyzes a crucial step in the fermentative route to produce propionate [22]. Therefore, the growth conditions of *P. freudenreichii* have been optimized for the production of vitamin B12, using substrates such as cereal matrices [23, 24], waste frying sunflower oil [25], tofu wastewater [26] and soybean agroindustry residue [27]. Moreover, *P. freudenreichii* has been genetically engineered to enhance vitamin B12 and propionate production [6, 28].

The production of vitamin B12, organic acids, trehalose and other metabolites, together with the safe use as cheese ripening starter and probiotic characteristics, make this bacterium attractive for several biotechnological and industrial applications [5, 6, 29, 30]. A wide range of genetic and environmental optimizations have been conducted to improve these properties [6, 29]. Moreover, some optimizations of the growth and processing conditions allowed the improvement of resistance towards storage and towards several industrial processes, such as freeze-drying and spray-drying [30–33].

3. Strain variability

The interesting properties of this bacterium, such as health-promoting features, and participation to industrial vitamin B12 and cheese production, were shown to be strain-dependent, suggesting the need for analysis that account for that variability [9]. As an example, some strains presented differences in nitrogen and sugar degradation, which had a genetic origin, probably resulting from horizontal transfers, duplications, transpositions and other mutations [13]. This strain diversity was confirmed at the genomic level by another study and attributed to transposable elements, in such a way that genome plasticity enabled bacterial adaptation to several environments [34].

In view of this strain-related variability, there have been efforts to specify criteria for the selection of probiotic strains. These criteria include tolerance to stresses encountered within the gastrointestinal tract, adhesion to host cells, antipathogenic activity, anticancer potential, immunomodulatory properties, industrial requirements and molecular characterization using omics methodologies [3]. Mounting evidence shows that *P. freudenreichii* fulfills these criteria [5].

4. Stress tolerance

Regarding stress tolerance and adaptation to the gastrointestinal tract (GIT), some *P. freudenreichii* strains presented adaptations, including morphological and proteomic modifications [35–37]. For example, those modifications were verified during the acid tolerance response in the strain *P. freudenreichii* SI41, which was investigated using a kinetic study of stress proteins production during acid adaptation [35]. As a result, biotin carboxyl carrier and proteins involved in DNA synthesis and repair were associated to early acid stress response, whereas chaperonins GroEL and GroES were associated to late acid stress response [35]. Analysis with the same strain showed that bile salts (a mixture of cholate and deoxycholate) triggered drastic morphological changes and induced proteins related to signal sensing and transduction, general stress and an alternative sigma factor [36]. The same strain was used in a follow-up comprehensive study that included heat, acid, and bile salts conditions to study *P. freudenreichii* tolerance. As a result, each form of stress induced specific proteins, but six of them were common to all stresses, including chaperones and proteins involved in energetic metabolism and oxidative

stress remediation [37]. An *in vitro* study that involved 13 strains of *P. freudenreichii* showed that most of them had high capacity of tolerance to simulated gastric juices with varying pH and small intestine conditions [38].

Moreover, this resistance was also evidenced *in vivo*. The mRNA of *P. freudenreichii* methylmalonyl-transcarboxylase was detected in human fecal samples using real time reverse transcriptase polymerase chain reaction (RT-PCR) [39]. Methylmalonyl-transcarboxylase is a key enzyme of the transcarboxylase cycle, only expressed when propionic fermentation is active, therefore its detection in fecal samples indicated that the bacterium survived and remained metabolically active, transcribing genes within the human digestive tract [39]. A multi-strain study using human microbiota-associated rats monitored intestinal microbiota composition and short-chain fatty acids production, confirming that *P. freudenreichii* stress tolerance in the GIT is also strain-dependent [40]. *P. freudenreichii* CIRM-BIA1 was shown to adapt metabolically and physiologically to the colon environment of pigs, with changes in carbohydrate metabolism, down-regulation of stress genes and up-regulation of cell division genes [41]. Furthermore, the use of food vehicles for *P. freudenreichii* delivery, such as cheese and fermented milk, improved its resistance towards the GIT stressing environment [15, 18, 19, 42, 43].

Other aspects of *P. freudenreichii* resistance to stress conditions have also been studied, such as long-term nutritional shortage [44, 45]. A screening was performed with eight *P. freudenreichii* strains, which were incubated for several days after the beginning of the stationary phase, without further supplementation of nutrients. They displayed high survival rates and no lysis, indicating that these strains adapt to long-term nutritional shortage, using a viable, yet nonculturable state [45]. The strain *P. freudenreichii* CIRM-BIA138 was further studied in these conditions of incubation, and it was shown that a high population was maintained, even after exhaustion of lactate, the preferred carbon source. RNA-seq analysis showed that several metabolic and information processing pathways were down-regulated [44].

Another important feature of *P. freudenreichii* during stress response is the accumulation of trehalose. A study that investigated this bacterium during adaptation to osmotic, oxidative and acid stress, showed that the trehalose-6-phosphate synthase/phosphatase (OtsA–OtsB) pathway, related to trehalose synthesis, was enhanced in these conditions [46]. Another study focused in stress response to low temperature (4°C), a condition that mimicked cheese ripening conditions. As a result, seven *P. freudenreichii* strains displayed a slowed-down cell machinery, cold stress response and the accumulation of trehalose and glycogen [47]. *P. freudenreichii* also accumulated glycine betaine, glycogen, trehalose and polyphosphates when cultured in hyperconcentrated media [48]. The accumulation of trehalose, together with glycine betaine, was further verified in a technological context, when bacterial viability was increased during spray drying and storage, through the optimization of the growth medium composition and thermal adaptation [31]. The ratio of the concentrations of these intracellular osmoprotectants, trehalose and glycine betaine, was further shown to modulate the stress tolerance during the technological processes of freeze-drying and of spray-drying [32].

5. Adhesion properties

Adhesion to host cells is another important feature of probiotics, which favors their local beneficial action. Early studies revealed the ability of several probiotic bacteria, including *P. freudenreichii*, to adhere to glycoproteins and mucus from human intestinal tract [49, 50]. In the case of *P. freudenreichii*, the adhesion of some

strains to pig ileum cells (IPEC-J2) was between 25 and 35%, and that proportion was higher with the addition of $CaCl_2$ [51]. In the case of human host, several bacterial strains were tested for adhesion to HT-29 colon cells *in vitro*; the most adhesive strain was *P. freudenreichii* CIRM-BIA129 and surface layer protein B (slpB) key role in adhesion was demonstrated by using gene inactivation [52, 53]. Another study demonstrated that bacterial adhesion to immobilized mucus could be synergistically improved by administration of strains combinations, such as *P. freudenreichii* ssp. *shermanii* JS in combination with *Bifidobacterium breve* or *Lactobacillus rhamnosus* strains [54].

6. Anti-pathogenic activity

There are also several evidences of an anti-pathogenic activity in this species. *P. freudenreichii* JS reduced by 39% the adhesion of *S. aureus* to human intestinal mucus and by 27% its viability, probably due to the production of organic acids [55]. *P. freudenreichii* PTCC 1674 was reported to secrete a lipopeptide biosurfactant with antimicrobial activity mainly against *Rhodococcus erythropolis*, and anti-adhesive activity mainly against *Pseudomonas aeruginosa* [56]. Moreover, *P. freudenreichii* DSM 20270 significantly inhibited *E. coli* O157:H7 growth *in vitro* [51].

P. freudenreichii also showed anti-pathogenic properties in animals. *P. freudenreichii* B-3523 and B-4327 impacted *Salmonella* strains multiplication, motility and adhesion to avian epithelial cells *in vitro* [57]. The follow up study indicated that the cell-free culture supernatants of the same probiotic strains were bactericidal against multidrug-resistant *Salmonella enterica* serovar Heidelberg [58]. *In vivo* assays further showed that the probiotic strains reduced the pathogen cecal colonization and dissemination to the liver in turkey poults [58]. *P. freudenreichii* consumption was furthermore shown to limit and to delay colonization of the mice intestinal tract by the pathogen *Citrobacter rodentium* [59].

In line with the synergies observed in terms of adhesion, probiotic combinations were proposed to improve anti-pathogenic activity, such as a combination of *P. freudenreichii* JS, *L. rhamnosus* GG and LC705, and *B. breve* 99, which promoted the inhibition, displacement and competition with several pathogenic species, such as *S. enterica*, *Listeria monocytogenes* and *Clostridium difficile* [60]. In another study, *P. freudenreichii* JS decreased the adhesion of *Helicobacter pylori* to Caco-2 intestinal cells when used individually, but also inhibited membrane leakage, improved epithelial barrier function and modulated inflammatory cytokines when used in combination with *L. rhamnosus* and *B. breve* strains [61].

7. Anticancer potential

Promising results, in the context of intestinal carcinogenesis, were also reported in this species. A pioneer study showed that *P. freudenreichii* ITGP18 and *P. freudenreichii* SI41 could induce apoptosis of cultured human colorectal carcinoma cell lines *in vitro* and this effect was mediated by short-chain fatty acids (SCFAs), such as propionate and acetate, acting on cancer cells mitochondria [62]. Following up, it was further clarified that the effect of SCFAs was modulated by extracellular pH shifts; and in acidic pH, cell death mode changed from apoptosis to necrosis in human colon HT-29 cells [63]. These effects were confirmed *in vivo*, with *P. freudenreichii* TL133 inducing the apoptosis of colon cells in human microbiota-associated rats treated with 1,2-dimethylhydrazine, yet not in healthy rats [64].

Another strain, *P. freudenreichii* ITG P9, was also employed for the development of a fermented milk with anti-oncogenic potential, since it induced apoptosis in cultured HGT-1 human gastric cancer cells *in vitro* [43]. Next, this fermented milk was proposed as an adjuvant in colorectal cancer therapy based on TNF-related apoptosis-inducing ligand (TRAIL), due to possible synergistic effect between the bacterium and TRAIL, which was confirmed with the enhancement of cytotoxic activity in HT-29 cells [65]. Another study investigated the crosstalk between bacterium and cancer cells: the latters produce lactate as a result of the metabolic shift referred as "aerobic" glycolysis or "Warburg effect"; lactate may then be used by this bacterium as a carbon source, stimulating its production of SCFAs [66].

8. Modulation of microbiota composition

Regarding the modulation of microbiota composition, consumption of dairy propionibacteria was shown to enhance intestinal populations of bifidobacteria in humans [67, 68]. In line with this, the stimulation of bifidogenic growth was observed in cell-free filtrate and cellular methanol extract derived from *P. freudenreichii* 7025 cultures [69]. Following analysis with the same strain allowed the purification of a bifidogenic growth stimulator component, the identification of its chemical structure (2-amino-3-carboxy-1,4-naphthoquinone, ACNQ) and the demonstration of its bifidogenic activity in the concentration of 0.1 ng/mL [70]. Another strain, *P. freudenreichii* ET-3 was reported to produce 1,4-dihydroxy-2-naphthoic acid (DHNA) in concentrations of 10 μg/mL, which also stimulated the growth of bifidobacteria [71]. The beneficial effect of DHNA was later confirmed *in vivo*, using mice with colitis induced by 2.0% dextran sodium sulphate (DSS). DHNA attenuated inflammation, through the modulation of intestinal bacterial microbiota and suppression of lymphocyte infiltration [72].

The bifidogenic growth stimulator derived from *P. freudenreichii* was also orally administrated to human patients in a pilot study, being promising for the treatment of ulcerative colitis [73]. Subsequent studies included optimizations of the production of bifidogenic growth stimulators, including an increased production by switching to aerobic growth conditions [74] and the use of lactic acid as a carbon source in a bioreactor system with a filtration device [75].

9. Immunomodulatory properties

There is mounting evidence, both *in vitro* and *in vivo*, that *P. freudenreichii* exerts immunomodulatory effects by several mechanisms, in a strain-dependent manner. For example, a screening for IL-10 induction in human peripheral blood mononuclear cells (PBMCs) was performed in 10 strains of *P. freudenreichii*, resulting in the selection of the two most anti-inflammatory strains: *P. freudenreichii* ITG P20 (equivalent to CIRM-BIA129) and SI48 [59]. In the same study, the strain *P. freudenreichii* SI48 was further tested *in vivo*, in mice with acute colitis induced by trinitrobenzenesulphonic acid (TNBS), lowering significantly inflammatory and histological markers of colitis [59]. Other studies also showed that the immunomodulatory properties were strain-dependent within the species *P. freudenreichii* [76]. An integrative strategy encompassing comparative genomics, surface proteomics, transcriptomics, assays of cytokines induction and genes inactivation, identified relevant proteins and strains specificities in immunomodulation [77]. Remarkably, surface proteins of the S-layer type were shown to be crucial in immunomodulation, but the immunomodulatory properties varied among strains, due to complex

Strain	Protein	Name/Description	Accession	Evidence level	Ref.
CIRM-BIA129 (ITG P20)	Ensemble of surface proteins			proteomic, *in vitro*	[82]
	GroL2	60 kDa chaperonin 2	CDP49125	genomic, proteomic	[77]
	HsdM3	Type I restriction-modification system DNA methylase	CDP48267	genomic, transcriptomic, mutant studies *in vitro*	[77]
	LacI1	Arabinose operon repressor	CDP47860	transcriptomic	[77]
	MerA	Pyridine nucleotide-disulphide oxidoreductase	CDP48574	genomic, proteomic	[77]
	Pep	Hypothetical protein	CDP48241	genomic, mutant studies *in vitro*	[77]
	PFCIRM129_04790	Hypothetical protein	CDP48736	genomic, transcriptomic	[77]
	PFCIRM129_10785	Hypothetical protein	CDP49252	transcriptomic	[77]
	PFCIRM129_10930	Hypothetical protein	CDP48242	genomic, transcriptomic	[77]
	SlpB	Surface layer protein B	CDP48273	genomic, transcriptomic, proteomic, mutant studies *in vitro*	[77]
				mutant studies *in vitro* and *in vivo*	[83]
				mutant studies *in vitro*	[52]
	SlpE	Surface protein with SLH domain	CDP48858	genomic, transcriptomic, proteomic, mutant studies *in vitro*	[77]
	SlpF	Surface protein with SLH domain	CDP49687	proteomic, mutant studies *in vitro*	[77]
CIRM-BIA 121	Acn	Aconitase, Aconitate hydratase	CEG89374	transcriptomic	[77]
	DcuA	C4-dicarboxylate transporter	CEG91776	genomic	[77]
	Eno1	Enolase 1	CEG91483	proteomic, mutant studies *in vitro*	[77]
	HtrA4	Serine protease	CEG91080	genomic, transcriptomic, proteomic, mutant studies *in vitro*	[77]
	PFCIRM121_08235	Hypothetical protein/unknown function	CEG91253	genomic, mutant studies *in vitro*	[77]
	SlpC1	Surface layer protein C	CEG91216	genomic, transcriptomic	[77]
UF1	LspA	Large surface layer protein A	n.a.	proteomic, *in vivo*	[84]
	DlaT	Dihydrolipoamide acetyltransferase	n.a.	proteomic, mutant studies *in vivo*	[11]

Legend: Ref.: references, n.a.: not available.

Table 1.
Proteins from P. freudenreichii *related to its immunomodulatory properties.*

combinations of molecular features [77]. The strain-specific export of surface proteins, adhesins and moonlighting proteins was confirmed in a different subset of *P. freudenreichii* strains [78]. Additionally, acute colitis induced by dextran sodium sulfate (DSS) in rats was ameliorated by *P. freudenreichii* KCTC 1063, which stimulated in intestinal cells the expression of MUC2, a main component of mucus [79].

The roles of *P. freudenreichii* in the modulation of host immunological response became even more relevant when a human commensal strain was identified. *P. freudenreichii* UF1 was demonstrated to be a component of the gut microbiota of preterm infants that were fed with human breast milk and to mitigate intestinal inflammatory diseases [11]. Moreover, this strain modulated the intestinal immunity of mice against pathogen challenge, specifically against systemic *L. monocytogenes* infection, by regulating Th17 cells [80]. This beneficial effect was confirmed in newborn mice, which were susceptible to intestinal pathogenic infection, but had their defense enhanced by this strain, particularly by the increase in protective Th17 cells and regulatory T cells [81].

Regarding the bacterial factors involved in immunomodulation, evidence points out mainly to surface proteins (**Table 1**). The strain *P. freudenreichii* CIRM-BIA129 had its proteome investigated, with the identification of surface-exposed proteins and their role in induction of IL-10 and IL-6 release by PBMCs [82]. Among the identified proteins, there were cell wall-remodeling proteins, transport proteins, moonlighting proteins and other proteins involved in interactions with the host [82]. The multi-strain and multi-omics study conducted by Deutsch et al. [77] clarified that cytoplasmic proteins might also be relevant in immunomodulation, but confirmed the key role of surface-layer proteins B (SlpB) and E (SlpE), particularly in strain *P. freudenreichii* CIRM-BIA129. SlpB was then shown to be crucial for bacterial adhesion to epithelial intestinal cells [52], and a mutation in its gene had pleiotropic effects, suggesting this protein could have a central role in cellular processes [53]. Additionally, *in vivo* assays that were conducted in mice with mucositis induced by 5-Flourouracil (5-FU), showed that SlpB protein is crucial for the cytokine modulation triggered by *P. freudenreichii* CIRM-BIA129 [83]. Moreover, the glycosylated large surface layer protein A (LspA) of the commensal strain *P. freudenreichii* UF1 was shown to regulate the interaction with SIGNR1 receptor, which regulates dendritic cells and counteracts pathogenic-driven inflammation, maintaining gut homeostasis [84]. Interestingly, some of these immunomodulatory proteins, including SlpB and SlpE, were recently identified in association with extracellular vesicles produced by the strain *P. freudenreichii* CIRM-BIA129, which serve as an alternative export system [85].

In addition to surface proteins, DHNA was also associated to immunomodulation. Beside its bifidogenic properties, DHNA inhibited the production of proinflammatory cytokines in intestinal macrophages of IL-10(−/−) mice treated with piroxicam [86]. Moreover, DHNA was also described as an activator of aryl hydrocarbon receptor (AhR), which is involved in the detoxification of xenobiotics and inflammation regulation [87, 88].

10. Functional foods

Importantly, the immunomodulatory properties of *P. freudenreichii* were preserved when food matrices were used as delivery vectors, including cheese [18, 19, 42, 89] and fermented milk [90–92], indicating a great potential for developing probiotic-based functional foods with immunomodulatory properties. As an example, a dairy product fermented by strain CIRM-BIA129 reduced the secretion of pro-inflammatory cytokines by colonic mucosa, improved food intake and growth of

piglets [92]. *P. freudenreichii* CIRM-BIA129 was also employed in the production of an immunomodulatory single-strain cheese, whose consumption by mice ameliorated colitis induced by TNBS, restoring the expression of tight-junction proteins and reducing the expression of markers of inflammation and of oxidative stress [89]. Similar protection, in the same colitis model, was observed using a two-strain model cheese containing *P. freudenreichii* and *L. delbrueckii* [20]. An industrial Emmental cheese was then produced using *S. thermophilus, P. freudenreichii* and *L. delbrueckii* [18] (**Figure 3**). Its consumption protected mice against colitis induced by DSS [18]. In healthy piglets, the consumption of the same CIRM-BIA129 strain associated to a cheese matrix was crucial in the preservation or enhancement of the immunomodulatory properties of the bacterium, including the induction of Th2 and Treg phenotypes [19]. The importance of the cheese matrix was also related to the protection of immunomodulatory protein SlpB against proteolysis in simulated gastrointestinal tract conditions [42]. These examples unveiled how appropriate food matrices protected or enhanced the beneficial properties of these traditional dairy propionibacteria, while establishing perspectives for the design of novel functional foods [91].

11. Safety assessments

The long history of safe production of fermented food, such as Emmental cheese, and the bacterium status of "generally recognized as safe" (GRAS) and "qualified presumption of safety" (QPS) assure the safety of *P. freudenreichii* consumption [5, 93]. However, additional assessments need to be conducted in different matrices and contexts. Probiotics included in humans trials are most frequently from genus *Lactobacillus* or *Bifidobacterium*; nevertheless, propionibacteria have also been tested [93]. For example, two clinical studies evaluated *P. freudenreichii* ET-3 culture medium safety in human adult subjects, the first one reported no differences in gastrointestinal symptoms between the groups and the other one reported differences in hematological parameters, although within the normal ranges [94]. *P. freudenreichii* strains SI 26 and SI 41 were given to adult healthy human volunteers without adverse effects, while a modulation of fecal bifidobacteria and of segmental colonic transit was observed [67]. In another study, *P. freudenreichii* strain SI 41 was given in capsules at the same dose to human volunteers without adverse effects, while an increase in fecal propionibacteria, concomitant with enhanced short chain fatty acids, was observed [95].

Moreover, several clinical trials tested multispecies probiotic supplementation containing propionibacteria. A complex formula that included *P. freudenreichii* JS, together with *L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* 99, and galactooligosaccharides prebiotics has been tested in several randomized, double-blind, placebo-controlled setups. The probiotic intervention was conducted in pregnant women and newborn infants, being safe and effective in the prevention of atopic eczema in children [96], increased children resistance to respiratory infections [97], protected Cesarean-delivered children from IgE-associated allergic disease [98], restored microbiota composition in children treated with antibiotics or born by cesarean procedure [99] and protected Cesarean-delivered children from allergic disease in a 13-year follow-up [100]. Finally, an integrative study analyzed adverse events associated with this probiotic combination in some of these trials, concluding that there was no association with adverse events in young and elderly subjects [101].

Importantly, probiotics supplementation is not recommended in cases of immunosuppression, such as during anticancer treatment [93]. Moreover, their beneficial effects and safety are conditioned to a complex interplay between peculiarities

of the host and of the probiotic strain or strains, which both encourages further research and suggests caution in some of its applications [93].

12. Postbiotics and beyond

As previously detailed, *P. freurenreichii* probiotic effect has been associated to several factors, including cytoplasmic and surface-exposed proteins [52, 77, 82, 83], short chain fatty acids [62, 63], metabolites [71, 72, 75] and culture supernatants [58, 94] (**Figure 4**). These probiotic-derived factors, which exert a beneficial effect on the host, have been referred as postbiotics [102]. "Postbiotic" is an emerging denomination that encompasses probiotic-derived cell-free metabolic products with health-promoting properties, including proteins, lipids, organic acids, vitamins, supernatants, among others [102–104]. The advantages of postbiotics over probiotics include purity, easy production and storage, industrial scalability, higher specificity in the mechanism of action and less adverse effects [102, 103].

In the case of *P. freudenreichii*, a remarkable example of postbiotic is SlpB protein, which was purified and exerted an immunomodulatory effect, i.e. induction of IL-10, in cultured human intestinal epithelial cells [83]. Another example that would fit into postbiotics definition is extracellular vesicles, which are membranous spherical nanostructures that transport molecules between cells [105, 106]. In probiotic bacteria, such as several *Lactobacillus* and *Bifidobacteriaum* strains, extracellular vesicles have been reported as immunomodulatory [107]. In the case of *P. freudenreichii*, we recently described their production by the strain CIRM-BIA129, which has been the first report with physicochemical, proteomic and functional characterization of extracellular vesicles in the species [85]. We identified relevant proteins in their cargo, including SlpB, and demonstrated their anti-inflammatory activity via the modulation of NF-κB pathway in cultured human intestinal epithelial cells [85].

Postbiotics hold promising perspectives for developing novel probiotic-derived products with enhanced safety and functionality [107]. Moreover, yield and cargo loading optimization are promising for modulating their properties, enhancing their beneficial effect and biotechnological applications [108]. Finally, clinical trials should be conducted in the near future to assure the

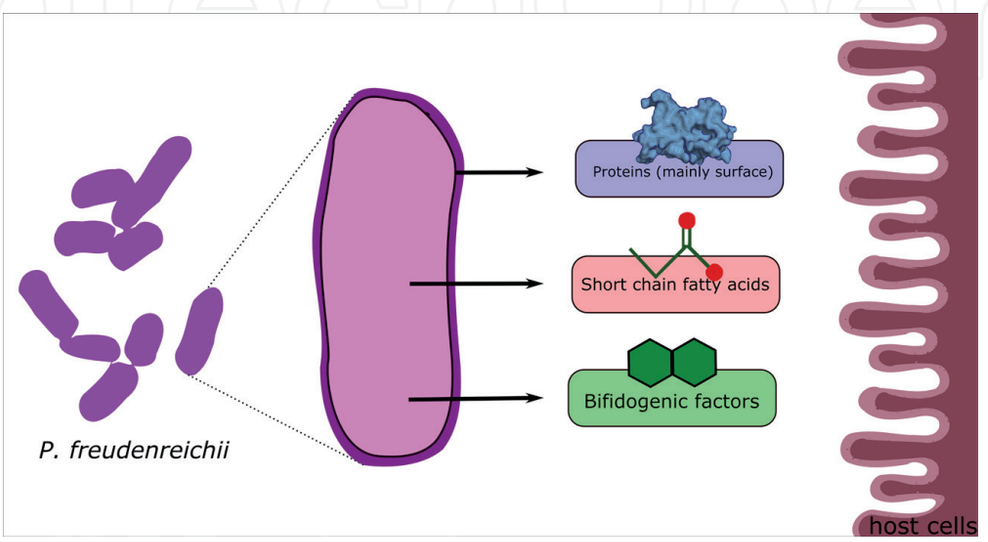


Figure 4.
Schematic summary of P. freudenreichii *probiotic traits at the molecular level.*

suitability of postbiotics and probiotics for therapy and prophylaxis, since they might exert a great impact in human health [93, 107, 109].

13. Conclusion

Overall, research on *P. freudenreichii* is consolidating its role as a probiotic, due to several outstanding features, such as the tolerance to stresses encountered in the gastrointestinal tract, adhesion to host cells, the anti-pathogenic activity, the anticancer potential and the immunomodulatory properties. Moreover, this species holds technological importance, due to long-established applications in the production of food, vitamin B12 and organic acids. Therefore, this is a promising 2-in-1 bacterium, with both fermentative and probiotic properties. New research on *P. freudenreichii* should allow the development of novel health-promoting fermented foods and should dive further into the characterization of strain diversity and of corresponding properties, as well as employ omics approaches to dissect the molecular mechanisms of its beneficial properties. Studies on this species hold a great potential for the development of novel technological approaches and therapeutic products directly impacting human health.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Vinícius de Rezende Rodovalho[1,2], Diego Lucas Neres Rodrigues[1], Gwénaël Jan[2], Yves Le Loir[2], Vasco Ariston de Carvalho Azevedo[1] and Eric Guédon[2*]

1 Laboratory of Cellular and Molecular Genetics, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil

2 INRAE, Institut Agro, STLO, Rennes, France

*Address all correspondence to: eric.guedon@inrae.fr

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