Interactions Between Probiotic Dairy Propionibacteria and the Intestinal Epithelium

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Abstract: Dairy propionibacteria are gram positive, aerotolerant and mesophilic bacteria found in dairy products, and in diverse habitats such as soil, plants, and digestive tracts of ruminants. They are essential as ripening culture in the manufacture of Emmental cheese and other types of Swiss cheeses so they are consequently ingested in high amounts by the consumers. Dairy propionibacteria are also considered for their probiotic use. Indeed, they are species with low nutritional requirements, and with high adaptability and tolerance toward stresses, including acid and bile salts. Some strains (species) were shown to survive in the human gut, where they may have health-promoting effects. In this review, we summarize the knowledge on the interactions between probiotic dairy bacteria and the intestinal epithelium. We focus on the metabolites that are likely to play a probiotic role in the colon, as well as propionibacteria adhesion to intestinal mucus and epithelial cells. Then, among the probiotic potentialities, the immunomodulatory properties of dairy propionibacteria are given detail in.

Keywords: *Propionibacterium*, propionibacteria, adhesion, immunomodulation, colon, probiotic.

I. INTRODUCTION

Dairy propionibacteria are consumed in high amounts in several fermented dairy products. Due to their remarkable adaptability and tolerance, they can be found at elevated populations within the human gut. Cutaneous propionibacteria, that are closely related to the dairy species have been extensively studied owing to their immunomodulatory properties. Indeed, cutaneous species of propionibacteria that can be found all over the body, are part of the skin and gut microbiota, and participate in the cross-talk between bacteria and the epithelium. However, cutaneous species can cause infections and are regarded as opportunistic pathogens. Similar and divergent features should thus be sought in the dairy species, recognized as probiotics. A particular interest is currently focused on interactions between probiotic dairy propionibacteria on one hand and the epithelial and immune components of the intestinal mucosa on the other. Several studies suggest that they may play a role in the context of epithelial homeostasis preservation.

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II. GENERAL OVERVIEW OF DAIRY PROPIONI-BACTERIA

Dairy propionibacteria have been known for a century. Orla-Jensen described, in 1898, the bacteria responsible for the formation of eyes in Emmental cheese. The first pure culture was obtained in 1906 [1] and the genus *Propionibacterium* was proposed by Orla-Jensen in relation with propionic fermentation occurring in the cheese with concomitant production of carbon dioxide [2]. The current classification derives from the work of Cummins and Johnson in 1972 [3] in which the genus *Propionibacterium* comprises both dairy species described by Orla-Jensen and cutaneous species previously classified as corynebacteria (Table **1**).

Propionibacteria are Gram-positive bacteria with a high genomic GC content. Their rRNA 16S gene sequences and their GC content (53 to 68%) allow classification in the actinobacteria [4]. More precisely, they are members of the actinomycetales order. Actinomycetales, which contain

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pathogenic, marine, plant symbiont and soil bacteria, are known for their prolific production of small molecules including antibiotics, anticancer agents and immunomodulatory compounds. This order is characterized by a remarkable diversity in terms of morphology, ecology, genome size, genomic GC content, and the number of coding sequences in the genome [5-7]. Several changes occurred in the classification of propionibacteria since the pioneering work of Van Niel in 1928. The current taxonomy of propionibacteria describes 12 species (http://www.bacterio.cict.fr/) including the four typical dairy species: *P. freudenreichii*, *P. acidipropionici*, *P. jensenii* and *P. thoenii*. The main dairy species considered for human consumption is *P. freudenreichii*. Its type strain CIRM-BIA1^T genome has recently been sequenced [8], constituting the first and unique dairy propionibacterium sequenced genome. The species *P. freudenreichii* is divided into 2 subspecies, *P. freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*, discriminated on the basis of lactose fermentation and nitrate reductase activity. However, a recent multilocus sequence typing scheme questioned these phenotypic distinctions, as it appeared that *P. freudenreichii* subspecies definition does not reflect the ancestral relationships between strains [9]. Only dairy propionibacteria will be further considered in this paper.

Table 1, Dairy and Cutaneous *Propionibacterium* Species

Dairy (Classical) Propionibacteria	Cutaneous Propionibacteria
P. acidipropionici	*P. acidifaciens*
P. cyclohexanicum	*P. acnes*
P. freudenreichii subsp. *freudenreichii*	*P. australiense*
P. freudenreichii subsp. *shermanii*	*P. avidum*
P. jensenii	*P. granulosum*
P. microaerophilum	*P. propionicum*
P. thoenii	*P. humerusii*

The species formerly known as *P. inoccuum* and *P. lymphophilum* have been reclassified as *Propioniferax innocua* [83] and *Propionimicrobium lymphophilum* [84] respectively.

Dairy propionibacteria are described as pleomorphic rods, non motile, non sporing, anerobic to aerotolerant and generally catalase positive, which grow in the temperature range 15 to 40°C and the pH range 5.1 to 8.5. They were long considered as bacteria restricted to dairy environments including cheese, dairy plants and raw milk. However, they are also isolated from soil, plants, fodder and from the rumen content [10-12] so that their natural habitat is probably the digestive tract of ruminants. They are heterofermentative and metabolize various carbohydrates (including glucose, galactose, fructose and lactose), alcohols (including glycerol) and organic acids (including pyruvate and lactate, the preferred substrate). Such substrates are available in the colon content in which ingested dairy propionibacteria keep an active metabolism and release their metabolites [13;14]. Metabolites released by dairy propionibacteria include the short chain fatty acids (SCFA) acetate and propionate, vitamins (B8, B9 and B12) and DHNA (1,4-dihydroxy-2-naphtoic acid). These metabolites are likely to play a probiotic role in the colon. DHNA is the bifidogenic compound known to modulate the human colon microbiota in favor of bifidobacteria [15;16]. It was also described as an anti-inflammatory compound reducing colitis in a mice experimental model [17]. Acetate and propionate are SCFA known to play a role in the modulation of proliferation and apoptosis of colon cancer cells [18;19]. Propionate was moreover described as having anti-inflammatory modulating NF-kB activity, immune related genes expression and cytokine release in cultured human colon cell lines [20].

III. ADHESION TO INTESTINAL EPITHELIUM

The epithelial surface lining the gastrointestinal tract is covered by a layer of mucus synthesized and secreted by globet cells. This layer limits the access of bacteria from the intestinal lumen, highly populated, to the epithelial cells and forms a microenvironment colonized by intestinal autochthonous bacteria [21]. The mucus contains high molecular mass glycoproteins responsible for the viscosity and gel-forming properties, which are of importance for the bacterial exclusion from the epithelium. However, interactions with these glycoproteins allow explaining, in part, how autochthonous bacteria as well as some pathogens and probiotic bacterial strains adhere to the intestinal wall and resist peristalsis and subsequent removal from the gut.

Bacterial adhesion not only increases the residence time of probiotic strains but is also one of the mechanisms by which some probiotics reinforce the structure of the mucosa barrier, compete for adhesion determinants with pathogens, preventing translocation to tissues and organs and modulate the immune response.

Bacterial cell adhesion to the intestinal wall is affected by the composition and structure of both interacting surfaces. The process is sometimes mediated by specific attachment of bacterial lectin-like proteins to carbohydrate residues on the target tissues [22]. However, a frequently proposed mechanism involves non-specific hydrophobic interactions with the epithelial surface [23]. Moreover, the production of extracellular substances by bacteria may also influence adhesion [24].

Adhesion Models

Different *in vitro* models (Table **2**) have been proposed as screening methods for the selection of bacteria with potential ability to adhere to the intestinal mucosa including the study of physicochemical properties and chemical composition of the bacterial surface, adhesion to cell-lines derived from human carcinoma, exfoliated normal intestinal cells, immobilized intestinal mucus or mucus glycoproteins. In the same way, *in vivo* studies in animals' models have been assayed. Whatever the methods used, clinical trials are always necessary to validate the results of persistence and safety of any probiotic strain in a human or animal host.

Dairy propionibacteria grow slowly in natural environments, even more in the challenging conditions of the intestine where the high concentration of bile salts and low availability of carbohydrates limit the growth of many bacteria. To exert many of their probiotic effects that depend on their survival and persistence in the gut, the ability to

Table 2. Adhesion of Dairy Propionibacteria to Gut Epithelium According to the Used Model

	Adhesion Models		
Dairy Propionibacteria Strains	**Human Immobilized Mucus (%)**	**Epithelial Cells (%)**	***In Vivo* Adhesion (Log CFU/g of Tissue of Cells)**
P. acidipropionici CRL 1198		[a] 62.2 ± 2.51 % [27]	[c] 7.46 ± 0.34 [27] [d] 5.7 ± 0.5 [39] [d] 5.9 ± 0.5 [40]
P. acidipropionici Q4		[a] 61.1 ± 7.94 % [27]	[c] 7.43 ± 0.62 [27]
P. acidipropionici DSM 4900	1.3 ± 0.2 % [38]		
P. freudenreichii subsp. *shermanii* DSM 4902	1.7 ± 0.4 % [38]		
P. freudenreichii subsp. *freudenreichii* DSM 20271	2.6 ± 0.1 % [38]		
P. freudenreichii subsp. *shermanii* JS (DSM 7067)	0.4 ± 0.2 % [23] 5.2 ± 3.6 % [36] 14 % [37] 0.9 ± 0.5 % [38] 11.3 ± 3.6 % [41] 0.9 ± 0.5 % [42] 1 % [43] 0.9 % [85]	[b] 12.2 ± 1.3 % [31]	
P. freudenreichii CRL 757		[a] 28.3 ± 2.35 % [27]	[c] 4.57 ± 0.53 [27]
P. freudenreichii F3		[a] 50.0 ± 9.40 % [27]	[c] 6.62 ± 0.50 [27]
P. freudenreichii G1		[a] 40.0 ± 4.00 % [27]	[c] 2.21 ± 1.33 [27]
P. freudenreichii P2	1.6 ± 0.5 % [23]		
P. freudenreichii P6	0.8 ± 0.2 % [23] 1.5 ± 0.3 % [38]		
P. freudenreichii subsp. *freudenreichii* P131	0.4 ± 0.2 % [23] 1.7 ± 0.5 % [38]		
P. jensenii DSM 20278	1.9 ± 0.3 % [38]		
P. jensenii TL 246		[a] 60.0 ± 3.35 % [27]	[c] 6.86 ± 0.36 [27]
P. jensenii 702		[b] 8 ± 3 % [33]	
P. thoenii DSM 20277	1.4 ± 0.4 % [38]		

(%) Values are expressed as a percentage of adhered bacteria as compared to the total propionibacterial population used in the assay.
[a]Adhesion was assessed on mouse intestinal exfoliated cells.
[b]Adhesion was assessed on Caco-2 human colon cancer line.
[c]Adhesion is expressed in log CFU/g of mouse intestinal exfoliated cells after feeding of dairy propionibacteria.
[d]Adhesion is expressed in log CFU/g of mouse cecal tissue after feeding of dairy propionibacteria.

interact and adhere to the intestine wall becomes an important feature. Therefore, the surface properties and many of the available methods to assess adhesion ability have been studied in dairy propionibacteria.

Autoaggregation, that appears to trigger a rapid adhesive response in some bacteria and hemagglutination ability, frequently used to detect the presence of lectin-like proteins on the bacterial surface, both have been associated to high cell adhesion in bifidobacteria [25]. Microbial Adhesion To Solvents (MATS) method has been extensively used to determine hydrophobic or hydrophilic properties of the bacterial cell surface. Affinity of bacterial cells to non-polar solvents like hexadecaene or p-xylene is considered as indicative of hydrophobic surface, while the affinity to polar solvents such as chloroform or ethyl acetate suggests hydrophilic character. A good correlation between hydrophobicity and adhesion has been reported for lactobacilli, bifidobacteria and streptococci. However, the relevance of the method to predict the adhesion to tissues is questionable as many researchers have demonstrated adhesiveness of bacteria with either hydrophilic or hydrophobic surfaces [26].

Related to dairy propionibacteria, Zárate *et al.* [27] demonstrated that only one strain of *P. freudenreichii*, among twenty-four assayed for their solvents affinities, had hydrophobic surface. Four strains of *P. freudenreichii* showed autoaggregation and only one *P. jensenii* had the ability to agglutinate erythrocytes. This study showed that hydrophobicity, autoaggregation and hemagglutination are not widespread features among dairy propionibacteria.

The relevance of the MATS method, autoaggregation and agglutination of erythrocytes assays to predict the adhesion ability, was determined by these researchers with other *in*

vitro assay, in which intestinal exfoliated cells (IEC) from mice intestines were used as a model. Hydrophilic strains showed higher adhesiveness to epithelial cells than the hydrophobic strain *P. freudenreichii* G1 [27;28]. Moreover, a direct correlation among autoaggregation or hemagglutination features and cell adhesion was not found. The adhesion mechanism in dairy propionibacteria proved to be very complex with many surface structures acting in a cooperative way [28]. The process involves proteins, different carbohydrates and teichoic acids [28;29]. Mice intestinal exfoliated cells constitute an appropriate model to assess adhesion ability as cells do not need to be cultured, but have the disadvantage to be a non host-specific model.

Other *in vitro* studies have used cell lines, like Caco-2 or HT-29 human derived adenocarcinoma cells, to evaluate the adhesion of different probiotics [30]. Fig. (**1**) illustrates interaction of *P. freudenreichii* ITG P18 with Caco-2 cells, observed using scanning electron microscopy. Lehto and Salminen [31] demonstrated that *P. freudenreichii* subsp. *shermanii* JS adheres to Caco-2 cells model in a similar proportion as the probiotic *Lactobacillus rhamnosus* GG which was used as a positive control. In turn, Huang and Adams [32] assayed the adhesion of propionibacteria to Caco-2BBe1 cells, a more differentiated culture derived from Caco-2 cell line that expresses characteristics of mature enterocytes. Thirteen strains of *P. freudenreichii*, *P. acidipropionici* and *P. jensenii* were assayed and only one strain, *P. jensenii* 702, adhered to the used cell model. In a further study, Moussavi and Adams [33] also studied the adhesion of *P. jensenii* 702 in combinations with *L. casei* 01, *L. plantarum* HA8, *L. rhamnosus* GG, *L. reuteri* ATCC55730 and *B. lactis* Bb12 to Caco-2 cells. They found an improvement in values of adherence in the combination of strains, even when in most cases the values were not statistically different. However, they also found a significant reduction in adhesion percentage of two strains of lactobacilli related to their adhesion level when assayed alone. The authors highlighted that adhesion differences due to the presence of other bacteria in addition to the probiotic strain should be considered when new probiotic formulations are designed. The HT-29 cell line has also been used in an adhesion assay of *P. acidipropionici* Q4, previously characterized as a non-autoaggregant, non-hemoagglutinant strain with hydrophilic surface. The strain showed adhesiveness to this cell line similar to that observed with *L. rhamnosus* GG which was used as a positive control (Zárate *et al*., unpublished).

Lenaerts *et al*. [34] compared the protein expression pattern of Caco-2 and HT-29 cells with the patterns of intestinal epithelial cells from human small and large intestine. Numerous characteristic proteins of human epithelium *in vivo* were expressed in the cultures of colon cell lines. However, over-expression and under-expression of certain proteins were also observed. The global expression of intestinal epithelial scrapings was different from that of intestinal epithelial cells lines apparently due to proteins synthesized during adaptation of cells to culture conditions and their tumor origin. Therefore, cultures of human cell lines of tumor origin are not always comparable to normal tissue cells, especially regarding surface components that interact with bacteria. Adhesion results obtained with cell lines are in fact indicatives of the ability of strains to interact with the human epithelium surface, but a comparison between strains with different percentage of adhesion may not be relevant as data are influenced by the type and amount of receptors expressed on the cultured cell surface. On the other hand, the lack of mucus covering Caco-2 cells layers is a drawback as results are not easily extrapolated to the intestinal environment.

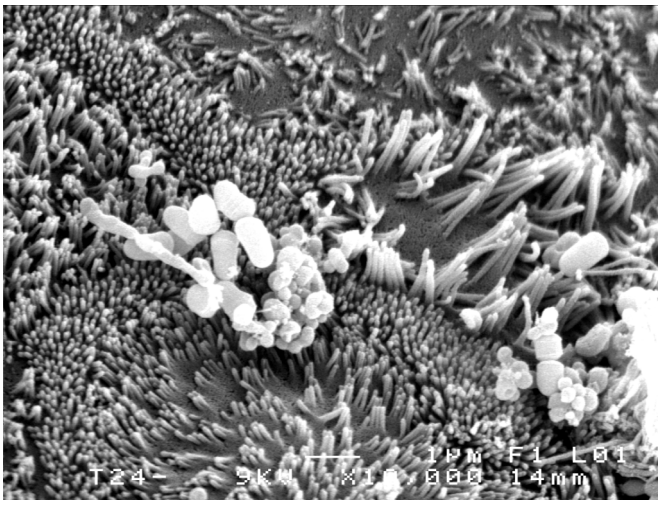

Fig. (1). Interactions between dairy propionibacteria and cultured human colon cancer cells. Caco-2 cells was co-cultured with *P. freudenreichii* ITG P18 during 24 hours and examined using scanning electron microscopy (magnification x 10,000).

Bacterial adhesion to mucus has also been studied in dairy propionibacteria. In a comparative assay, Zárate *et al.* [28] reported that *P. acidipropionici* CRL1198 adheres either well to either either mice IEC or the mucus layer covering them. In this study, adhesion to mucus was observed by Scanning Electron Microscopy (SEM). Ouwehand *et al.* [23] found that four strains of *P. freudenreichii* exhibited low to moderate adhesion to immobilized human mucus (0.2 to 6.5%), that was attributed to non-specific interactions between bacteria and mucus. An increased adhesion was observed when the mucus was pre-incubated with *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12 or *Bifidobacterium infantis* Bbi, depending on the propionibacteria strain assayed. In a study of Collado *et al.* [35], the ability of two strains of lactobacilli (*Lactobacillus rhamnosus* GG and LC705), one of *Bifidobacterium* (*Bifidobacterium breve* 99*)* and one of *P. freudenreichii* subsp. *shermanii* JS to adhere to immobilized mucus was evaluated. Strain combinations showed improved adhesion to mucus then each strain was individually assayed, suggesting a synergic effect in the strain combinations. With respect to *P. freudenreichii* subsp. *shermanii* JS, its adhesion was very low (0.9% ± 0.5) but was enhanced by association with any other of the probiotic strains used, either alone or in combinations. Moreover, combinations of *P. freudenreichii* JS with *L. rhamnosus* GG or LC705 showed good coaggregation properties with percentages higher than 10%.

Human ileostomic glycoproteins have been used by Tuomola *et al.* [36] in assessing *in vitro* adhesive probiotic strains. The adhesion percentage reported for *P. freudenreichii* JS, 5.2 ± 3.6, was higher than that observed when the entire mucus was used [23;35]. The higher adhesion to glycoproteins obtained from ileostomy effluents,

compared to colonic mucus obtained by gently scrapping of the mucosa, may be related to differences in the composition of free mucus and the mucus layer firmly adhered to the epithelium that contains a mix of gel-forming mucins and membrane-bound mucins.

Ouwehand *et al.* [37] assessed adhesion of various probiotics after heat, u.v. and γ-irradiation treatments. They found that heat or u.v. inactivation increased the adhesiveness of *P. freudenreichii* JS. In turns, Zárate *et al.* [28] and Thiel *et al.* [38] studied the effect of low pH values, gastric and pancreatic enzymes and bile salts, used individually or in a sequential model, on the adhesiveness of different strains of dairy propionibacteria. An increased adhesion to mucus was found by Thiel *et al.* in most of the strains of *P. freudenreichii* and one strain of *P. acidipropionici* at low pH and in the presence of bile salts. Pepsin and pancreatin treatments reduced the adhesiveness of strains to a different extent with the only exception of *P. freudenreichii* JS, not affected by these enzymes treatments. Zárate *et al.* [28] reported a lower adhesion percentage for *P. acidipropionici* CRL1198 to mice IEC after incubation in an artificial gastric juice containing 3 mg mL^{-1} pepsin at pH 3, and no effect after incubation with simulated pancreatic juice with bile that contained 0.15% bile salts, 1 mg mL^{-1} trypsin and α-chemotrypsin at pH 8. The results confirmed that different structures may be involved in the adhesion of a particular strain, as they are affected to a different extent by the treatments. However, a comparison between studies is not possible as different adhesion models were used.

Murine models have been used to assess adhesion of different strains of dairy propionibacteria to the intestinal tissues. *P. acidipropionici* CRL1198 administered in the drinking water of mice during seven days, in daily doses of 8-9 log bacteria.mL^{-1}, were found adhered to caecum in a level of 5.7 ± 0.5 log UFC g^{-1} of tissue after an overnight period of fasting [39]. Similar counts were obtained when this strain was administered in a milk suspension (5.9 ± 0.6 log UFC.g^{-1} of tissue) and in an experimental cheese (5.0 ± 0.8 log UFC.g^{-1} of tissue) [40]. Mice from these studies showed counts near of 10^9 UFC.g^{-1} in the cecal contents independently of the way to deliver the strain. A comparison of the ability to adhere to tissues *in vivo* of two strains of *P. acidipropionici*, one of *P. jensenii* and three of *P. freudenreichii* was performed by Zárate *et al.* [27]. Strains were administered *ad libitum* to mice during three days in a level of 10^9 bacteria.mL^{-1} of milk and recovered in IEC suspensions obtained by scrapping washed small bowel tissues. Results expressed as UFC.g^{-1} of IEC were higher than that observed by the authors in the same tissues in other studies as feeding protocols and procedures for the recovery and counting of bacteria were different. Adhesion ability was strain dependent but a correlation between adhesion percentages *in vitro* and recovery of strains *in vivo* from IEC was found. Only one strain of *P. freudenreichii* with autoaggregation property exhibited less *in vivo* recovery than that expected from the *in vitro* assay. In conclusion, *in vitro* assays with IEC have proven to be a useful and valuable tool to predict the behavior of propionibacteria *in vivo*, when the same intestinal segment is analyzed. However, to extrapolate results to humans, *in vitro* assays should be carried out by using cells obtained from resected intestinal tissues.

Competition with Pathogenic Bacteria for Adhesion Sites

Competition with pathogens for adhesion to the mucosal surface may help in protecting the host against infections. Assessing the ability of probiotics to inhibit, compete or displace pathogens from a biological surface requires the availability of appropriate *in vitro* models, in such a way, to involve a response of the host immune system.

Vesterlund *et al.* [41] studied the ability of *P. freudenreichii* subsp. *shermanii* JS and several strains of lactobacilli and enterococci to displace, exclude or compete with *Staphylococcus aureus* for adhesion sites in immobilized mucus. Significant displacement of *Staphylococcus* was observed with only two strains of lactobacilli and with *P. freudenreichii* subsp. *shermanii* JS. Collado *et al.* [42] assessed the effect of a combination of *L. rhamnosus* GG, *L. rhamnosus* LC705 and *P. freudenreichii* subsp. *shermanii* JS, with the addition of *Bifidobacterium breve* 99 or *B. lactis* Bb12, in the adhesion to immobilized mucus to exert a negative effect on several pathogens adhesion. Pathogens used in the study were *Bacteroides vulgatus*, *Clostridium histolyticum*, *C. difficile*, *Escherichia coli* K2, *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*. Pre-incubations of mucus with probiotics or pathogens and the simultaneous additions of probiotics combinations and each pathogen were used to determine inhibition, displacement or competition for the adhesion sites respectively. The authors found that inhibition or displacement of pathogens is very specific of strains and that this ability is not directly related with the degree of adhesion of the probiotics strains. Moreover, displacement and inhibition profiles were different, suggesting that different mechanisms may be involved in both events. Better effects were observed with the combination containing *B. breve* 99. In a further study, Collado *et al.* [43] assessed the ability of 12 commercial probiotic strains, including *P. freudenreichii* subsp. *shermanii* JS, to exclude eight selected pathogens. They observed that all the probiotics tested were able to adhere to mucus and inhibit and displace *Bacteroides*, *Clostridium*, *Staphylococcus* and *Enterobacter* by different mechanisms.

L. rhamnosus GG, *L. rhamnosus* LC705, *P. freudenreichii* subsp. *shermanii* JS and *Bifidobacterium breve* 99 were also assayed, each individually or combined, to inhibit the adhesion of *Helicobacter pylori* to Caco-2 cell line [44]. *P. freudenreichii* subsp. *shermanii* JS inhibited 76% *H. pylori* adherence but only at the highest concentration tested (10^9 CFU.mL^{-1}). At this cell density, *P. freudenreichii* was more effective than the probiotic combination. Moreover, combined strains increased the levels of IL-8, PGE_2 and LTB_4 released by the epithelial cells infected by the pathogen suggesting that other effects on cells should be investigated before using probiotic combinations.

Recently, the human HT-29 cell line was used to assess the ability of *P. acidipropionici* Q4 to prevent the adhesion of *Salmonella* Enteritidis and *Escherichia coli* (Zarate *et al.*, unpublished). The adherence of both pathogens was inhibited by propionibacteria (82% and 30% respectively).

Competition with pathogens for adhesion sites in the intestinal wall is a desirable feature in a probiotic strain and

dairy propionibacteria have demonstrated the ability to protect the host by means of this mechanism. However, more investigations are needed in this field in normal physiological conditions of the intestinal environment.

IV. DAIRY PROPIONIBACTERIA AND IMMUNOMODULATION

Besides the above-mentioned ability of dairy propionibacteria to interact with the gut, their immune aspects are also highly suggested to confer the role of these food/cheese inhabitant microorganisms as probiotics. Indeed, among the probiotic potentialities of dairy propionibacteria, scientific evidences indicate their immunoenhancing effects or their immunomodulatory and anti-inflammatory capacity (Table **3**). Distinct studies performed *in vitro*, *ex vivo*, and *in vivo* in animal models as well as in humans sustained such effects, although clinical studies have mainly reported on the use of dairy propionibacteria in complex bacterial mixtures and rarely with propionibacteria alone. This, in addition with the bifidogenic role of propionibacteria, leads to delicate interpretations of their direct and own impact on immune function(s).

P. freudenreichii has been observed to modulate the immune system *in vitro* through inhibition of *H. pylori*-induced IL-8 and PGE_2 release in human intestinal epithelial cells [44]. These anti-inflammatory effects did not persist when *P. freudenreichii* subsp. *shermanii* JS was used in combination with two lactobacilli and one bifidobacterium. In addition, *P. freudenreichii* and *P. acidipropionici* were reported to induce NKG2D ligand MICA/B expression in human activated T lymphocytes without affecting this expression on resting peripheral blood cells. However, this effect was also observed with propionate alone and to a lesser extent by acetate [45]. Considering the production of cytokines, the strain *P. freudenreichii* subsp. *shermanii* JS was able to induce TNF-α and IL-10 production in human peripheral blood mononuclear cells (PBMCs) [46]. Interestingly, *P. freudenreichii* subsp. *shermanii* JS induced only weakly the expression of the pro-inflammatory cytokine IL-12, suggesting useful implication to treat colitis as reported earlier [47]. More recently, this anti-inflammatory effect was shown to be shared by several dairy propionibacteria strains, but to a highly strain-dependent extent [48]. *E. coli* DH5α-induced IFN-γ production was also reduced when it was combined with *P. freudenreichii* subsp. *shermanii* JS [46]. Thus, considering the anti-inflammatory actions of IL-10, these *in vitro* data, although rare/scarce, suggest an immunomodulatory potential that could be helpful in the treatment of inflammatory conditions or diseases.

Considering gut inflammation, colonic infusion with *P. acidipropionici* TL15 or TL223 [49], as well as oral supplementation with a milk whey culture of *P. freudenreichii* ET-3 [50] was shown to reduce the severity of TNBS-induced colitis in rats. Similar protective effect of *P. freudenreichii* SI48 and CIRM-BIA118 was observed towards experimental colitis, induced either by the chemical TNBS, or by the pathogen *Citrobacter rodentium* [48]. Interestingly, healing of TNBS-induced colitis was also observed with oral administration of propionate [50]. In therapeutic and preventive studies, the 1,4-dihydroxy-2-naphtoic acid (DHNA), a compound released by propionibacteria, improved the survival rate and histological damage scores of mice with DSS-induced colitis [17]. DHNA not only attenuated colonic inflammation by balancing intestinal bacterial ecosystem but also by suppressing lymphocyte infiltration.

Considering immunomodulation, Perez-Chaia *et al.* observed an improvement in carbon-clearance in mice fed with *P. acidipropionici* CRL 1198, indicating an enhanced phagocytic function of the reticuloendothelial system [39]. Administration of this strain prior to *S. typhimurium* pathogen inoculation led to an increase in both anti-*S. typhimurium* IgA levels and numbers of cells producing the antibody [51]. Okada *et al.* reported that DHNA had an anti-inflammatory effect on NSAID-induced colitis in IL-10-knockout mice through increased numbers of *Bifidobacteria* and suppression of inflammatory cell infiltration [52]. Moreover, *P. acidipropionici* CRL 1198 enhanced the phagocytic activity of isolated mouse peritoneal macrophages [39], which was higher in mice fed propionibacteria than in the controls. This immunostimulating effect was also triggered by oral administration of *P. acidipropionici* isolated cell wall but not with isolated peptidoglycan [53]. Immune system modulation by dairy propionibacteria may be related to the chemical composition of the cell walls, particularly molecules protruding from the surface. Oral treatment of mice with *P. freudenreichii* subsp. *shermanii* JS in combination with *Lactobacillus rhamnosus* GG increased T-cell and B-cell proliferation after stimulation with concanavalin A (T-cell mitogen) and lipopolysaccharide (B-cell mitogen) respectively [54]. The authors suggest that these results may indicate that the splenic lymphocytes acquired a higher tolerance to the cytotoxic effects of the mitogens. They suggest that they are related to the capacity of dairy propionibacteria to bind this lectin *in vitro*, as cited above. Another study showed a higher T-cell proliferation of splenic lymphocytes with a *P. jensenii* strain in mice receiving soluble *Mycobacterium tuberculosis* antigens [55]. The strain has been patented as an adjuvant for oral vaccines [56;57].

Recently, Kekkonen *et al.* reported that a probiotic intervention with *P. freudenreichii* subsp. *shermanii* JS in healthy adults led to a reduction in the serum level of C-reactive protein (CRP) compared to a placebo control [58]. CRP being a sensitive inflammation marker, this result confirms the anti-inflammatory potential of dairy propionibacteria. In a randomized, placebo-controlled, double-blind trial performed in Helsinki on infants at high risk of allergy, a probiotic mixture containing *L. rhamnosus* GG, *L. rhamnosus* LC-705, *B. breve* Bb99 and *P. freudenreichii* subsp. *shermanii* JS was given daily for 6 months after birth and compared to a placebo [59-61]. Two years after birth, less antibiotic prescription and fewer respiratory infections were reported in the supplemented infant group, whatever the mode of delivery [61]. In addition, less eczema and less atopic eczema were diagnosed in the treated group [60]. Five years following birth, significant differences were detected among caesarean-delivered children: less IgE-associated disease occurred, particularly eczema, and less IgE sensitization was detected [59]. Thus, during the first stages of life, probiotic

Table 3. Effects of Dairy Propionibacteria Reported on Immune System

Conditions Tested	Tested Microorganisms	Results/Effects	Ref.
In vitro			
Caco-2 cells	*P. freudenreichii* ssp. *shermanii* JS	Inhibition of *H. pylori*-induced IL-8 and PGE2 release	[44]
Human-activated T lymphocytes	*P. freudenreichii* and *P. acidipropionici*	Induction of NKG2D ligand expression	[45]
PBMCs	*P. freudenreichii*	Induction of TNF-α and IL-10 release	[46]
	P. freudenreichii and *P. jensenii*	Induction of IL-10 release	[48]
In vivo			
TNBS-induced colitis in rats	*P. acidipropionici*	Healing of TNBS-induced colitis	[49]
	P. freudenreichii ET-3	Healing of TNBS-induced colitis	[50]
	P. freudenreichii BIA118 and SI48	Healing of TNBS-induced colitis	[48]
Citrobacter rodentium-induced colitis in rats	*P. freudenreichii* BIA118 and SI48	Attenuation of bacterial colitis severity	[48]
Mice	*P. acidipropionici* CRL 1198	Stimulation of mice peritoneal macrophage phagocytic activity	[39]
	P. acidipropionici cell wall		[53]
Mice	*P. freudenreichii*	Stimulation of splenic lymphocyte proliferation	[54]
	P. jensenii		[55]
Mice	*P. acidipropionici* CRL 1198	Increase of intestinal IgA production	[51]
Clinical studies			
Healthy subjects receiving probiotics during 3 weeks	*P. freudenreichii* ssp. *shermanii* JS	Decrease in CRP serum levels	[58]
IBS patients receiving probiotics during 6 months	*L. rhamnosus* GG and Lc705	Alleviation of the irritable bowel syndrome symptoms	[86, 87]
	B. breve Bb99		
	P. freudenreichii ssp. *shermanii* JS		
IBS patients receiving probiotics during 5 months	*L. rhamnosus* GG and Lc705	Alleviation of the irritable bowel syndrome symptoms	[88]
	B. animalis ssp. *lactis* Bb12		
	P. freudenreichii ssp. *shermanii* JS		
Children with atopic dermatitis receiving probiotics during 4 weeks	*L. rhamnosus* GG and Lc705	Induction of IL-4 secretion in infants PBMCs with CMA	[64]
	B. breve Bb99		
	P. freudenreichii ssp. *shermanii* JS		
Probiotics consumed by pregnant women two or four weeks before delivery and by infants during 6 months after birth	*L. rhamnosus* GG and Lc705	Prevention of IgE-associated allergy in caesarean-delivered children	[59]
	B. breve Bb99		
	P. freudenreichii ssp. *shermanii* JS		
Probiotics consumed by pregnant women two or four weeks before delivery and by infants during 6 months after birth	*L. rhamnosus* GG and Lc705	Increase in the resistance to respiratory infections during the first two years of life	[61]
	B. breve Bb99		
	P. freudenreichii ssp. *shermanii* JS		
Probiotics consumed by pregnant women two or four weeks before delivery and by infants during 6 months after birth	*L. rhamnosus* GG and Lc705	Inverse association between atopic diseases and colonization of the gut by probiotics	[60, 62, 63]
	B. breve Bb99		
	P. freudenreichii ssp. *shermanii* JS		

P.: Propionibacterium *L.: Lactobacillus* *B.: Bifidobacterium.*

supplementation including propionibacteria seems to promote immune system maturation, preventing infections and allergies. In addition, such supplementation would further counteract disorders linked to caesarean delivery such as delayed colonization of the gut by bifidobacteria and lactobacilli. Another randomized, placebo-controlled, double-blind trial tested the same probiotic mixture in infants with atopic eczema-dermatitis syndrome (AEDS) and suspected cow's milk allergy (CMA). Soluble E-selectin and plasma IL-10 levels were higher after probiotic supplementation than after placebo treatment [62] and fecal IgA levels tended to be higher in the probiotic group [63]. Another study showed that a mixture of *P. freudenreichii* subsp. *shermanii* JS, *L. rhamnosus* GG and LC-705 and *B. breve* Bbi99 increased IL-4 secretion and tended to stimulate IFN-γ secretion in PBMCs of infants with CMA [64]. This may offer clinical benefits in the treatment of allergic diseases by immunologic means. Altogether, these clinical data indicate beneficial immunomodulation by a mixture of Gram-positive bacteria including *P. freudenreichii* subsp. *shermanii* JS, with a reported anti-inflammatory effect of the latter. Further clinical data should pin down the role of dairy propionibacteria *per se*.

Probiotic properties "in general" and especially immune effects of strains including bifidobacteria and lactobacilli differ from one species to another and are strain-dependent [65-68]. Although the ways in which probiotic bacteria affect the human immune system *in vivo* and *in vitro* are not fully assumed, understanding the cytokine patterns elicited by probiotics may help to design probiotics for specific preventative or therapeutic purposes [46]. Therefore, it is necessary to screen a large number of dairy propionibacteria in order to select strains with the best potentialities for dedicated applications. In this context, such an approach to study immunomodulation by a large set of dairy

propionibacteria strains both *in vitro* (PBMC) and *in vivo* (experimental colitis and infectious mice models) has been initiated [48] and will be enlarged. The probable mechanisms that are involved in the immune-mediated probiotic effects are supposed to be multiple and probably involve several specific ways. First, it may involve induction of cytokines that further regulates innate and adaptive immune responses, directing immune responses to either the Th1 type or the anti-inflammatory side [46]. We can also speculate that dairy propionibacteria may jointly or separately release cell-wall constituents and/or soluble metabolites which are able to modulate pro-inflammatory mediators or key inflammatory inducers such as NF-κB. Implication of surface components may be responsible for some of the probiotic effects seen inside as well as outside the gut. Indeed, propionibacteria surface extractible components are involved in immunomodulation [48], and lactobacilli surface proteins were reported to be involved in both adhesion [69] and in immunomodulation [70]. The potential impact of the newly described *P. freudenreichii* surface polysaccharide, whose occurrence is highly strain-dependent [71;72], is also to be considered. Besides the classical screening methods, the first dairy propionibacterium genome from a *P. freudenreichii* subsp. *shermanii* strain is now available. Genomic data will allow new mechanistic investigations of its probiotic potential [73]. This will in turn confirm propionibacteria usefulness in the context of Inflammatory Bowel Diseases (IBD) and Irritable Bowel Syndrome (IBS), as well as other gastrointestinal infection diseases, cancers and allergies.

V. BENEFICIAL METABOLITES OF PROPIONIBACTERIA AND MAIN GUT EPITHELIAL FUNCTION

Dairy propionibacteria secrete a variety of compounds, such as vitamins, trehalose, conjugated linoleic acid (CLA), bacteriocins, anti-fungal and anti-viral compounds, which can be regarded as beneficial [74]. The main end-product of propionibacteria metabolism is propionate. Propionate, being an SCFA, has been investigated for its health effects. The suggested beneficial effects of propionate suggest similar properties for dairy propionibacteria.

Dairy propionibacteria may modulate intestinal functions. The mechanisms remain unclear, but probably implicate the local production of metabolites. *Ex vivo*, with rats intestine sections in an Ussing chamber model, Bouglé *et al*. [75] observed an enhancement of iron absorption by the proximal colon, due to the presence of *P. freudenreichii* or its metabolites (propionate and acetate). In addition, these SCFA may protect gut epithelium by improving mucus secretion [76].

Propionibacteria may contribute to the reduction of risk factors for colon cancer, which is the second most fatal cancer in Europe [77]. In fact, *P. freudenreichii* and *P. acidipropionici* have the ability to induce apoptosis of colorectal carcinoma cells owing to the action of short-chain fatty acids, especially propionate, on cancer cell mitochondria [18;78]. In addition, *P. freudenreichii* and *P. acidipropionici* induced NKG2D ligand expression on various cancer cells and the authors speculate that the pro-apoptotic effect may also be mediated by this over-expression [45]. Lan *et al.* reported that *P. freudenreichii* TL133 increased induction of apoptosis in colonic mucosal crypts of human microbiota-associated rats treated with the carcinogen 1,2-dimethylhydrazine (DMH) [19]. The administration of propionibacteria alone did not increase the number of apoptotic cells in healthy colonic mucosa. This study demonstrates the ability of *P. freudenreichii* to favor apoptotic depletion of damaged cells at an early stage of malignant cell transformation in rats. Strain TL133 also increased the concentrations of acetate, propionate and butyrate in rat cecal contents [14]. Metabolic activity of *P. freudenreichii* has been also confirmed in the gastrointestinal tract of human microbiota-associated rats [14]. Transcriptional activity within the intestine was demonstrated by the presence of *P. freudenreichii*-specific transcarboxylase mRNA. Transcarboxylase is involved in propionic acid metabolism. In another study, mice fed *P. acidipropionici* CRL 1198 showed enhanced propionic acid levels in the caecum [40]. Moreover, propionate prevented colon cancer cell colonization [79]. In addition, Matthews *et al.* reported that propionate induced apoptosis of gastric cancer cells *in vitro* [80]. These *in vitro* and *in vivo* data suggest that the use of dairy propionibacteria could reduce the incidence of colon cancer or help to treat this cancer, by modulating the proliferation/apoptosis balance of gut epithelial cells.

VI. USE OF PROPIONIBACTERIA AS PROBIOTICS: PRESENT AND FUTURE

In westernized countries, changes in environmental conditions, including diet, smoking, alcohol, hygiene and physical activity, are involved in the increased prevalence of various pathologies including colon cancer, obesity, inflammatory diseases and immune disorders. In these syndromes, deregulation of the digestive microbiota (dysbiosis) and of its cross-talk with the host is observed.

In this context, probiotic propionibacteria offer interesting perspectives by their ability to modulate the gut microbiota and its enzymatic activities, intestinal motility, inflammation and immune system and may have potential satiety effects [81]. These food grade bacteria are consumed without side effects in elevated amounts [82]. The elucidation of their promising probiotic effects and of the corresponding mechanism is still in its infancy.

The future research work will aim at screening a large series of strains for their robustness, activity within the gut, and to acquire evidences of beneficial effects for the most promising ones. The recent sequencing of a whole propionibacterial genome will in this context, allow identification of molecular mechanisms involved in interactions between dairy propionibacteria and intestinal epithelium. This should lead to preclinical and clinical studies aimed at establishing *in vivo* potential of these bacteria in the remediation of physiological disorders.

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ABBREVIATIONS

AEDS	=	Atopic eczema-dermatitis syndrome
CLA	=	Conjugated linoleic acid
CMA	=	Cow's milk allergy
CRP	=	C-reactive protein
DHNA	=	1,4-dihydroxy-2-naphtoic acid
DMH	=	1,2-dimethylhydrazine
IBD	=	Inflammatory bowel diseases
IBS	=	Irritable bowel syndrome
IEC	=	Intestinal exfoliated cells
MATS	=	Microbial adhesion to solvents
PBMC	=	Peripheral blood mononuclear cells
SCFA	=	Short chain fatty acid
SEM	=	Scanning electron microscopy

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