Review

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Review

Dairy Propionibacteria: Probiotic Properties and Their Molecular Bases

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Abstract: Dairy propionibacteria are commonly ingested through the consumption of raw milk cheeses or Swiss type cheeses in which they are added as starter cultures. Some strains of the species *Propionibacterium freudenreichii* or their culture media have been commercialized in multi-strain probiotic preparations or to supply bioactive substances such as short chain fatty acids, bifidogenic molecules and vitamins, respectively. In recent years, many more mechanisms of action of dairy propionibacteria as probiotics for different novel applications were discovered and are summarized in this descriptive review. Strains of *P. freudenreichii* mitigated inflammatory bowel diseases (IBDs), mucositis and prevented necrotizing enterocolitis (NEC) in preterm newborns. Moreover, these bacteria exerted immunomodulation, particularly in food allergy, anti-obesity, anti-diabetic, anti-cancerogenic effects, inhibition of osteoclastogenesis in rheumatoid arthritis, and infection mitigation in animal models. Most of the observed effects were mediated by cell surface proteins or extracellular vesicle (EV) proteins such as the surface layer (S-layer) protein SlpB, DlaT and GroEL. Based on the available information, these bacteria do not present safety issues but investigations on the presence of transferable antibiotic resistance traits should be specifically assessed both phenotypically and genotypically. In most possible applications the confirmation of beneficial effects in clinical trials still need to be carried out to allow their use as health promoting agents.

Keywords: Dairy propionibacteria; *Propionibacterium freudenreichii*; *Acidipropionibacterium* species; disease mitigation; immunomodulation; short chain fatty acids; S-layer proteins; extracellular vesicles; beneficial metabolites; safety

1. Introduction

Dairy propionibacteria are a taxonomically heterogeneous group of actinomycetes, i.e. high G+C Gram-positive bacteria, associated to food and feed environments that belong to the genera *Propionibacterium* and *Acidipropionibacterium*. There two genera were previously both classified as *Propionibacterium* that comprised both food associated species and cutaneous species of clinical significance. The currently recognized species associated to food products are *Propionibacterium freudenreichii*, *Acidipropionibacterium acidipropionici*, *A. jensenii*, *A. thoenii* and the less frequently isolated *P. cyclohexanicum*, *A. microaerophilum*, *A. damnosum*, *A. olivae*, *A. virtanenii*, and *A. timonense*. The species *P. freudenreichii* was previously separated into the subspecies *freudenreichii* and *shermanii* on the basis of lactose fermentation and nitrate reducing ability, a distinction no more valid since whole genome sequencing highlighted that the first trait was encoded by an integrative-conjugative element (ICE) acquired by horizontal gene transfer (HGT), while the second trait was lost in intra-species groups following a frameshift mutation [1-4].

Dairy propionibacteria are rod shaped, anaerobic or microaerophilic aerotolerant for their endowment of enzymes that protect against oxidative stress such as a superoxide dismutase, a

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catalase and a cytochrome *bd* oxidase. Moreover, they express heme biosynthesis proteins and functional electron transport chains and some strains are able to grow in aerobic conditions. Their central metabolism leads to the production of propionate, acetate and CO_2 from lactate through the Wood-Werkman cycle, involving succinate decarboxylation, and they can utilize different carbohydrates, including mannose, arabinose, and xylose, and glycerol as carbon sources [5 - 7]. *P. freudenreichii* typically produces SCFAs mixtures in which the amount of propionate produced is typically at least double compared to acetate [8,9].

These bacteria have been isolated from milk, acid whey, different traditional cheeses, feed flour and silage mixed or not with grains, soil, fermented vegetables, barley grains, goat rumen, chicken intestine and fecal samples from breast-fed preterm infants [9-15]. Moreover, one study indicated that they could survive the transit in the gastrointestinal tract when supplied in cheese [16]. Dairy propionibacteria, mainly *P. freudenreichii,* have an essential role in the Swiss Type cheese technology in which they allow to obtain the distinctive "eyes", given by CO_2 accumulation during ripening, and the production of flavors. In other cheese types these bacteria are unwanted since they can cause defects such as anomalous blowing and colors so their presence in milk should be kept under control. However, only a few studies considered their distribution in milk and reported that their presence is very frequent, ranging between 50% and 95.6%/97% [17-19]. Carafa et al. reported an increase of the percentage of samples positive for dairy propionibacteria in milk from cows led to Alpine pastures compared to the milk of cows reared in permanent farms [20].

The relevance of dairy propionibacteria as probiotic organisms, i.e. "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [21] on human and animal health was documented by a body of research that dates back to the eighties according to a short review on the first findings from in vivo trials published in 1995. Until that time propionibacteria had been used as growth promoters for farmed animals such as calves, piglets and hens, mostly combined with lactic acid bacteria but also as pure cultures. Some *P. freudenreichii* were effective in increasing weight gain, decreasing feed conversion ratio and improving intestinal health. Though not adhering, *P. freudenreichii* showed a good survival rate in simulated gastrointestinal transit. Mixed cultures of dairy propionibacteria with bifidobacteria and lactic acid bacteria had shown anticholesterolemic and β-galactosidase activity and fermented milk containing two strains of *P. freudenreichii* and *Lactobacillus acidophilus* improved the overall health status in elderly people in a small-scale clinical trial. Other properties of propionibacteria were the capacity to stimulate the development of bifidobacteria and lactobacilli. At that time dairy propionibacteria were already industrially exploited to produce vitamin B12 and propionic acid [22].

Dairy propionibacteria are among the bacterial species involved in dairy technology and able to exert health promoting effects in vivo [23]. According to the latest review article regarding their probiotic properties published in 2017, *P. freudenreichii* and *A. acidipropionici* transiently survive and maintain metabolic activity in the gastrointestinal tract, and adhere to intestinal cells. These two species of dairy propionibacteria favor the increase of intestinal bifidobacteria by producing the bifidogenic molecules 1,4-dihydroxy-2-naphtoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ), and also induce a decrease of toxin producing Bacteroides and *Clostridium difficile*. Moreover, *P. freudenreichii* selected strains exerted immunomodulation with anti-inflammatory effects in human cell-lines and in mice with induced colitis or mice fed on a high fat diet (HFD). The in vivo beneficial effects of *P. freudenreichii* were shown to be mediated by surface-layer proteins (Slps). In addition, *P. freudenreichii* favored the apoptosis in human cancer cell lines and in a mice tumor model for the ability to produce short chain fatty acids (SCFAs) propionate and acetate [24].

Currently, *P. freudenreichii* and *A. acidipropionici* are included in the list of microbial species with qualified presumption of safety (QPS) status for use in food and feed of the European Food Safety Authority (EFSA). Therefore, based on the taxonomic identification, body of knowledge and potential safety concerns, representatives of these species must not undergo a full safety evaluation process and must only satisfy the requirement of not harboring "any acquired antimicrobial resistance genes

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to clinically relevant antimicrobials" before use in food and feed [25]. In addition, the species *P. freudenreichii*, *A. acidipropionici*, *A. jensenii* and *A. thoenii* are included in the list of the microbial species with safety demonstration by the International Dairy Federation (FIL-IDF) [26].

This descriptive review summarizes the state-of-the-art of knowledge on the beneficial effects of dairy propionibacteria on disease prevention and mitigation and their molecular bases to obtain a complete and up-to-date overview of their probiotic potential. To this end, scientific articles regarding the evaluation of the probiotic properties of dairy propionibacteria were sought in the databases GoogleScholar (https://scholar.google.com/schhp?hl=it, accessed on 25 January 2025) and Embase (https://www-embase-com.bibliosan.idm.oclc.org/, accessed on 25 January 2025) by relevance sorting with the search strings "*Propionibacterium* probiotic" or "*Acidipropionibacterium* probiotic". For the GoogleScholar search results 80 pages were screened to select pertinent titles since two subsequent pages did not contain any further items relevant to the study. In Embase 705 sources were retrieved and evaluated for pertinence. The articles finally selected after elimination of duplicates, abstracts screening and critical judgment of originality and novelty are commented in this review. Among articles regarding probiotic mixtures, only those indicating distinct actions of propionibacteria were considered.

2. Molecular Traits of Dairy Propionibacteria Relevant for Probiotic Activities

Dairy propionibacteria are rather understudied because of technical difficulties in their isolation, due to the long incubation times of up to seven days, poor selectivity of the isolation media [17,19], and difficulties in the outcomes of genetic tests determined their high G+C genome content higher than 67% [27]. Indeed, for polymerase chain reaction (PCR) tests and sequencing the addition of reagents that facilitate strand and secondary structure dissociation are needed for these bacteria [28]. Whole genome sequences became available rather recently for these bacteria with 125, 19, 11 and 1 annotated sequences for *P. freudenreichii*, *A. jensenii*, *A. acidipropionici*, and *A. thoenii*, respectively, available in GenBank [https://www.ncbi.nlm.nih.gov/nuccore, accessed on 24 January 2025].

A recent study used the *P. freudenreichii* species as a representative of high G + C genome content organisms to evaluate the performance of different long-read sequencing methods in providing complete sequence data, and highlighted the necessity to adopt appropriate sequencing strategies to obtain optimum completeness and contiguity, correct assemblies, and correct gene content determination from these bacteria. For three *P. freudenreichii* strains, TL19, TL29 and TL110, the genome dimension varied even for the same strain depending on the sequencing technique and assembling method adopted and ranged between 2,497,808 bp and 2,566,603 bp, while the number of coding sequences (CDS) was comprised between 2158 and 3236 [29].

The largest study on the genome features of dairy propionibacteria was carried out for 20 strains of *P. freudenreichii* isolated from milk, barley and cheese by using the long read sequencing with the PacBio RS II sequencing platform. Eight strains showed an additional genome deriving from duplication driven by transposable elements. The presence of these transposable elements, ICEs and prophages, the number of accessory and unique genes varied among the strains and inversions or other rearrangements were observed though the level of co-linearity was high. The composition of the accessory genome highlighted variability in probiotic properties, stress tolerance and safety. Variable genomic islands encode a pilus locus, rhamnose utilization, heat shock proteins, immunity related clustered regularly interspaced short palindromic repeats Cas (CRISPR-Cas) systems, restriction/modification systems, resistance to heavy metals and putative antibiotic resistance determinants. Some of these regions were probably acquired by horizontal gene transfer from *A. acidipropionici*. Putative plasmids were detected only in two strains but these lack a replication origin. Among the S-layer protein genes involved in the anti-inflammatory properties of *P. freudenreichii*, *slp*A and another *slp* gene were present in all the strains, but on genomic islands in some of them, the S-layer protein precursor *ctc*, *slp*E, and *slp*B were present in 13, 12 and 2 strains, respectively [2].

All the strains harbored the gene cluster for the production of vitamin B12 that comprises 33 genes and only in one strain some genes of the cluster showed mutations. The three riboswitches that

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regulate this gene cluster were also conserved. Pilus genes were found in all the strains and encode putative subunits of fimbriae anchored to the cell surface, fimbriae major subunits and a putative sortase C. However, the number of genes and gene sequence varied and only one strain showed pilus appendages in electron microscopy observation and exhibited mucus binding capacity [2].

The production of exopolysaccharides (EPS), which is commonly considered a trait involved in adhesion and immunomodulation [30], was analyzed in 68 *P. freudenreichii* strains and found to vary. PCR tests showed that all the strains carried a glucosyltransferase *gtf* gene homologous to the *ttf* gene responsible for EPS production in *Streptococcus pneumoniae*. However, only 24 strains agglutinated when exposed to a *P. freudenreichii* EPS specific antiserum. Optical and electron microscopy showed that in some of the agglutinating strains a capsule was present. The Gtf protein was highly conserved among strains with changes only in six amino acid positions. Its disruption in the *P. freudenreichii* type strain hindered EPS production and its heterologous introduction in *Lactococcus lactis* IL1403 conferred a polysaccharide production capacity to this strain, thus showing that *gtf* is the only gene required for EPS production in *P. freudenreichii*. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) highlighted that different levels of EPS production were correlated with the *gtf* transcription level. This was between 18 and 264 higher in agglutinating and a number of *gtf* transcript copies of at least 10^7/100 ng of RNA conferred the capsular phenotype. The presence of two putative transposase genes 194 upstream to the *gtf* coding region could be responsible for the overexpression of *gtf* in the agglutinating strains [31].

It was later reported that the presence of a capsule hinders the immunomodulation effects of *P. freudenreichii* by masking the surface molecules responsible for the immune response induced. About 30% of the strains of this species produce EPS constituted by (1→3,1→2)-β-D-glucan. Strains with an inactivated *gtf*F gene showed a better biofilm formation capacity than the respective wild types but not enhanced adhesion to Caco-2 cells. The wild type strains were able to induce the anti-inflammatory interleukin IL-10 production by these cells but this property was enhanced in the Δ*gtf*F mutants. The presence of the EPS did not increase the survival of *P. freudenreichii* in the mouse gastrointestinal tract [32].

A study on the cell surface proteins obtained by guanidine hydrochloride extraction from *P. freudenreichii* CIRM BIA 129 (ITG P20) indicated the presence of the internalin A (InlA), S-layer proteins SlpA, SlpB and SlpE, the large surface protein A LspA, and proteins involved in adhesion (BopA), penicillin binding, solute binding, resuscitation (RpfB factor) and cytoplasmic proteins among which heat shock proteins and translation/elongation factors. Shaving with trypsin retrieved a subset of the proteins non-covalently bound to the cell surface among which the S-layer proteins. SlpB was the most abundant protein present in the guanidine hydrochloride extracts and its molecular mass was experimentally determined to be 54,147 Da. The surface protein extract induced a dose-dependent release of the immunomodulatory cytokines IL-10 and IL-6 by peripheral blood mononuclear cells (PBMCs) [33].

Mono-dimensional electrophoresis (1-DE) of proteins secreted by 27 strains of *P. freudenreichii* and *A. acidipropionici* showed different patterns for strains of dairy origin and those of cereal origin. Among the secreted proteins and with variability at the strain level a 27-kDa transglycosylase was in common between the two genera. Moreover, SlpA, RpfB, and a NlpC/P60 family peptidase were the most abundant secreted proteins. In addition, stress-response proteins and central metabolism enzymes were detected with differences in identity among the strains. Beyond the above listed proteins, two-dimensional electrophoresis (2-DE) followed by fluorescent staining allowed to identify a d-alanyl-d-alanine carboxypeptidase involved in peptidoglycan synthesis, and the lipoprotein OppA, involved in oligopeptide transport, more abundant in the dairy strains, InlA, and a metallo-endopeptidase M23B. Proteins involved in adhesion, such as a fimbrillin subunit FimB, were detected for the cereal strain *P. freudenreichii* JS14. This strain showed the capacity to adhere to a hydrophobic material and bovine serum albumin (BSA), while the dairy strain *P. freudenreichii* JS22 showed minimal adhesion and a clumping phenotype. None of the strains studied adhered to mucus [34].

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Among the surface proteins of *P. freudenreichii* JS14 SlpB and the aminopeptidase N (PepN) were specifically identified [34]. The gene encoding the latter protein is present in the genomes of all the dairy propionibacteria type strains but *A. microaerophilum* was found to harbor two paralogs of *pep*N [35] and the genome sequences made available later show the same also for *A. acidipropionici* [https://www.ncbi.nlm.nih.gov/nuccore, accessed on 20 February 2025]. For *P. freudenreichii* JS22 a putative arabinose isomerase AraI, the Clp family ATPase ClpB and LspA predominated among the secreted proteins [34].

A *P. freudenreichii* CIRM BIA 129 *slp*B deletion mutant (Δ*slp*B) showed reduced cell surface electronegativity and reduced hydrophobicity determined as adhesion capacity to hydrocarbon solvents xylol, chloroform, and ethyl acetate. Moreover, the Δ*slp*B mutant showed reduced tolerance to acidic, bile salts and heat stress. Proteome analysis identified 27 proteins expressed only in the mutant strain and involved in metabolism, replication, recombination and repair, while a carboxylic ester hydrolase was expressed only in the wild type strain. The downregulated proteins in the Δ*slp*B mutant included an exported enolase known to be involved in immunomodulation and adhesion, and the surface-layer (S-layer) proteins SlpA, SlpD and SlpE and InlA [36].

The anti-inflammatory properties of *P. freudenreichii* were first demonstrated for the strain JS that reduced the expression of the pro-inflammatory interleukin IL-8 in Caco-2 cells infected with *Helicobacter pilori* and increased the expression of the anti-inflammatory cytokine IL-10 in PBMC. This effect was found to be strain-dependent since only *P. freudenreichii* CIRM BIA 129 among the 23 strains studied by Deutsch et al. [32] induced this cytokine at levels comparable to the anti-inflammatory probiotic *Bifidobacterium longum* BB536. Varying with the strain, the pro-inflammatory cytokines interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) were very weakly induced and no induction of interleukine IL-12 occurred. Shaving of *P. freudenreichii* cells with guanidine hydrochloride highlighted diversity in surface protein composition and a total number of 174 proteins unique for single strains. Proteins SlpA, SlpB, SlpE, LpsA containing the surface-layer homology (SLH) domain for anchorage to the cell wall, and a protein with glucosaminidase domain, were found in one or more strains [32].

Genomic, transcriptomic and surface proteome indicated respectively 158, 161 and 11 genes positively associated with IL-10 induction, with SlpE identified with all three data sets. Among fifteen genes with the strongest positive or negative association with IL-10 induction, the *slpB*, *slpE*, *slpF*, *hsdM3*, *pep*, *htrA4*, *eno1* and *pouf* were successfully inactivated in different strains so it could be demonstrated that the inactivation of *slpB, slpE* and *hsdM3* in *P. freudenreichii* CIRM BIA 129 induced a remarkably lower secretion of IL-10 by PBMCs, the inactivation of *slpB, slpF* and *pouf 10925* reduced the secretion of tumor necrosis TNF-α and IFN-γ, and the inactivation of *slpE* reduced the secretion of TNF-α only. Inactivation of *eno1, htrA4* and *pouf 08235* in the weakly anti-inflammatory strain CIRM BIA 121 increased the secretion of IL-10, TNF-α and IL-6 [37].

The SlpB protein is also involved in adhesion of *P. freudenreichii* to human intestine epithelial cells HT29. This protein was detected in different amounts in four among seven *P. freudenreichii* strains tested for adhesion capacity. The *P. freudenreichii* CIRM BIA 129 strain showed highest adhesion rate and strain CIRM BIA 118, also expressing SlpB, showed about half its adhesion rate. The strain with the lowest adhesion rate did not express S-layer proteins. The *P. freudenreichii* strains were not internalized in the intestinal cells and scanning electron microscopy highlighted their localization on the brush border. The adhesion capacity was lost after enzymatic shaving with trypsin and after deletion of the *slp*B gene [38]. In HT29 cells *P. freudenreichii* CIRM BIA 129 or its S-layer proteins attenuated the upregulation of the pro-inflammatory interleukins IL-8 and IL-12 and TNF-α induction by the *E. coli* lipopolysaccharide (LPS). Moreover, the *P. freudenreichii* cells induced an upregulation of IL-10 both in presence and absence of LPS, while and its Slps stimulated the increase of the tumor growth factor-β (TGF- β) [39].

A Δ*slp*B mutant of the strain *P. freudenreichii* CIRM BIA 129 showed decreased adhesion compared to the wild type to intestinal epithelial barrier (IEB) in vitro models constituted by Caco-2 cell monolayers. Moreover, after inflammation induction, it did not reduce the trans-epithelial

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electrical resistance (TEER) and did not prevent the paracellular permeability to sulfonic acid-FITC, and the transcellular permeability to horse radish peroxidase (HRP) as the wild type strain. Western blotting highlighted that, differently from the wild type, it did not prevent the decrease of the zonula occludens 1 (ZO-1) protein expression. The organic acids produced by *P. freudenreichii* CIRM BIA 129 and its Δ*slp*B mutant did not induce significant changes on inflammation-induced permeability thus proving the essential role of SlpB in mitigating the inflammation induced effects [40].

Extracellular vesicles (EVs) are produced by cells of most organisms and are nanoparticles delimited by a lipid bilayer membrane that contain cell proteins, nucleic acids, and metabolites. EVs are implicated in the transfer of virulence and antibiotic resistance genes but also in the interaction of probiotic microorganisms with the host cells. Beneficial effects exerted by EVs were observed for the probiotic bacteria *Lacticaseibacillus paracasei* BL23 and *rhamnosus* GG, *Lactiplantibacillus plantarum* WCFS1 and *Bifidobacterium longum* KACC 91563. The EVs produced in ultrafiltered (UF) milk by *P. freudenreichii* CIRM BIA 129 were spherical with a diameter of about 84 nm, and found to contain 319 proteins representing 11% of the whole cell proteome. The proteins found in EVs were involved in energy metabolism, carbohydrate and amino acid transport, translation, ribosome structure and formation, cell envelope synthesis, protein turnover and signaling. Proteins involved in immunomodulation such as the enolase EnoI, the internalin InlA, the aconitase Acn, the glutamine synthetase Gln1, the glucose-6-phosphate isomerase Gpi, the triosephosphate isomerase Tpi, the surface-layer proteins SlpB and the chaperonin GroEL were detected in EVs [41].

The in silico prediction of protein-protein interactions of *P. freudenreichii* EV-associated proteins machine learning-based (interSPPI, http://zzdlab.com/intersppi/index.php, accessed on 30 March 2025) and homology-based (interolog), highlighted interactions with human proteins involved in metabolism, signal transduction, infectious diseases and immunity. Among these, the p105 subunit of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and members of its signaling pathway, such as adapters and Toll-like receptors, showed the highest number of interactions. A dose-dependent effect of *P. freudenreichii* EVs on the reduction of NF-κB activation was experimentally demonstrated in HT29/kb-seap-25 cells in which an inflammatory response was induced with the *E. coli* LPS but not in those treated with the inflammation inducers TNF-α and IL-1β. In addition, *P. freudenreichii* EVs induced a dose-dependent reduction of the pro-inflammatory cytokine IL-8 [41].

EVs produced in UF milk by *P. freudenreichii* CIRM BIA 129 more efficiently inhibited the nuclear factor NF-kB regulatory activity in inflammation induced by *E. coli* LPS in HT29 cells than those formed in yeast extract lactate medium (YEL). The two EV types shared 358 proteins but the UF milk EVs also contained proteins involved in carbohydrate and amino acid metabolism, DNA processing and immunomodulation targets in the NF-kB signaling pathway [42]. In addition, proteins involved in the production of secondary metabolites, metabolism in different environments, oxidative phosphorylation, peptidoglycan synthesis, ribosome composition, protein export, quorum sensing and proteins SlpB, SlpE, EnoI, aconitase Acn, GroEL2 and a membrane hypothetical lipoprotein, previously shown to participate to the immunomodulatory properties, were identified [43].

EVs produced by *P. freudenreichii* CIRM BIA 129 contain a correctly conformed SlpB protein. Those purified by size exclusion chromatography inhibited the increase in paracellular permeability to sulfonic acid-fluorescein isothiocyanate (FITC) in a cell monolayer, thus confirming the role of SlpB in mitigating the inflammation-induced changes [40].

3. In vivo Beneficial Effects of Dairy Propionibacteria

Dairy propionibacteria were shown to exert a multiplicity of health promoting effects in animal models for the prevention and treatment of different conditions including intestinal inflammations, colorectal cancer, bone diseases and infections. The best studied species was *P. freudenreichii* for which different strains were tested in vivo for the molecular mechanisms of action [14,44-71].

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The capacity to exert in vivo beneficial effects is determined by the ability of dairy propionibacteria to survive during transit in the gastrointestinal tract (GIT). In particular, *A. jensenii* 702 was fed to rats and retrieved from feces in high numbers [72] and the strain *P. freudenreichii* JS was recovered in high numbers from the feces of healthy subjects who consumed fruit juice enriched with whey containing this bacterium though gut colonization was transient [73]. Use of the *in vitro* dynamic DIDGI® digestion system allowed to observe that *P. freudenreichii* CIRM BIA 129 included in a cheese matrix did not lose viability during the gastric and small intestine phases and remained viable at high levels until the end of the process. After in vitro static digestion, sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) separation and Western blotting allowed to observe that SlpB remained intact until the end of digestion of cheese containing *P. freudenreichii*, while it was degraded after the gastric phase when the strain was supplied in UF milk. In the dynamic digestion system SlpB remained intact for longer in the duodenum phase when supplied with cheese compared to UF milk indicating that the assumption of *P. freudenreichii* with cheese favors the preservation of its probiotic properties [39].

The type strains of *P. freudenreichii*, *A. acidipropionici*, *A. jensenii* and *A. thoenii* adhered to normal cells of the ileal mucosa IPEC-J2 at rates comparable to *L. reuteri* 12002 used as positive control with *P. freudenreichii* showing the highest adhesion percentages enhanced in the presence of calcium. This finding was in accordance with some, though not all, results from previous studies. The *A. acidipropionici* strain showed the lowest viability loss at pH 2.5 since its decrease was negligible after 24 h, while the other species showed a viability loss of about 30% after 24 h. None of the strains showed a significant reduction after 3 h at this pH value [74]. However, literature data are not concordant, thus indicating strain-specificity for tolerance to low pH [75]. The tolerance to bile salts was also variable since high tolerance or high sensitivity was reported to 0.3% (w/v) of these compounds that could be explained with not uniform testing conditions [74]. Ibrahim et al. [74] observed that some strains were able to survive in presence of 1% bile salts for 48 h with little viability loss.

Growth promotion experiments in farm animals highlighted the beneficial effects of dairy propionibacteria on the health status and their capacity to maintain viability in GIT. Use of *A. acidipropionici* P169 as direct-fed microbial (DFM) in Holstein calves post-partum allowed to increase milk yield in a dose dependent manner and reduce weight loss [76]. *A. jensenii* 702 was used as DFM in calves since it was previously reported that dairy propionibacteria induce the increase propionate and butyrate in the rumen increasing feed conversion and growth rate and it was recovered from feces ant about 5 Log CFU/g during the treatment period when administered in daily doses of 9 Log CFU/kg. The group of calves treated with *A. jensenii* 702 showed a faster weight gain and the difference was statistically significant until 18 weeks after treatment. No adverse effects of the supplied propionibacteria emerged from hematological values [77]. The same strain was supplied to layer chicken in doses of 7 Log CFU per day and determined an egg weight increase of 4.2% compared to the control and no adverse effects. The *A. jensenii* strain was proven to survive the transit in the chicken GIT [78]. *A. acidipropionici* LET105 and LET107 isolated from chicken intestine were administered to 1 – 14 days aged chicks daily in a 6 Log CFU dose with no adverse effects or difference in growth rate. The group of animals receiving the propionibacteria showed a better development of the intestinal mucosa with increased crypt length, goblet cell number and mucus production [13].

3.1. Prevention and Mitigation of Intestinal Inflammatory Diseases

Inflammatory bowel diseases (IBD) conditions are accompanied by intestinal dysbiosis, i.e. a less diverse and more unstable intestinal microbiota than in healthy subjects and a reduction of microbial components with an immunomodulatory role [50]. Therefore, one of the expected functions of probiotics is the re-establishment of microbiota components with beneficial roles and the reduction of those with detrimental effects. Among dairy propionibacteria, *A. jensenii* 702 has been used in mixed commercial probiotic preparations since it favored the increase of bifidobacteria [79]. An in vitro study showed that *P. freudenreichii* W200 (Winclove Probiotics, Amsterdam, The Netherlands)

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was the only probiotic that did not increase *Bacteroides-Prevotella* spp. in fecal cultures and induced a higher production of lactate among the SCFAs compared to probiotic strains belonging to the species *Bacillus coagulans*, *B. subtilis*, *Levilactobacillus reuteri* and *Lacticaseibacillus rhamnosus* [80].

In murine colitis induced with sodium dextran sulfate (DSS), the *P. freudenreichii* DHNA metabolite attenuated the expression of the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) that favors leucocyte infiltration, and determined a lower colon mucosa damage score reduction in the number of β7 integrin positive cells that mediate the adhesion of leucocytes to vascular endothelial cells, significantly suppressed mRNA levels of IL-1β, IL-6, and TNF-α, and significantly increased the number of lactobacilli and the amounts of SCFAs [45].

One mechanism of colitis inhibition observed for *P. freudenreichii* ET-3 involves the aryl hydrocarbon receptor (AhR) transcriptional factor that recognized xenobiotics and is involved in their detoxification by activating genes with a xenobiotic-responsive element (XRE) consensus sequence in the promoter. It was also reported that the activation of AhR-regulated pathways in gut by agents such as some probiotic bacteria, suppresses IBDs. A screening of metabolites produced by probiotic bacteria for AhR-regulated pathways activation identified the DHNA derived from *P. freudenreichii* ET-3 as a suitable substance able to increase the transcription level of the cytochrome P450 family 1 subfamily A member 1 (CYP1A1) induced by AhR in Caco2 cells without affecting their viability. This effect occurred also in vivo, mainly in the upper intestine of mice [46]. The Ahr-induced anti-microbial C-type lectins RegIII inhibit DSS-induced colitis and some members of this family increased in mice receiving DHNA. Finally, DHNA inhibited the production of IL-6 upon exposure to LPS in bone marrow macrophages without showing toxicity for these cells. Therefore, DHNA could represent a nontoxic Ahr activator to be exploited in colitis prevention [46].

The surface proteome of *P. freudenreichii* CIRM BIA 129 grown in a cheese comprising only this strain included as most abundant components identified by MS/MS the proteins SlpA, SlpB and SlpE, InlA, the chaperonins Hsp20, GroEL1 and groEL2 previously shown to be involved in immunomodulation by *P. freudenreichii*. Administration of the cheese to mice with 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis significantly reduced the body weight loss, disease scores and the inflammation marker IL-6 in blood. The attenuation of colon inflammation was indicated by the increased expression of *Pparγ* encoding the peroxisome proliferator-activated receptor γ. Attenuation of the oxidative stress induced by TNBS in the colon was indicated by the decreased expression of the gene *cox2* for cytochrome c oxidase subunit 2. Moreover, the restoration of the intestinal barrier was indicated by an increased expression of the *Zo*1 gene for the zonula occludens-1, or occluding, a tight junction protein [47]. The increased expression of tight junction proteins counteracts the increased gut permeability occurring in IBDs such as ulcerative colitis (UC) and Crohn's disease (CD) that can lead to inflammation with further damages induced by IFN-γ and TNF-α and reduced expression of ZO-1 and Claudin-2 [40].

When *P. freudenreichii* CIRM BIA129 was supplied to mice with TNBS induced colitis in a cheese co-fermented with *Lactobacillus delbrueckii* subsp. *lactis* it enhanced the protective effect of the lactobacillus by reducing weight loss and the inflammatory signs, with expression levels of *IL-10* and *ifnγ* similar to healthy mice, and an increase of *Pparγ* expression. This cheese induced a decrease of oxidation markers cytochrome oxidase 2 (*Cox2*) and heme oxygenase 1 (*Hmox*) and protected the intestinal barrier by maintaining expression levels of *Zo1* similar to those of healthy mice. The effect on the intestinal microbiota was an increase of Rikenellaceae and a decrease of Ruminococcaceae. Based on previous reports, the increase of Rikenellaceae was considered a consequence of the inhibition of the NF-kB mediated inflammatory response [48].

P. freudenreichii CIRM BIA 129 reached levels of 9/10 Log CFU/g as single strain in an experimental cheese and in industrial Emmental cheese in which also *S. thermophilus* and *L. delbrueckii* were present. Both *P. freudenreichii* cheeses mitigated the weight loss and the disease activity index (DAI) in mice with DSS-induced colitis. Moreover, the industrial Emmental cheese reduced mucosa alterations and cell infiltration, increased the expression of ZO-1 and reduced the level of IL-6 and IL-17. Both *P. freudenreichii* cheeses decreased immunoglobulin IgA production in the small intestine

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indicating a lower disturbance of the intestinal barrier. Therefore, beneficial effects on intestinal barrier maintenance and innate immunity were demonstrated [49].

P. freudenreichii CIRM BIA 129 was used to ferment UF, skim milk or whole milk and administered to mice with DSS-induced colitis. Both *P. freudenreichii* fermented milks alleviated colon shortening and the decrease of goblet cell number and crypt length. However, only the fermented whole milk kept the disease score significantly lower than the positive control. In addition, the *P. freudenreichii* fermented milk reduced the increase in gut permeability caused by DSS determined as passage of radiolabelled diethylenetriamine pentaacetic acid (DTPA) in blood. The fermented milk preparations, and whole milk at a higher extent, decreased the expression of pro-inflammatory genes *il1b* and *il*6 and of the nitric oxide synthase 2 *nos*2, while the peroxisome proliferator activated receptor gamma *pparγ* was upregulated [50].

P. freudenreichii CIRM BIA 129 administered to mice in the form of fermented milk ultrafiltrate (MUF) prior to colitis induction with DSS decreased the disease indexes, bleeding and stool consistency but not weight loss. Gut permeability, measured by administering fluorescein-5,6-sulfonic acid (sulfonic acid-FITC) and determining its concentration in plasma, was significantly lower in the *P. freudenreichii* group. The intracellular permeability, measured by determining the activity of administered HRP in plasma, was also reduced in the *P. freudenreichii* group. Histopathological observation showed that in the *P. freudenreichii* group crypt length was not decreased [40].

Different metabolites produced by dairy propionibacteria are able to alleviate the symptoms of IBDs and the propionate produced by the Swiss-type cheese isolate *P. freudenreichii* ET-3 in whey cultures was the first proven to alleviate colitis induced by TNBS in rats [44]. Moreover, the SCFAs acetate, butyrate and, above all, propionate in *P. freudenreichii* KCTC 1063 culture supernatant at 10% (v/v) concentration was not cytotoxic for LS 174T goblet cells and stimulated the expression of MUC2 as ascertained by RT-qPCR and enzyme linked immunosorbent assay (ELISA). The intestinal mucus layer represents a physical barrier against pathogens and harmful chemicals that also protects from IBD. It is constituted by highly glycosylated *O*-glycoproteins called mucins that are secreted by the epithelial goblet cells. Among twenty mucins, the gel-forming mucin MUC2 is the most abundant in the human intestine [51].

When administered to rats with DSS-induced colitis, *P. freudenreichii* KCTC 1063 and its culture supernatant determined, respectively, a histological disease score equal to the negative control with no induced disease and less damaged crypt structure with lower leucocyte infiltration degree compared to the disease positive control. Both *P. freudenreichii* treated groups showed a number of goblet cells similar to the healthy control. The expression of MUC2 was not significantly different from the healthy control or higher for live *P. freudenreichii* and culture supernatant, respectively, according to RT-qPCR and immunohistochemistry (IHC), respectively. The body weight, initially decreased in all groups, later increased more rapidly in those treated with living *P. freudenreichii* and its culture supernatant that experimented less severe diarrhea and bleeding and had a normal colon length. Moreover, these groups presented lower levels of *TNF-α*, *IL-6*, and *IL-1β* in the distal colon and higher propionate and butyrate SCFAs fecal contents. After administration for seven days at a level of 8 Log CFU, *P. freudenreichii* remained at levels of 5 Log CFU/g of feces until the end of the experiment at day 29, thus indicating gut colonization [51].

In the genesis of ulcerative colitis (UC) the intestinal microbiota plays an important role by inducing changes in the intestinal mucosa and modulating pro- or anti-inflammatory cytokines. In UC harmful bacteria exceed beneficial bacteria in this microbiota and the equilibrium can be restored by supplying probiotics. *P. freudenreichii* B1 was compared with *Bifidobacterium bifidum* H3-R2 and *Clostridium butyricum* C1-6 for the effects in DSS-induced colitis mice model. This strain decreased colon shortening and the DAI as the other two probiotics, induced a similar decrease of the pro-inflammatory cytokines IL-8, IL-1β and TNF-*α* and increase of IL-10 in the colon. However, expression of the tight junction proteins occludin ZO-1and claudin-1 was enhanced for *P. freudenreichii* and *C. butyricum*. *P. freudenreichii* B1 induced a decrease in the level of TLRs 2 and 4 and

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an increase of TLR-5 as the other two probiotics but also an enhanced expression of R-spondin-3 (Rspo3) active in the repair of damaged tissues. *P. freudenreichii* and *B. bifidum* more efficiently downregulated of RHO kinase ROCK-1 and Axin2 that inactivate the Wnt/β-catenin pathway for the regeneration of the intestinal epithelium. *P. freudenreichii* and *C. butyricum* better preserved the diversity of the intestinal microbiota, not significantly different from the positive control group gavaged daily with mesalazine (5-AS). The genuses *Lactobacillus* and *Bifidobacterium* were enriched in all the probiotic groups to different extents. *P. freudenreichii* induced the most pronounced effect on the increase of SCFAs whose amount is negatively correlated with the level of *Escherichia-Shigella*, *Staphylococcus* and *Enterobacter* genuses and proinflammatory indexes and positively correlated with anti-inflammatory molecules including IL-10 and TLR-5 [52].

Mucositis is a severe condition occurring in cancer patients treated with radiotherapy or chemotherapy and is characterized by the inflammation of the small bowel with degeneration of enterocytes and goblet cells, infiltration of leucocytes in the lamina propria, increased production of mucus, atrophic villi and hypoplastic crypts. Symptoms are diarrhea and weight loss and therapy with antimicrobials and anti-inflammatory agents may be ineffective and not well tolerated so treatment with probiotics should be considered an alternative. *P. freudenreichii* CIRM BIA 129 and its ΔSlpB mutant were evaluated in vitro and in vivo for anti-inflammatory activities and the effects in mice with mucositis induced by the chemotherapy drug 5-fluorouracile (5-FU). In HT29 cells exposed or not to *E. coli* LPS The *P. freudenreichii* wild type strain induced IL-10, as the purified SlpB protein also did, and inhibited IL-8 induction by LPS and *ifnγ* and *tfna* expression. Moreover, it induced the toll-like receptor gene *tlr2* and repressed *tlr4*. The gene *tlr9* was less expressed in LPS-treated cells in presence of the *P. freudenreichii* wild type [51]. The *P. freudenreichii* wild type significantly reduced weight loss and protected the intestinal mucosa with a reduction of leucocyte infiltration and ulceration, partially restored height of villi and lower reduction of granular density in the Paneth cells. This strain reduced the gut permeability induced by 5-FU, based on the extraintestinal translocation of radiolabeled 99mTc-DTPA. Indeed, this the expression of the claudin-1 *cld1* gene was increased and the expression of IL-17a, IL-12 and IL-1β in the intestinal mucosa was reduced. These effects were not induced by the Δ*slp*B mutant [53].

Newborns are particularly susceptible to infections because of an immature immune system and an insufficient number of protective T cells. This risk is extremely high in preterm neonates who are exposed to inflammation induced by maternal pathogens that can lead to the deterioration of the intestinal microbiota and to the onset of neonatal necrotizing enterocolitis (NEC). The establishment of a protective intestinal microbiota stimulates the development of an immune system able to contrast enteric pathogens [53,54]. Breast milk reduces the risk of NEC by introducing beneficial bacteria that stimulate immune regulation. Indeed, a metagenome analysis of the intestinal microbiota of 20 preterm newborns fed with breast milk and 20 preterm newborns fed with formula milk, showed a higher microbial diversity and balance in the first group and presence of Actinobacteria among which the genus *Propionibacterium*, represented by the species *P. freudenreichii*, predominated and increased during time. These bacteria were isolated and one strain was obtained, *Propionibacterium* P. UF1, that showed highest genome identity with *P. freudenreichii* DSM 20271. When administered to C57BL/6 mice, this strain transiently colonized gut for about six days but its colonization lasted longer in germ-free mice [14].

The transfer of the intestinal microbiota of breast-fed newborns to mice did not increase IL-1β in colon dendritic cells (DCs), while the microbiota from formula fed newborns increased this pro-inflammatory cytokine. In addition, the microbiota from breast-fed infants increased T helper 17 (Th17) cells and anti-inflammatory regulatory T cells (Tregs) expressing IL-10. Similar effects were observed in mice receiving the microbiota of formula fed newborns and gavaged with *P. freudenreichii* P.UF1. Treatment with the *Propionibacterium* isolate decreased the level of γ-proteobacteria. This strain alone did not cause an increase of IL-1β in colon tissues and DCs, while it induced an increase of Th17 cells, in particular those IL-10^{+}IFN-γ^{-}, and IL-10^{+} Tregs [14].

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Dihydrolipoamide acetyltransferase (DlaT) was the main component of guanidine hydrochloride extracts from the surface of *P. freudenreichii* P.UF1 able to induce IL-10$^+$ Th17 cells. This is a cytoplasmic enzyme of pyruvate decarboxylase complex. The major histocompatibility complex II (MHC II) was found to mediate the differentiation of Th17 cells from CD4$^+$T cells induced by *P. freudenreichii* in *MHC II*$^{+/+}$ mice since this differentiation did not occur in *MHC II*$^{-/-}$ mice. A Δ*dlaT* mutant of *P. freudenreichii* P.UF1 did not induce the differentiation of IL-10$^+$ Th17 cells in the mouse colon and complementation of this deletion mutant with three peptides of DlaT restored the capacity to induce differentiation of Th17 cells and the induction of IL-10$^+$ Tregs. The C-type lectin receptor (CLR) SIGNR1 recognizes bacteria from their surface composition and stimulate DCs to induce T-cell differentiation. SIGNR1 in the colon and DCs was upregulated in mice receiving *P. freudenreichii* P.UF1. The involvement of SIGNR1 in the attenuation of inflammation induced by *P. freudenreichii* P.UF1 was demonstrated by observing that mice in *Signr1*$^{-/-}$ infected with a *Listeria monocytogenes* strain in which the *act* gene was inactivated to avoid its systemic spread, differentiation of Th17 cells and induction of IL-10$^+$ Tregs did not occur, while it was observed in *Signr*$^{+/+}$ mice [14].

The colon microbiota of germ-free mice gavaged with *P. freudenreichii* P.UF1 was enriched with *Lactobacillus* and *Ruminococcus* genera, showing the ability of this strain to modify the intestinal microbiota and its metabolites. Since the intestinal microbiota of the mother influences the immunity system of the newborn, *P. freudenreichii* P.UF1 was administered to pregnant mice. The newborn from these mothers presented an increase in DCs expressing the transforming growth factor TGF-β and IL-10, of IL-10$^+$Th17 and IL-10$^+$ Tregs. In addition, weight and survival rate increased in the newborns submitted to NEC-like injury induced by gavage with a mixture of commensal bacterial from adults and exposure to hypoxia. In these newborns the transcription of the inflammatory inducible nitric oxide synthase *iNOS*, and interleukins *Il-1b*, *Il-6*, and *Il-23*, was reduced. Moreover, the innate lymphoid cells 3 (ILC3) expressing IL-17A and also IL-22, that protects from bacterial infections and mediates tissue repair, increased in the small intestine in newborn mice gavaged with *P. freudenreichii* P.UF1 [14].

P. freudenreichii P.UF1 was further tested for an effect in murine systemic infection with the strain *L. monocytogenes* 10403S with a mutation in the *inl*A gene that allowed to control the infection kinetics. Compared to mice gavaged with the Δ*dlaT* mutant of *P. freudenreichii* P.UF1 and later infected with *L. monocytogenes*, those receiving the wild type strain showed a reduction of IL-1β, IL-6, and IL-12/IL23p40 produced by DCs and Th1 cells producing IFNγ and an increase of Th17 cells and IL-10$^+$ Treg cells. Moreover, the latter showed an increase of intestinal bifidogenic bacteria. Cloning of three DlaT peptides in the *L. monocytogenes* and subsequent infection of mice with this strain showed similar immunomodulatory effects as observed in mice receiving *P. freudenreichii* P.UF1, thus confirming that DlaT was responsible for the increase of Th17 cells and IL-10$^+$ Treg cells. In mice receiving *P. freudenreichii* P.UF1 *L. monocytogenes* was cleared from feces and tissues. The same effects were not observed in *Signr1*$^{-/-}$ mice. Therefore, it was demonstrated that *P. freudenreichii* P.UF1 mitigated the inflammation induced by the pathogen both locally and peripherally [14].

The role of the Th17 cells induced by *P. freudenreichii* P.UF1 in mitigating *L. monocytogenes* infection was demonstrated by neutralizing the IL-17A produced by these cells with specific antibodies injected in the mice peritoneum. In this case the pathogen persisted also in mice that received *P. freudenreichii* P.UF1. Moreover, infection mitigation by *P. freudenreichii* P.UF1 failed in mice defective in the recombination-activating gene required for the generation of T and B cells. In the induced Th17 cells the upregulation of pathways for the production of extracellular matrix components involved in tissue healing and genes involved in immune regulation was observed by transcriptome analysis. Principal coordinate analysis (PCoA) of the intestinal microbiota showed that mice receiving *P. freudenreichii* P.UF1 had higher numbers of Lactobacillaceae and Clostridiaceae able to produce SCFAs and lower levels of *Prevotella* species. Moreover, vitamins B2, B5 and B9 were significantly increased, while the proinflammatory molecules prostaglandine E1 and 20-Hydroxyleukotriene E4 decreased [54].

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Table 1 summarizes the effects exerted by *P. freudenreichii* strains in vivo in intestinal inflammation models.

Table 1. In vivo beneficial effects of dairy propionibacteria in intestinal inflammations with indication of the bacterial strain, disease treated, animal host, beneficial effects and molecules involved.

Strain	Disease	Animal model	Active molecule	Induced effects	Reference
P. freudenreichii ET-3	TNBS-induced colitis	rats	Propionate	Ulcer healing	[44]
P. freudenreichii ET-3	DSS-induced colitis	mice	DHNA	Reduction of molecules that favor leucocyte infiltration and vascular adhesion, Downregulation of IL-1β, IL-6, and TNF-α, Increase of lactobacilli and SCFAs	[45]
P. freudenreichii ET-3	DSS-induced colitis	mice	DHNA	Activation of AhR-regulated pathways with increase of colitis suppressing C-type RegIII lectins	[46]
P. freudenreichii CIRM BIA129	TNBS-induced colitis	mice	Cell surface proteins	Reduction of disease scores, Attenuation of inflammation (increased expression of *Pparγ*) and oxidative stress (decreased expression of *cox*2 and *Hmox*), Restoration of the intestinal barrier (increased expression of Zo1), decrease of IL-1β, IL-6 and IL-17	[40,47-50]
P. freudenreichii KCTC 1063	DSS-induced colitis	rats	SCFAs	Reduction of disease scores, Reduced crypt damage, Reduced leucocyte infiltration, Maintenance of the mucin MUC2 expression level	[51]

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P. freudenreichii B1	DSS-induced colitis	mice	Not investigated	decrease of IL-8, IL-1β and TNFα, increase of ZO-1, claudin-1 and Rspo3, downregulation of the RHO kinase ROCK-1 and Axin2 that inactivate the Wnt/β-catenin regeneration pathway	[52]
P. freudenreichii CIRM BIA 129	mucositis induced with 5-FU	mice	SlpB	reduction of leucocyte infiltration and ulceration, restored height of villi, reduction of gut permeability (increased expression of *cld*1), decreased expression of IL-17a, IL-12 and IL-1β	[53]
P. freudenreichii P.UF1	NEC-like injury	Newborn mice	DlaT	Downregulation of nitric oxide synthase *iNOS*, and interleukins *Il-1b*, *Il-6*, and *Il-23*, Increase of ILC3 expressing IL-17A and IL-22	[14]
P. freudenreichii P.UF1	Infection with *L. monocytogenes*	Mice	DlaT	reduction of IL-1β, IL-6, and IL-12/IL23p40 produced by DCs, reduction of Th1 cells producing IFNγ and an increase of Th17 cells and IL-10$^+$ Treg cells	[14]

3.2. Immunomodulation

P. freudenreichii CIRM BIA 129 was selected among other ten strains for the anti-inflammatory effects in vitro. UF milk fermented by this strain administered to piglets improved food intake and growth rate. Moreover, it induced a decrease of IL-8 and TNF-α in the colon mucosa [8]. Supply of 11 Log CFU of *P. freudenreichii* CIRM BIA 129 in culture or in cheese determined the presence of this bacterium at about 7 Log CFU/g and the increase of bifidobacteria in piglet feces. After 14 days of administration total SCFAs and branched chain fatty acids (BCFAs) were significantly higher in the

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animals receiving *P. freudenreichii*. Slps from this bacterium increased IL-10 and reduced TNFα and IFNγ production in piglet PBMC and mesenteric lymph node immune cells (MLNC) in which inflammation was induced with *E. coli* LPS or concanavalin A. In MLNC cells *P. freudenreichii* supplied with cheese enhanced the expression of the regulators GATA binding protein 3 (GATA3) and forkhead box P3 (FoxP3) that have a role in immune tolerance [81,82].

Regardless of the method of administration, *P. freudenreichii* increased the ratio between anti-inflammatory Treg and pro-inflammatory Th17 cells. Ex vivo stimulation with LPS of PBMC from piglets treated with *P. freudenreichii* showed an increased secretion of IL-10, while stimulation with concanavalin A induced also an increased IFNγ secretion only in MLNC cells from the group treated with *P. freudenreichii* supplied in cheese. Importantly, IFNγ is responsible for the intestinal immune response against pathogens by macrophage activation and increase of Th1 cells [81]. The same strain was used to ferment sweet milk whey to obtain a functional food from a by-product. Supply of the *P. freudenreichii* fermented whey to piglets for 14 days did not influence growth or food intake but determined a significant increase of bifidobacteria in feces. MLNCs isolated after the treatment showed an upregulation of T-box expressed in T cells (T-bet) regulator of the Th1 response [83].

A. jensenii 702 isolated from raw cow milk in Australia was tested as adjuvant for an oral vaccine constituted by *Mycobacterium tuberculosis* H37Rv culture filtrate administered to rats. The *A. jensenii* strain survived the passage through the gastrointestinal tract and was recovered from feces during the whole trial. This strain induced an activation of spleen T-cells higher than the cholera toxin which is a strong mucosal adjuvant. The IFNγ levels, 2 to 3 Log higher than IL-4 in the *A. jensenii* groups, indicated a Th1 response that is effective in protection from tuberculosis [55].

The *Caenorabditis elegans* worm is used as an animal model to study the effects of different agents on innate immunity and ageing. Feeding on a *P. freudenreichii* KCTC 1063 cell lawn prolonged by 13% its lifespan compared to conventional feeding on *E. coli* OP50, increased the body movements that depend on muscular strength and decreased the accumulation of lipid granules indicators of aging. The immunity related pathways of DNA binding ligand (DBL)/TGF-β, P38 mitogen-activated protein kinase (MAPK) involved in innate immunity, Daf-2/DAF-16 involved in the response to insulin/insulin-like growth factor-1 (IGF-1) signalling (IIS) and the *lys-7* and *lys-8* genes for antimicrobial peptides were upregulated. Use of *C. elegans* mutants defective in innate immunity and life span extension related genes highlighted that *P. freudenreichii* regulates the p38 MAPK and TGF-β pathways. Moreover, activation of the p38 MAPK pathway by *P. freudenreichii* led to increased resistance to the infection by *Salmonella* Typhimurium [56].

P. freudenreichii JS induced an increase in the number of natural killer (NK) cells in the liver but also their decrease in the spleen with an increase of IFN-γ production in NK cells from the spleen in mice. Moreover, this strain induced serum IL-6 levels significantly higher than the control, a lower than control Th1/Th2 average ratio in the spleen, a %Th17 cells in CD4+T cells higher than the control both in liver and spleen, a % Treg cells in CD4+T cells higher than the control in the spleen, and a %Treg cells/%Th17 cells lower than the control in both liver and spleen, indicating a pro-inflammatory effect for most parameters [84].

In allergic reactions Th2 cells are induced and produce cytokines that promote the proliferation of B cells and immunoglobulin class switching to IgE that induce the release of histamine and other inflammatory substances from mast cells and basophils. A new exposure to the allergen induces the production of mediators that lead to the proliferation of Th2 cells and the activation of DCs towards the reduction of allergen-specific Treg cells and activation of mast cells. The intestinal microbiota is involved in the balance between the inflammatory reactions and immune tolerance by influencing innate and adaptive immune responses, as demonstrated with experiments in germ-free mice. SCFAs produced by components of the intestinal microbiota stimulate the production of ILC that distribute in peripheral tissues and promote homeostasis during inflammation [57].

P. freudenreichii CIRM BIA 129 administered at a daily dose of 9 Log CFU to mice with food allergy sensitized with orally supplied wheat gliadins prevented the increase in body temperature and in gliadin-specific IgE and IgG1 in serum, while it induced an increase of gliadin-specific IgG2.

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The level of mucosal mast cell protease-1 (mMCP-1) activation marker decreased in this group. Moreover, the levels of Th2 cells infiltrated in mesenteric lymph nodes were not significantly different from the control and the associated cytokines IL-5 and IL-13 were lower than in the food allergy controls. Treg cells that regulate Th1 and Th2 responses increased in the group treated with *P. freudenreichii* CIRM-BIA 129. Therefore, it was concluded that this bacterial strain exerted an anti-inflammatory effect by limiting the Th2 response. In addition, it prevented an increase of ILC2 in Peyer's patches and significantly reduced the intestinal paracellular and transcellular permeability determined by measuring the translocation of fluorescein-5.6 sulfonic acid and HRP, respectively. Indeed, the expression of ZO-1 was unaltered. Co-cultures of *P. freudenreichii* CIRM-BIA129 with human PBMCs from healthy volunteers showed an increase of TGF-β lvels. Furthermore, this strain increased IL-10 and decreased IFN-γ production by monocyte-derived DCs (MoDCs). The role of SlpB in these effects was demonstrated by the comparison with the ΔSlpB *P. freudenreichii* CIRM-BIA129 mutant strain [57].

Potential protective effects of *P. freudenreichii* on the respiratory system from infections was suggested by the ability of strain PF-24 to induce the oxidative burst in bovine alveolar lavage cells (BAL) represented mainly by macrophages more intensely than other probiotics. Flow cytometry coupled with use of fluorescent antibodies showed that this strain also induced a higher percentage of cells expressing CD14 necessary for the recognition of Gram-negative bacteria and an increase, though not significant, of cells expressing the CD205 dendritic cell marker that modulate the immune response to microbial infections [85], thus showing a general capacity to enhance leukocyte functions [86].

Table 2 summarizes the effects on immune response observed in vivo for dairy propionibacteria.

Table 2. In vivo immunomodulation effects of dairy propionibacteria with indication of the bacterial strain, disease treated, animal host, beneficial effects and molecules involved.

Strain	Disease	Animal model	Active molecule	Induced effects	Reference
A. jensenii 702	Administration of *M. tuberculosis* culture filtrate	rats	Not defined	IFNγ levels 2 to 3 log higher than IL-4 indicative of a Th1 response effective in protection from tuberculosis	[55]
P. freudenreichii KCTC 1063	No disease	*C. elegans*	Not defined	Upregulation of innate immunity related pathways DBL/TGF-β, P38 MAPK and Daf-2/DAF-16 involved in IIS signalling, upregulation of antimicrobial peptide genes *lys-7* and *lys-8*	[56]
P. freudenreichii CIRM BIA 129	wheat gliadin sensitization	mice	SlpB	Prevention of the increase of the body temperature, Prevention of increase of gliadin-specific IgE and IgG1 in serum,	[57]

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		increase of gliadin-specific IgG2, prevention of intestinal permeabilization

3.3. Effects on Obesity

A high fat diet (HFD) predisposes to chronic conditions such as obesity preceded by low level intestinal inflammation and insulin resistance, type 2 diabetes, cardiovascular diseases and fatty liver disease. The effects of *P. freudenreichii* on ApoE*3Leiden transgenic mice receiving a HFD were demonstrated for the strain JS. These mice express a mutated human *APOE3* gene associated with human dys-beta-lipoproteinaemia and also harbor a human *APOC1* gene and a human promoter that determine high levels of lipoproteins and triglycerides in plasma. In these mice *P. freudenreichii* JS reduced the weight gain and the increase of gonadal adipose tissue compared to groups receiving *L. rhamnosus* GG, a HFD diet comprising also fibers or only the HFD. Moreover, in the *P. freudenreichii* group the level in plasma of the adhesion molecule VCAM-1, a marker of vascular inflammation, mast cell numbers and TNF-α level was lower. Like *L. rhamnosus* GG, also *P. freudenreichii* JS decreased ALT values and left the adiponectin levels unaltered [58].

Live and heat-killed *P. freudenreichii* MJ2 exerted anti-obesity effects and reduction of insulin resistance in mice in which obesity was induced with an HFD. It was at first shown that preadipocytes 3T3-L1 exposed to MDI (3-isobutyl-1-methylxanthine, dexamethasone, and insulin) and incubated with heat-killed *P. freudenreichii* MJ2 (hkMJ2) even at numbers as high as 8 Log CFU/ml did not lose viability, as determined the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Then it was observed that hkMJ2 reduced fat accumulation in a dose-dependent manner and more efficiently of an anti-obesity *L. plantarum* KACC15357 probiotic. Moreover, hkMJ2 induced an increase in the expression of the transmembrane protein preadipocyte factor-1 (Pref-1), that favors the maintenance of the preadipocyte stage, and a decrease in the expression of genes encoding the adipogenic proteins PPARγ, CCAAT/enhancer-binding protein alpha (C/EBPα), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and acetyl-CoA carboxylase (ACC) [59].

Treatment of HFD induced obese mice with live *P. freudenreichii* MJ2, hkMJ2 and *L. plantarum* KACC15357 led to decreases in body weight of 31%, 22.8%, and 18.5%, respectively, and a decrease of the food efficiency ratio. *P. freudenreichii* MJ2, hkMJ2 decreased the expression of *PPARγ*, *FAS*, *SCD-1*, and *ACC* in epididymal white adipose tissue (eWAT) and increased the expression of the lipolytic enzymes adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and carnitine palmitoyltransferase 1α (CPT-1α), and the enzyme involved in β-oxidation of fatty acids peroxisomal acyl-coenzyme A oxidase 1 (ACOX1). These findings were confirmed by Western blot analysis. Moreover, treatment with *P. freudenreichii* MJ2 and hkMJ2 decreased the adipocyte size in eWAT [59].

The effects on serum lipids were a significant decrease of low-density lipoprotein cholesterol (LDL) in the *P. freudenreichii* MJ2 group and the increase of the ratio high-density lipoprotein cholesterol (HDL)/total cholesterol (TCHO) in all probiotic groups. The fasting glucose levels were decreased in the *P. freudenreichii* MJ2 group and insulin levels decreased for all the probiotic treated groups with decreased homeostasis model assessment insulin resistance (HOMA-IR) scores. Staining with hematoxylin and eosin showed a significantly decreased lipid accumulation in hepatocytes of these groups and significantly lower levels of serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) indexes of hepatic damage [59].

Obesity is caused by a remodeling of the adipose tissue induced by the imbalance between energy intake and expenditure. In this condition the adipocytes originating from mesenchymal stem cells (MSCs) become hypertrophic and hyperplastic so molecules that impair this process could be useful to treat the condition. Preadipocytes 3T3-L1, in which lipid accumulation was induced with MDI, were treated with hkMJ2, cell wall (CW) and cytoplasmic (Cyto) fractions of the *P. freudenreichii*

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MJ2 cells during differentiation to adipocytes and a reduction of lipid levels was observed in cells treated with hkMJ2 and the CW fraction and the separated surface protein component (SP) but not with the CW fraction deprived of the SP fraction. The fractions CW and SP caused a reduction in the expression level of the lipogenesis factor *Fas* [60].

The main protein components of the SP fraction of *P. freudenreichii* MJ2 were chaperones, carbon metabolism proteins and ribosomal proteins. SP components were separated by Q fast, anion exchange and size exclusion chromatography and it was found that the protein fraction with molecular weight above 35 kDa was probably implied in the reduction of lipid accumulation. Among the proteins included in this fraction the chaperonin 60 (Cpn60), otherwise designated heat shock protein 60 (Hsp60) or GroEL, was expressed as recombinant protein, tested on 3T3-L1 cells and found to reduce adipogenesis at a dose-dependent extent. The observed effect was most likely attributable to the decreased expression of genes *Pparγ*, *Cepba*, *Fas*, and *Scd1*. Indeed, the terminal differentiation stage from MSC to adipocytes is mediated by the master regulators PPARγ and C/EBPα that induce the lipogenesis genes FAS and SCD1. The expression levels of the respective coding genes and the content of lipid droplets were progressively reduced during eight days of adipocyte differentiation for the reduced PPARγ translocation to the nucleus and binding to the promoters of the target gene [60].

Since *Pparγ* and *Cebpa* genes are positively regulated by C/EBPβ in MDI-induced adipogenesis, the level of C/EBPβ in the nucleus and its binding to the *Pparg* and *Cebpa* promoters was determined and found to decrease after 24 h of exposure to the *P. freudenreichii* GroEL as determined by Western blot and chromatin immunoprecipitation (ChIP), respectively. In addition, the upregulation of genes *GATA*2 and *GATA*3, encoding adipocyte differentiation suppressors that inhibit the expression of *Cebpa* and *Pparg* by binding to C/EBPβ, was observed. Therefore, the mechanism of adipogenesis inhibition by the GroEL protein of *P. freudenreichii* MJ2 was elucidated [60].

Cholesterol lowering ability is a trait of some probiotics that could help to prevent cardiovascular diseases. *A. acidipropionici* C03B-STR isolated from goat milk degraded cholesterol in vitro thus lowering its uptake by Caco-2 cells [87]. Cholesterol lowering activity was demonstrated in vivo in mice and cows for strains of dairy propionibacteria. One mechanism for cholesterol lowering by these bacteria is bile salt deconjugation and co-precipitation of cholesterol with the deconjugated bile salts followed by excretion. Of seven strains tested, all were able to deconjugate sodium glycocholate and two strains of *P. freudenreichii* and one *P. jensenii* also deconjugated sodium taurocholate. In the broth medium containing oxgall, cholesterol precipitation occurred mostly for one *P. freudenreichii* strain, one *P. jensenii* strain and one *P. thoenii* strain [88].

A. acidipropionici OB7439 was found to increase the SCFA levels in plasma, cecum and feces of HFD obese C57BL/6J mice. Moreover, administration of this strain in high levels induced an increase of insulin secretion and consequent suppression of blood glucose rise after its oral administration. These effects were not observed for *A. acidipropionici* JCM6427, thus proving strain-specific. In addition, mice fed with *A. acidipropionici* OB7439 showed a lower weight, lower white adipose tissue and liver weight gain than the control at 16 weeks of administration. Plasma triglycerides and total cholesterol levels were also reduced. Based on previous research, the observed effects were attributed to the increase of propionate intake. The mRNA levels of *Tnfα*, macrophage marker *F4/80*, the fibrosis marker *Col*Iα, and *Fas* and *Chrebp* for fatty acid synthesis decreased in the liver, while those for the peroxixome proliferator-activated receptor α *Ppara* for lipid metabolism increased. Energy regulation via the G protein-coupled receptor GPR41 was involved in the observed effects, as demonstrated by the lack of similar outcomes in *GPR41*$^{-/-}$ mice [61].

Table 3 summarizes the effects exerted in vivo by dairy propionibacteria in obesity animal models.

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Table 3. In vivo effects of dairy propionibacteria in the mitigation of obesity clinical signs with indication of the bacterial strain, disease treated, animal host, beneficial effects and molecules involved.

Strain	Disease	Animal model	Active molecule	Induced effects	Reference
P. freudenreichii JS	HFD induced obesity	ApoE*3Leiden transgenic mice	Not investigated	Reduced weight gain and gonadal adipose tissue, Decreased level of VCAM-1 vascular inflammation marker, Decreased mast cell number and TNF-α level	[58]
P. freudenreichii MJ2	HFD induced obesity	Mice	GroEL	Reduced fat accumulation, Pre-adipocyte stage maintenance by upregulation of Pref-1and downregulation of PPARγ, C/EBPα, FAS, SCD-1, ACC and lipolytic enzymes	[59,60]
A. acidipropionici OB7439	HFD induced obesity	Mice	Not investigated	Increase of insulin secretion, Decrease of *Tnfα*, *F4/80*, *Col*Iα, *Fas* and *Chrebp* and increase of *Ppara* in the liver	[61]

3.4. Anti-Cancer Effects

Colorectal cancer (CRC) is the third most prevalent cancer type worldwide mainly in developed countries but increasing also in developing countries with risk factors that are both genetic and linked to dietary habits, smoking and alcohol consumption. A rise of CRC incidence in young persons aged 20-50 is in course [62]. The first anticarcinogenic activity reported for strains of dairy propionibacteria regarded one *A. acicipropionici* strain and one *P. freudenreichii* strain isolated from Swiss type cheese that were found to decrease the β-glucuronidase activity, that releases toxic and carcinogenic compounds by hydrolyzing the glucuronic acid conjugates formed in the liver, in mice feces. The effect persisted also one week after that treatment with propionibacteria ceased [63].

It was demonstrated that the anticarcinogenic effect exerted by the propionibacteria *A. acidipropionici* CNRZ80, *P. freudenreichii* ITG18, and SI41 in vitro was attributable to the production of SCFAs able to induce the apoptosis of cancer cells such as Caco-2, HeLa, and HT29. A medium fermented by *P. freudenreichii* CIRM BIA 138 induced death in HT29 colon cancer cells, and an optimal SCFA concentration was 0.75 g L^{-1} of acetate and 2.7 g L^{-1} of propionate [89]. Lan et al. showed that propionate induced the death of HT29 colon cancer cells by apoptosis or necrosis in the pH range 6.0-7.5 or at pH 5.5, respectively [90]. Moreover, *P. freudenreichii* TL133, able to survive and be metabolically active during transit in the gastrointestinal tract, exerted this effect also in the colon of rats exposed to the genotoxic agent 1,2-dimethylhydrazine (DMH). This chemical induced apoptosis

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in all crypt zones and in different colon regions but also an increase of the proliferative index. The administration of *P. freudenreichii* further increased the number of apoptotic cells, mostly in the middle crypt zone, but decreased the proliferative index [64].

TNF-related apoptosis-inducing ligand (TRAIL), a cytokine of the TNF superfamily, selectively kills cancer cells. To increase the effectiveness of TRAIL on CRC cells, synergistic treatments with chemotherapy agents or IFN-γ are required. As observed by DNA microarray analysis, culture supernatants or fermentation products from *P. freudenreichii* TL133 (CIRM BIA 138) induced 2180 genes in HT29 cells and co-treatment with TRAIL increased the number of induced genes to more than three thousand. The genes induced by all the treatments included those encoding apoptosis pathways and immune response elements such as nucleotide-binding oligomerization domain (NOD)-like receptors and interactions between cytokines and receptors. These were more numerous for the association TRAIL-fermentation products. Importantly, culture supernatants or fermentation products alone induced the TRAIL death receptor TRAIL-R2/DR5 and the presence of these proteins in the HT29 cell membrane was confirmed by flow cytometry combined with antagonistic mouse monoclonal antibodies [64].

The exposure of HT29 cells to the combination of TRAIL with *P. freudenreichii* culture supernatants or fermentation products induced 57% cell mortality while healthy epithelial colon cells HIEC were not sensitive to this treatment. The treatment with *P. freudenreichii* culture supernatants or fermentation products combined or not with TRAIL induced the acetylation of histone H3, thus indicating a histone deacetylase inhibition activity. In addition, the expression of the apoptosis inhibitors FLIPL and XIAP decreased. Milk and milk ultrafiltrate fermented by *P. freudenreichii* CIRM BIA 138 induce the death of HT29 cells in a similar manner. The fermented milk ultrafiltrate induced the caspases 3, -8 and -9. Apoptosis was confirmed by flow cytometry combined with propidium iodide staining, by the translocation of phosphatidylserine to the external layer of the cell membrane, the decrease of the mitochondrial membrane potential $\Delta\Psi m$, accumulation of superoxide anion O_2^- and release of cytochrome *c* into the cytoplasm. The changes induced in HT29 cells by TRAIL and *P. freudenreiichii* metabolites indicated that the extrinsic apoptotic pathway mediated by the activation of death receptors and caspase 8, and the intrinsic pathway, involving mitochondria and caspase 9, were activated [64].

Lectins are proteins or glycoproteins that bind to cell surface carbohydrates and are abundant in foods of plant origin. Concanavalin A, the lectin present in Jack bean, binds to cells in the intestinal epithelium causing alterations that reduce nutrient adsorption and impair mucus secretion with an increase of mechanical stress [65]. Moreover, the lectins concanavalin A and peanut agglutinin stimulate the proliferation of epithelial cells in rat intestine and colon cancer cell lines and could have cancerogenic effects beyond an anti-nutritional function [91]. *A. acidipropionici* CRL 1198, a dairy strain able to survive in the gastrointestinal tract, when supplied together with concanavalin A, mitigated some detrimental effects observed with the administration of concanavalin A in a mouse model such as epithelial cell proliferation in the intestine, structural alterations of microvilli and the increase of enterobacteria and enterococci [65].

The lectins wheat germ agglutinin, the agglutinin from *Artocarpus integrifolia* , soybean agglutinin, *Ulex europaeus* agglutinin and concanavalin A were tested on cancer cell lines such as HCT-15, LoVo, SW837 and HT29 and all except the wheat germ agglutinin induced cell proliferation with a more pronounced effect of the *Artocarpus integrifolia* agglutinin and concanavalin A [91]. *A. acidipropionici* CRL 1198 was incubated in media containing these two lectins and the derived supernatants reduced the cell proliferation effect of lectins on SW837 cells indicating binding of these compounds by the propionibacteria. The adhesion capacity to cells of *A. acidipropionici* was decreased in presence of lectins and was restored after addition of sugars able to bind to the lectins (haptens). This was an indication of the lectin binding capacity of *A. acidipropionici* mediated by cell surface carbohydrates [91].

Previous evidence that the SCFAs acetate, propionate and butyrate favored the arrest of the cell cycle, inhibition of histone deacetylase and apoptosis in cancer gastric cells led to testing the effect of

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milk ultrafiltrate fermented by *P. freudenreichii* CIRM BIA 138 on HGT-1 cells. Fluorescence microscopy with Hoechst 33342 staining showed typical apoptotic nuclei and bodies in these cells exposed to the fermented milk with a higher degree of DNA fragmentation compared to the positive control with campothecin apoptosis induction. The fraction of cells in sub-G1 phase after treatment with the fermented milk wascomparable to that observed with campothecin. In the fermented milk treated cells the combination of Annexin labelled with Fluorescein isothiocyanate (Annexin V-FITC) staining and flow cytometry demonstrated the translocation of phosphatidylserine to the outer cell membrane leaflet in up to 80% cells, indicating apoptosis, while the number of necrotic cells was very low according to staining with 7-Amino Actinomycin D (7-AAD). Apoptotic mitochondrial modifications were indicated by the decrease in $\Delta\Psi m$ membrane potential based on DiOC(6)3 and JC-1 probes fluorescence, accumulation of O_2^- indicator of the production of reactive oxygen species (ROS) measured with dihydroethidium (DHE) and cytochrome *c* release into the cytoplasm determined by immunoblotting. Western blotting and determination of enzymatic activity demonstrated the activation of caspases 3, 8 and 9 in HGT-1 cells exposed to the fermented milk and to a mixture of propionate/acetate 2:1. The *P. freudenreichii* fermented milk doubled the cell killing potential of campothecin [92].

P. freudenreichii DSM 20271 showed the capacity to produce SCFAs in the culture medium used for CRC derived RKO cells, most probably stimulated by the lactate produced by these cells from glucose fermentation (warbourg effect). Culture broths of the *P. freudenreichii* DSM 20271 strain inhibited the proliferation of CRC cells at different extents depending on the growth medium and previous adaptation to simulated digestive stress. Flow cytometry coupled with propidium iodide staining demonstrated the accumulation of CRC cells with cell-cycle arrested at the apoptotic sub-G1 phase or G2/M phase indicating the inhibition of their proliferation [9].

The mitochondrial activity assay with 3--2,5-difeniltetrazolium bromide (MTT assay), used to investigate cell viability, showed that *P. freudenreichii* DSM 20271 inhibited the proliferation of HCT116 CRC cells in a dose-dependent manner in vitro. In vivo effects exerted by this strain included the mitigation of oxidative stress induced with azoxymethane (AOM) in the colon of rats as determined by measuring the concentration of malondialdehyde (MDA). Moreover, this strain reduced the formation of aberrant crypt foci induced by AOM to less than half and reduced the abnormalities observed in crypts at cell and tissue level. Supplementation with *P. freudenreichii* preserved the diversity of the intestinal microbiota compared to treatment with AOM alone. The enriched microbial groups were different from the control not treated with AOM and included lactobacilli and bifidobacteria [62].

To date, clinical studies on the indirect anti-cancer effects of dairy propionibacteria regarded only the association *P. freudenreichii* JS/*Lacticaseibacillus rhamnosus* LC705 (Valio, Finland) both originated from cheese and commonly used in the production of semi-hard cheese [93]. In one study this strain association was shown to decrease the risk of hepatocellular carcinoma (HCC) in Chinese young men exposed to aflatoxin B1 known to potentiate the liver cancer risk associated with hepatitis B virus (HBV) infection [94]. This strain association was previously shown to bind aflatoxin B1 and to decrease the level of this mycotoxin in the feces of healthy volunteers after two or three weeks of dietary supplementation with a preparation containing the bacteria [95]. In the double-blind trial 90 subjects were equally distributed in the intervention or the placebo group and the two groups did not significantly differ in the fecal concentration of aflatoxin B_1-N^7-guanine (AFB-N^7-guanine) marker of aflatoxin B1 exposure. Probiotic administration led to a significant decrease of this marker in the urine, up to 55%, after 5 weeks but this effect disappeared after the end of the intervention [94].

Another double-blind, placebo-controlled trial with the same bacterial association involving 38 men aged 24-55 years examined the effects on the activities of β-glucosidases, β-glucuronidases and β-galactosidases, produced mainly by clostridia and *Bacteroides*, that release potentially carcinogenic compounds and urease that is responsible for the formation of toxic and mutagenic substances from ammonia. The assumption of the two bacterial strains did not cause adverse effects and a 10% decrease of the β-glucosidase activity occurred in the intervention group and the decrease was

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negatively correlated with the number of propionibacteria. A 13% decrease of urease activity was also observed in the intervention group that was not correlated with the number of lactobacilli or propionibacteria [93].

Table 4 summarizes the effects exerted in vivo by dairy propionibacteria on cancer prevention in animal models.

Table 4. In vivo cancer prevention effects of dairy propionibacteria in animal hosts.

Strain	Carcinogen	Animal model	Induced effects	Reference
P. freudenreichii TL133	DMH	Rats	Increased cell apoptosis and decreased proliferation in crypts	[90]
A. acidipropionici CRL 1198	Concanavalin A	Mice	Reduced proliferation of intestinal epithelial cells, preservation of microvilli structure	[91]
P. freudenreichii DSM 20271	AOM	Rats	Reduced formation of aberrant crypt foci	[62]

3.5. Effects of Propionibacterium Freudenreichii on Bone Health

Rheumatoid arthritis is an autoimmune disease that causes chronic inflammation of joints and can extend to most organs and the nervous system. This condition involves bone loss caused by enhanced osteoclast differentiation and the available treatments do not allow a definitive recovery [66,67]. The apoptosis regulator RANKL, ligand of the RANK receptor activator of NF-kB, is one of the factors stimulating osteoclast differentiation from macrophages [66,68]. Osteoclasts are multinucleated cells with a major role in bone-destruction. These create an acidic microenvironment in which they secrete proteins involved in bone destruction such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (Ctsk), and calcitonin receptor (Calcr). Moreover, these cells produce pro-inflammatory cytokines and chemokines [66]. Osteoprotegerin (OPG) is a receptor that binds to RANKL and inhibits osteoclastogenesis by hindering the RANK-RANKL interaction. Heat-killed *P. freudenreichii* MJ2, an isolate from raw milk, inhibited osteoclastogenesis and mitigated collagen-induced arthritis (CIA) in a mouse model by increasing the *OPG*/*RANKL* expression ratio [68].

This effect was mediated by *P. freudenreichii* MJ2 surface proteins extracted with guanidine hydrochloride. This substance did not disrupt cells, that remained viable, so the extraction of cytoplasmic proteins was considered to be negligible. The surface proteins were tested at concentrations of 2.5, 5, and 10 μg/mL on murine macrophages RAW 264.7 that can differentiate in osteoclasts and the latter concentration was selected for further experiments. Each addition of 10 μg/mL *P. freudenreichii* MJ2 surface proteins at different days significantly inhibited osteoclast differentiation. Moreover, TRAP+ osteoclasts decreased after 4 days of treatment with *P. freudenreichii* MJ2 surface proteins in a dose-dependent manner as well as the expression of osteoclastogenic genes induced by RANKL and encoding RANK, c-fos, NFATc1, and NF-κB with consequent lower levels of the genes regulated by the NFATc1 master regulator of osteoclast differentiation [66].

The transcriptome of cells treated with *P. freudenreichii* MJ2 surface proteins comprised 888 upregulated or downregulated genes among which *lcn*2 (lipocalin 2), with immunity function, showed the highest expression level with 2426-fold upregulation. Moreover, 128 genes involved in

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osteoclast differentiation were differentially expressed. STRING protein-protein interaction and functional enrichment analysis of the genes with higher degrees of differential expression highlighted that Lcn2 directly interacts with the tumor necrosis factor (*Tnf*) which in turn interacts with the *RANK* gene and the *Nfatc*1 gene thus influencing the expression of downstream genes *Cst*1, *Ctsk*, *Mitf*, and *TRAP*. Therefore, it was hypothesized that *lcn*2 might be involved in the inhibitory mechanism of *P. freudenreichii* MJ2 surface proteins on osteoclast differentiation. Inhibition of *lcn*2 expression by small interfering RNA (siRNA) silencing led to TRAP activity and TRAP(+) increase and formation of the F-actin ring responsible for bone resorption also in cells treated with *P. freudenreichii* MJ2 surface proteins, suggesting that these inhibit osteoclast differentiation by upregulating *lcn*2. Protein expression analysis showed that the effect was due to the downregulation of c-fos, and NFATc1 with its downstream genes. TRAP activity in osteoclasts was decreased also by surface proteins from *P. freudenreichii* MJ2 cells treated at 100°C for 30 min or the same proteins separated with trypsin from the cell surface. In the latter case TRAP activity inhibition was slightly enhanced, thus showing that the effect does not necessitate of entire proteins. Liquid chromatography tandem mass spectrometry (LC-MS/MS) separation of trypsin treated surface proteins highlighted that chaperonins and heat shock proteins were the main components of the surface protein extracts [66].

It was investigated if the EVs from *P. freudenreichii* MJ2 were involved in the inhibition of osteoclastogenesis in a murine model of rheumatoid arthritis. This strain produced spherical EVs with average diameter of 171 nm and containing 585 proteins involved in metabolism, cell structure composition and binding of various molecules such as ATP, nucleotides, ions, carbohydrates, cyclic and heterocyclic compounds. The effect of *P. freudenreichii* EVs on RAW 264.7 murine macrophages differentiation in osteoclasts when exposed to RANKL were investigated. The EVs were not toxic to the RAW 264.7 cells even at a number of 8 Log CFU/mL of *P. freudenreichii* so this level was used in the in vitro and in vivo experiments. RAW 264.7 cells exposed to *P. freudenreichii* EVs showed a decreased differentiation into TRAP osteoclasts and TRAP activity was significantly decreased compared to the control. Therefore, it was concluded that osteoclast differentiation induced by exposure to RANKL was inhibited. Experiments with fluorescent antibodies coupled with microscopy showed reduced binding of RANKL to RANK in EV-treated cells [67].

P. freudenreichii EVs significantly decreased the arthritis score in CIA mice that presented reduced bone erosion and synovial inflammation, a lower number of TRAP(+) osteoclasts and collagen-specific IgGs. In addition, the levels of pro-inflammatory cytokines IL-6, TNF-α, and IL-17 decreased, while the anti-inflammatory IL-10 increased in serum. The in vivo data confirmed the decreased expression of genes associated to osteoclastogenesis and indicated an increase of the *OPG*/*RANKL* ratio. EVs administration did not increase aspartate transaminase (AST) and alanine transaminase (ALT) levels, indicating the absence of hepatotoxicity [67].

Table 5. In vivo effects of dairy propionibacteria in bone diseases, bacterial strains involved, induced disease, animal host, beneficial effects and molecules involved.

Strain	Disease	Animal model	Active molecule	Induced effects	Reference
P. freudenreichii MJ2	CIA	Mice	Surface proteins extracted with guanidine hydrochloride	Increased *OPG*/*RANKL* expression ratio	[65,66]
P. freudenreichii MJ2	CIA	Mice	EVs	Decrease of IL-6, TNF-α and IL-17, Increase of IL-10,	[67]

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	Increased *OPG/RANKL* expression ratio

4. Antimicrobial Properties of Dairy Propionibacteria

P. freudenreichii JS, commercialized by Valio (Finland), a strain with a high capacity to adhere to intestinal mucus, prevented the adhesion of *Staphylococcus aureus* RN4220 by 39% and significantly reduced the viability of adhered *S. aureus* to 72% by producing non-bacteriocin antimicrobial substances [96]. One pathogen inhibiting substance produced by *P. freudenreichii* PTCC 1674 is a surfactant/emulsifier of lipopeptide nature showing slight inhibitory activity and prevented surface adhesion of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* strains at different extents in culture plates [97].

Dairy propionibacteria were reported to inhibit *Escherichia coli* and *Shigella sonnei* strains, but not *Listeria monocytogenes*, in vitro for an effect of the low pH of the growth medium [74]. Two *P. freudenreichii* dairy strains B3523 and B4327 of dairy origin showed high percentages of adhesion to Budgerigar Abdominal Tumor Cells (BATCs) and their cell-free culture supernatants inhibited MDR *S.* Heidelberg, *E. coli* O157:H7 and *L. monocytogenes*. The *P. freudenrichii* strains did not invade BATCs and were not hemolytic [98].

The strain *P. freudenreichii* B3523 exhibited inhibitory activity towards three *Salmonella* serotypes, *S.* Enteriditis, *S.* typhimurium and *S.* Heidelberg, reducing pathogen motility, adhesion and invasion of avian epithelial cells, and multiplication in the cecal contents of turkeys. The *P. freudenreichii* strain was able to adhere to the epithelial cells and its cell-free extracts inhibited growth and motility of *S.* Heidelberg [99]. When this strain was supplied at about 10 Log CFU daily to turkey poults of 2 to 12 weeks of age challenged with the multidrug resistant (MDR) *Salmonella* Heidelberg GT2011, that caused a foodborne outbreak from turkey meat in the United States in 2011 [70], it induced a reduction in the levels of the pathogen in cecal content of 1 to 2 Log CFU/g. This effect was linked to the changes induced by *P. freudenreichii* in the intestinal microbiota composition of growing poults such as the increase of actinobacteria and the genus *Subdoligranulum* and the decrease of *Streptococcus* after 2 days of treatment but not later. In finishing turkeys, supplementation with *P. freudenreichii* induced an increase of lactobacilli and Ruminococcaceae after 2 days and an increase of the genera *Lactococcus*, *Erysipelatoclostridium*, *Leuconostoc* and *Butyricicoccus* after 7 days, while the *Salmonella* Heidelberg group showed a higher abundance of *Turicibacter* and *Streptococcus* in different groups and sampling times. Similar results were obtained for finishing turkeys in a separate experiment. The genuses enriched in the *P. freudenreichii* treatment groups are responsible for the formation of SCFAs that promote gut health, whereas the genus *Streptococcus* comprises opportunistic pathogens and the genus *Turicibacter* was associated to intestinal inflammatory diseases so the results indicated a protective effect of *P. freudenreichii* against intestinal dysbiosis induced by the pathogen *Salmonella* Heidelberg [69].

It was later reported that in treatments of turkeys with *P. freudenreichii* B3523 the dissemination of *S.* Heidelberg in liver and spleen was reduced, depending on the age of animals and treatment time, from to 20% - 60% [70]. The same *P. freudenreichii* strain was used to treat turkeys challenged with drug resistant field turkey isolates of *S.* Agona, *S.* Saintpaul, and *S.* Reading and proved equally effective with a *Salmonella* vaccine in the reduction of the pathogen in cecum, and even more effective when combined with the vaccine. All the liver and spleen samples from animals receiving the vaccine and *P. freudenreichii* were negative for *Salmonella* despite the fact that *P. freudenreichii* alone showed a relatively low percentage of *Salmonella* negative organ samples. However, the reason for this higher efficacy in comparison with the vaccine alone and the vaccine combined with a *Ligilactobacillus salivarius* strain was not investigated [71].

A. acidipropionici Q4, isolated from a Swiss-type cheese made in Argentina adhered to HT29 cells and prevented the adhesion of an *E. coli* and a *S.* Enteriditis strain mediated by its cell surface proteins (CSP) that separately exerted similar effects [100]. *A. jensenii* B-6085, and *A. thoenii* B-6082 showed

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moderate inhibitory activity against the intestinal pathogens *E. coli* ATCC 25922, *S. enterica* ATCC 14028, *S. aureus* ATCC 25923, *P. aeruginosa* B6643, *P. vulgaris* ATCC 63, and *L. monocytogenes* ATCC 7644 and the strain *P. freudenreichii* B-11921 showed a weaker activity compared to the *Acidipropionibacterium* spp. against *L. monocytogenes* [101].

P. freudenreichii DSM 20271 and SS10 (Optim PropioniBacter, Laboratoire Optim, Bionoto sprl., Belgium), and *A. acidipropionici* DSM 20272 showed very slight inhibiting effects against wound pathogens in vitro, confirming previous results for these species. However, moderate antimicrobial activities had been previously reported for *Propionibacterium jensenii* B-6085 and *Propionibacterium thoenii* B-6082 [102].

The cheese isolates *P. freudenreichii* AS2 and AS51, and the malt isolate *A. virtanenii* JS278 exerted anti-quorum sensing (QS) activity and inhibition of QS-dependent biofilm formation toward *Chromobacterium violaceum* ATCC 31532 with *P. freudenreichii* exerting the most pronounced effect. This activity depended on the SCFAs acetic and propionic acid produced that inhibited the synthesis of the auto-inducer (AI) acyl-homoserine lactone (AHL) as demonstrated by the comparison with a deletion mutant for the gene *cvil* for QS-dependent biofilm formation [103].

5. Production of Beneficial Metabolites

The role of *A. jensenii* 702 to correct B12 vitamin deficiency in vivo was demonstrated in rats fed with a diet deficient in B12 vitamin for three months. Supplementation with 10 Log CFU of the bacterium daily for two months restored the B12 vitamin levels before administration of the B12 vitamin deficient diet. After three months of *A. jensenii* 702 administration the B12 vitamin levels were comparable to those obtained by direct supplementation of the vitamin showing that its deficiency can be corrected by supplying the bacterium through fermented food [104].

A contrasting result was obtained with the generally recognized as safe (GRAS) certified strain *P. freudenreichi* ATCC 6207. This was added in 8 Log CFU/g to yogurt administered to 30 volunteers with 18 to 50 years of age at the KEM Hospital, Pune, India, in a double-blind placebo trial aimed to the prevention of vitamin B12 deficiency. No significant differences were found in vitamin B12 concentration in venous blood samples in the *P. freudenreichii* group compared to the placebo group, though the strain is a producer of vitamin B12. The causes of the missing beneficial effect were not investigated [105].

Four *P. freudenreichii* isolates from goat milk, selected on the basis of growth capacity and tolerance to gastrointestinal and technological stresses, were used to ferment the Scotta dairy product from two industries. The formation of vitamin B9 vitamers, cobalamin-derivatives and folates was demonstrated, though at extent that differed between the two dairies [15]. A *P. freudenreichii* PS-4 strain (Christian Hansen, Denmark) was used for in situ production of up to 6.4 mg/g of fat conjugated linoleic acid (CLA), a substance with antioxidant, anti-cholesterolemic, anti-inflammatory and anti-carcinogenic properties, in a yogurt supplemented with inulin [106].

Studies on fermented food products of both plant and animal origin such as sourdough bread, tofu and fermented milk have demonstrated the potential of dairy propionibacteria as single strains or combinations to enrich the diet with vitamin B12, SCFAs, folate and bioactive peptides [107-113]. Moreover, *A. acidipropionioci* LET 120, a strain with high β-galactosidase activity, produces prebiotics from lactose and lactulose, namely oligosaccharides from lactose (GOS) and lactulose (OsLu). *P. freudenreichii* strain DSM 4902 was utilized to produce vitamin B12 on spent beer yeast [114,115].

6. Safety of Dairy Propionibacteria

The only known virulence factor of dairy propionibacteria is β-hemolytic activity expressed by red pigmented strains of the species *A. jensenii* and *A. thoenii* that produce granadaene. Despite adverse events caused by granadaene producing strains were never reported, it is preferable that these bacteria are not present in food. No detrimental activities such as biogenic amine formation in cheese were reported for dairy propionibacteria. [116,117]. Granadaene production is encoded by the

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cyl gene cluster comprising the genes *acpC, cylZ, cylA, cylB, cylE*, homologous to those found in *Streptococcus agalactiae* and essential for hemolytic activity, in *A. thoenii*, *A. vitanenii* and *A. jensenii*. The link between the red pigmentation and hemolytic activity was confirmed for *A. jensenii*, *A. thoenii* and *A. virtanenii* strains and not pigmented strains, including *P. freudenreichii* strains, did not show any hemolytic activity. A conserved region in the *cyl*G gene was selected for PCR-based diagnosis of hemolytic activity in dairy propionibacteria [117]. The safety of the pigmented *Acidipropionibacterium* strains has not yet been assessed in vivo.

No safety issues were reported for the strains tested as probiotics. In particular, *A. jensenii* 702 was fed to rats for 81 days and did not cause adverse effects, it did not affect body and organ weight and faecal β-glucuronidase activity. Moreover, extra-intestinal translocation was not observed [71]. The culture medium of *P. freudenreichii* ET-3, approved for use in form of tablets produced by Meiji and able to selectively stimulate the growth of bifidobacteria in human gut, when supplied to rats in high amounts of 6 g per kg per day did not cause any clinical sign and toxicity for different organs at macroscopic and microscopic level and no alterations of hematological and chemical values were observed. Moreover, the preparation was not mutagenic for *Salmonella* and *E. coli* indicators and for Chinese hamster lung cells [118]. Clinical safety assessment was carried out for participants receiving the culture medium for one week and no statistically different hematological and chemistry parameters were found compared to the placebo group and the baseline. In participants receiving the culture medium for 13 weeks there was a significant decrease of total blood protein levels, white blood cells, hemoglobin, mean corpuscular hemoglobin concentration and increased medium red cell volume and urine pH from the baseline but the values remained in the normal ranges. No gastrointestinal symptoms could be attributed to the *P. freudenreichii* culture medium. Therefore, no adverse effects were attributed to its dietary supplementation [119]. *P. freudenreichii* JS used in combination with *L. rhamnosus* GG did not induce gastrointestinal or respiratory disorders or infections in randomised, double-blind, placebo-controlled clinical studies carried out with 1,909 healthy participants [120].

Regarding the aspect of antibiotic resistance, it was reported that *P. freudenreichii* strains showed intrinsic resistance to aminoglycosides, quinolones, including levofloxacin, oxacillin, metronidazole, and kanamycin, and no transferable antibiotic resistance was described based on plasmid-curing experiments [116]. Phenotypic resistance tests carried out by disc diffusion for the antibiotics ampicillin, benzylpenicillin, carbenicillin, polymyxin, streptomycin, gentamicin, clotrimazole, chloramphenicol, tetracycline, neomycin, and kanamycin indicated for *P. freudenreichii* B-11921 moderate resistance to gentamicin and neomycin, sensitivity to polimixin and resistance to all the other antibiotics, for *A. acidipropionici* B-5723 moderate resistance to benzylpenicillin, gentamicin, and clotrimazole, sensitivity to polymyxin and tetracycline and resistance to all the other antibiotics, for *A. jensenii* B-6085 moderate resistance to carbenicillin, polymyxin, and tetracycline and high resistance to all the other antibiotics, and for *A. thoenii* B-6082 moderate resistance to streptomycin, clotrimazole, and chloramphenicol and resistance to all the other antibiotics [101]. However, the genetic bases of resistance were not investigated.

Testing with the broth microdilution method according to the ISO 10932:2010 norm [121] indicated that 47 dairy propionibacteria isolated from goat milk, cheese and rumen in the of Sardinia island, Italy, were resistant to amoxicillin. For the species *P. freudenreichii*, 71%, 48%, 24% and 13% of the isolates were resistant to spectinomycin, ciprofloxacin, erythromycin, and tetracycline, respectively, while four isolates were resistant to kanamycin. Among the 18 *Acidipropionibacterium* strains screened seven were resistant to tetracycline, three to ciprofloxacin, two to kanamycin and one to clindamycin according to the cut off values fixed for bifidobacteria and non-enterococcal lactic acid bacteria [122]. Four *P. freudenreichii* strains and one *A. acidipropionici* strain showed multiple antibiotic resistances and only three strains of *P. freudenreichii* and six *Acidipropionic*i spp. strains did not show any antibiotic resistance [15].

Mutations G2294A and G2295A in the 23S rRNA that could determine resistance to macrolide antibiotics were observed in the *P. freudenreichii* strain T82 but these are non-transferable [123].

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Proteins related to antibiotic resistance, namely a mitomycin radical oxidase, a protein with tetracyclin repressor domain and a puromycin resistance protein Pur8 were found to be encoded by a genomic island in *P. freudenreichii* JS8 [2].

Phenotypic resistance of some *Propionibacterium* and *Acidipropionibacterium* strains was reported towards vancomycin and ciprofloxacin, and reversible resistance was observed for tetracycline [74].

The lectin binding strain *A. acidipropionici* LET103 intended for use as avian growth promoter in a multi-strain preparation, did not show any expression of virulence factors or antibiotic resistance [124].

7. Discussion

Based on the studies consulted for this review, dairy propionibacteria can have a wide spectrum of applications as probiotics with proven benefits in IBDs, obesity, rheumatoid arthritis, allergy and infections in animal models [14,44-71]. Despite these bacteria were less investigated than lactobacilli and bifidobacteria, they also demonstrated multiple disease mitigation activities often associated to the activation of anti-inflammatory components of the immune system. The capacity to produce vitamin B12, folate and CLA represents an additional benefit that could derive from supplementation of substrates or foods fermented by of dairy propionibacteria.

A fact that should be considered in favor of conducting new studies on the dietary supplementation of dairy propionibacteria is the total absence of infection case reports with their involvement, differently than found for lactobacilli [125], bifidobacteria [126] and the widely used probiotic *Saccharomyces boulardii* [127,128]. This might depend on their less frequent use in living probiotic supplementation for humans, that was limited to the strain *P. freudenreichii* JS [93]. However, it must be considered that the frequent occurrence of dairy propionibacteria in cheeses, naturally or intentionally added, represents an exposure source for which adverse effects were never reported. Another evidence in favor of the incapability of dairy propionibacteria to be harmful is the absence of adverse effects and extraintestinal translocation in farm animals and in animal disease models [71]. Moreover, studies aimed at evaluating the toxicity of dairy propionibacteria did not report any adverse effect and those regarding survival in the gastrointestinal tract never indicated long persistence after the administration period that could be a consequence of transient adhesion to intestinal epithelial cells or mucus. On the other hand, the inability of *P. freudenreichii* to invade intestinal epithelial cells was demonstrated in vitro [38,98]. All these indications should encourage the execution of clinical trials to explore the possible beneficial effects of dairy propionibacteria for humans.

Studies on *P. freudenreichii* ET-3 supplied as culture medium concentrate and not as living probiotic demonstrated that the production of postbiotics from dairy propionibacteria is also a possibility. A further indication supporting this opportunity are the beneficial effects obtained by using the purified immunomodulatory proteins or EVs that were comparable with those of the living bacteria in animal hosts [14,40,44,47-50,53,57,59,60].

The molecular basis of dairy propionibacteria interactions with the immune system of host cells and tissues were clarified for strains of the species *P. freudenreichii* able to induce host responses that led to inflammation mitigation and tissue integrity protection and were found to be strain-dependent. In particular, the gene encoding SlpB, one of the proteins with highest immunomodulatory effect produced by *P. freudenreichii*, was found in the genome of only two among 20 *P. freudenreichii* strains included in a genome sequencing and comparison study [2]. Other proteins involved in the probiotic effects of *P. freudenreichii* were the central metabolism protein DlaT and the chaperonin GroEL [14,57]. These proteins are known to belong to the core genome of bacteria and, in the case of *P. freudenreichii*, they function as mediators of probiotic activities only in some strains. This could be explained with their overexpression determined by the presence of more than one gene copy or to rearrangements in the regulatory regions that lead to increased expression. The genome duplication observed in some *P. freudenreichii* strains could be at the origin of the increased expression of some intracellular proteins found to be more abundant in cell surface protein extracts or EVs [2].

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The identification of the protein mediators of the probiotic activities offers the possibility to design screening tests targeted on their encoding genes for the selection of new *P. freudenreichii* strains to be tested for probiotic activities. Similarly, tests aimed at determining the *gtf* gene expression level could allow to exclude the strains whose surface proteins are likely to be shielded by a polysaccharidic capsule [31].

In one *P. freudenreichii* strain among 20 studied a pilus structure was found to be expressed and promote binding to mucus [2]. Since this character is known as a main factor favoring adhesion and, if overexpressed, even to increase the capability of probiotics to cause infections [125], its occurrence in dairy propionibacteria and its involvement in persistence in the gastrointestinal tract should be evaluated.

No molecular investigations were carried out for the *Acidipropionibacterium* genus, despite the health-promoting effects observed in animal trials and in farm animals. Therefore, this research field should be explored for a possible expanded application of different dairy propionibacteria or derived culture products as food and feed supplements. From a one health perspective, the studies on the inhibition of *Salmonella* infections in turkeys demonstrated that use of dairy propionibacteria can reduce the risk of human exposure to zoonotic pathogens by decreasing the infection levels in farm animals [69,70].

A research area that must still be explored for a safe use of dairy propionibacteria as probiotics is the study of the genetic bases for the antibiotic resistance phenotypes observed in some studies and the definition of ecological cut off (ECOFF) values of antibiotic resistance by examining a large number of isolates. This would permit a more reliable distinction between intrinsic and acquired resistance than it is currently possible. Indeed, despite no transferable antibiotic resistance was reported for these species, up to date studies specifically focused on this aspect should be carried out considering the increasing trend of antibiotic resistance genetic determinants spread among bacteria.

5. Conclusions

Dairy propionibacteria are a component of the microbiota of cheeses naturally occurring in those produced with raw milk or added as selected cultures in industrial Swiss-type cheeses. Infections caused by these bacteria in human beings or in animals were never reported and their probiotic activities, demonstrated at the molecular level and in vivo, could help to multiple different disease conditions. In addition, dairy propionibacteria can enrich foods or culture media with their main metabolites, the SCFAs propionate and acetate, shown to favor the apoptosis in cancer cell lines, vitamin B12, folate and CLA. However, they are not yet widely exploited as commercial probiotics or probiotic foods. Aspects to be further investigated comprise the molecular bases of the probiotic properties exerted by *Acidipropionibacterium* spp. and the development of adequate procedures for the evaluation of phenotypic and genotypic antibiotic resistance in these bacteria. Based on the currently available knowledge, dairy propionibacteria exert beneficial effects on IBDs, obesity, rheumatoid arthritis, immunomodulation, CRC and infection mitigation in vivo but, based on published literature, these properties must still be examined in clinical studies.

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Abbreviations

The following abbreviations are used in this manuscript:

ACNQ	2-amino-3-carboxy-1,4-naphthoquinone
BAL	Bovine alveolar lavage cells
BCFA	Branched chain fatty acid
CFU	Colony forming unit
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats
CW	Cell wall
DAI	Disease activity index
DBL	DNA binding ligand
DC	Dendritic cell
DFM	Direct fed microbial
DHNA	1,4-dihydroxy-2-naphtoic acid
DSS	Dextran sulfate
DTPA	Diethylenetriamine pentaacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme linked immunosorbent assay
EPS	Exopolysaccharide
EV	Extracellular vesicles
FIL-IDF	International Dairy federation
FITC	Fluorescein isothiocyanate
5-FU	5-fluorouracile
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HDL	High-density lipoprotein cholesterol
HFD	High fat diet
HGT	Horizontal gene transfer
HOMA-IR	Homeostasis model assessment insulin resistance
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
ICE	Integrative-conjugative element
IEB	Intestinal epithelial barrier
IFN-γ	Interferon γ
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
LDL	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MAPK	Mitogen-activated protein kinase
MHC II	The major histocompatibility complex II
MLNC	Mesenteric lymph node immune cell
mMCP-1	Mucosal mast cell protease-1
MoDC	Monocyte-derived DC
MSC	Mesenchymal stem cells
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NEC	Necrotizing enterocolitis
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
QPS	Qualified presumption of safety
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction

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SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SLH	Surface layer homology
Slp	S-layer protein
SP	Surface protein
T helper	Th
TEER	Trans-epithelial electrical resistance
TGF- β	tumor growth factor-β
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor α
Treg	Regulatory T cell
UC	Ulcerative colitis
UF	Ultra-filtered
XRE	Xenobiotic-responsive element
YEL	Yeast extract lactate

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