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Dairy Propionibacteria: Less Conventional Probiotics to Improve the Human and Animal Health

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1. Introduction

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts. In the last decades there has been a great interest from food and pharmaceutical industries to develop products containing probiotics due to the great demands of healthy foods and alternatives to conventional chemotherapy.

Although the great bulk of evidence concerns lactobacilli and bifidobacteria, since they are members of the resident microbiota in the gastrointestinal tract, other less conventional genera like *Saccharomyces*, *Streptococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* and *Propionibacterium* have also been considered.

The genus *Propionibacterium* has been historically divided, based on habitat of origin, into “dairy” and “cutaneous” microorganisms which mainly inhabit dairy/silage environments and the skin/intestine of human and animals, respectively. Dairy propionibacteria are generally recognized as safe microorganisms whereas members of the cutaneous group have shown to be opportunistic pathogens in compromised hosts. In consequence, the economic relevance of propionibacteria derives mainly from the industrial application of dairy species as cheese starters and as biological producers of propionic acid and other metabolites like exopolysaccharides and bacteriocins to be used as thickeners and foods preservers, respectively.

However, the ability of dairy propionibacteria to improve the health of humans and animals by being used as dietary microbial adjuncts has been extensively investigated. In this sense, our research group has been studying for the last two decades the probiotic properties of dairy propionibacteria isolated from different ecological niches. In the present article the

current evidences supporting the potential of dairy propionibacteria to be used as probiotics are reviewed focusing in a less studied mechanism such as the protection of the intestinal mucosa by the binding of dietary toxic compounds.

Nowadays there are clear evidences that propionibacteria used alone or combined with other microorganisms can exert beneficial effects in the host. Dairy propionibacteria have proven to possess many promising properties such as the production of nutraceuticals like vitamin B₂, B₁₂, K and conjugated linoleic acid, and their health promoting effects could be attributed to one or more of the following modes of action: *i*) influence on gut microbial composition and exclusion of pathogens; *ii*) modulation of the metabolic activities of the microbiota and host, and *iii*) immunomodulation. The most documented probiotic effects for propionibacteria within these categories include: bifidogenic effect in the human gut, improvement of nutrients utilization, hypocholesterolemic effect and anticarcinogenic potential immune system stimulation.

Different studies have also described the ability of dairy propionibacteria to bind and remove toxic compounds from different environments such as the gut and food. Some of them have focused in the removal of mycotoxins, like Aflatoxin B and Fusarium sp. toxins by *in vitro*, *ex vivo* and *in vivo* assays whereas others have reported the binding of cyanotoxins and some heavy metals like cadmium and lead. It has been proposed that probiotic microorganisms may reduce by binding, the availability of free toxic compounds within the intestinal tract which reduces in turn, their negative effects. In this respect, in recent years we have been investigating the potential of dairy propionibacteria to protect the intestinal mucosa from the toxic and antinutritional effects of some common dietary substances like the plant lectins from the *Leguminosae* family. By *in vitro* and *in vivo* studies we have determined that certain strains are able to bind and remove different dietary lectins from media, preventing their cytotoxic effects on intestinal epithelial cells. Daily ingestion of *P. acidipropionici* CRL 1198, a dairy strain studied in our laboratory, at the same time than concanavalin A prevented the deleterious effects caused by this lectin on some morphological and physiological parameters related to intestinal functionality in mice. Propionibacteria reduced the incidence of colonic lesions, the enlargement of organs, the disruption of brush border membranes and the decrease of their disaccharidase activities. Since consumption of suitable propionibacteria may be an effective tool to avoid lectin-epithelia interactions, further investigations on their potential as probiotic detoxifying agents are actually ongoing.

With regard to animals' health it has been reported that dairy propionibacteria directly fed to farm animals increased weight gain, food efficiency and health of many animals like chickens, laying hens, piglets and cows. With a wider insight, propionibacteria may be assayed as probiotics for other ruminants like goats and sheep since their milk-derived products are highly appreciated by consumers.

It should be emphasized that much of the health benefits described above could be related to the ability of propionibacteria to remain in high numbers in the

gastrointestinal tract by surviving the adverse environmental conditions and adhering to the intestinal mucosa.

On the basis of the GRAS status of dairy propionibacteria and the positive results obtained by us and other authors, further studies are encouraged in order to select the appropriate strains for developing new functional foods that include these bacteria for human and animal nutrition.

2. The genus *propionibacterium*

2.1. General features and taxonomy

Propionibacteria are Gram positive, catalase positive, high G+C%, non spore forming and non motile pleomorphic bacteria [1, 2]. In general, microorganisms of the genus *Propionibacterium* are anaerobic to slightly aerotolerant and morphologically heterogeneous including rod-shaped and filamentous branched cells that may occur singly, in pairs forming a V or a Y shape, or arranged in “Chinese characters”. They have a peculiar metabolism leading to the formation of propionic acid as main end-product of fermentation.

Although in 1861, Louis Pasteur demonstrated that propionic fermentation was due to the biochemical activity of microorganisms, the first studies about the morphology and physiology of propionibacteria were carried out by Albert Fitz (1879) [3], who observed that organisms from cheeses with “eyes” ferment lactate to propionic and acetic acids and liberate carbon dioxide.

By the beginning of the XXth century, E. Von Freudenreich and Sigurd Orla-Jensen (1906) [4] isolated the bacteria responsible for the “eyes” formation in Emmental cheese and some years later, the name *Propionibacterium* was suggested by Orla-Jensen [5] for referring to bacteria that produced large amounts of propionic acid. Although several strains were isolated during the following years these microorganisms were not included in the Bergey’s Manual of Determinative Bacteriology till the third edition published in 1930. Since then, new species were described on the basis of their morphological and biochemical characteristics such as their typical pattern of Chinese characters, propionic acid production, and carbohydrate fermentation profile.

In 1972, Johnson and Cummins [6], classified strains with several common features into eight homology groups based on DNA-DNA hybridization and peptidoglycan characteristics. This study was the basis for the classification of propionibacteria into “dairy or classical” and “cutaneous” groups included in the 8th edition of Bergey’s Manual of Determinative Bacteriology (1974). Four dairy species were recognized in this edition: *P. freudenreichii* and their three subspecies (*freudenreichii*, *shermanii* y *globosum*), *P. thoenii*, *P. jensenii* and *P. acidipropionici* whereas other four species that inhabit the human skin were ascribed to the cutaneous propionibacteria: *P. acnes*, *P. avidum*, *P.*

lymphophilum and *P. granulosum*. The same scheme was followed in the first edition of Bergey's Manual of Systematic Bacteriology [1]. In 1988, on the basis of 16S rRNA sequences, the species *Arachnia propionica* was reclassified as *Propionibacterium propionicus* [7]. Then, in Bergey's Manual 9th edition (1994), the classification of previous edition was maintained but the subspecies *P. freudenreichii* subsp. *globosum* was removed without justification. Other species like *P. innocuum* and *P. lymphophilum* were then also reclassified as *Propioniferax innocua* [8] and *Propionimicrobium lymphophilum* [9], respectively.

In the last two decades six new species were isolated: *P. cyclohexanicum* was obtained from spoiled orange juice [10]; *P. microaerophilum* was isolated from olive mill wastewater [11]; *P. australiense* came from granulomatous bovine lesions [12] and *P. acidifaciens* from human carious dentine [13]. Recently, a new species isolated from human humerus, *P. humerusii*, has been proposed [14].

At present, the genus *Propionibacterium* is classified as Actinobacteria with a high G+C content, that make them more related to corynebacteria and mycobacteria than lactic acid bacteria. The current taxonomic position of propionibacteria is the following [2]: **Phylum** Actinobacteria; **Class** Actinobacteria; **Subclass** Actinobacteridae; **Order** Actinomycetales; **Suborder** Propionibacterineae; **Family** Propionibacteriaceae; **Genus** *Propionibacterium*.

In the more conventional and general way, propionibacteria are divided based on habitat of origin, in two main groups:

- “Dairy or classical propionibacteria” that inhabit dairy environments and silages, and
- “Cutaneous propionibacteria” that inhabit the skin and the intestine of humans and animals.

Classical propionibacteria include among their main habitats: raw milk and cheese [1, 2] but have been obtained also from silages and vegetables for human consumption [15], and from ruminal content and feces of cows and calves [16]. Furthermore, they are not limited to the gastrointestinal tract of ruminants being also isolated from the intestine of pigs and laying hens [17].

On the other side, cutaneous species are found mainly in the human skin, but have been isolated also from the intestine of humans, chicken and pigs [1, 2, 18], being best represented by the acne bacillus, *Propionibacterium acnes*.

The 13 species known up to now are listed in Table 1.

From a safety point of view, classical species have a long history of safe application on industrial processes whereas members of the cutaneous group are commonly considered opportunistic pathogens in compromised hosts. In consequence, the economic relevance of propionibacteria derives mainly from the industrial application of dairy species as cheese starters and as biological producers of propionic acid and other metabolites with a more recent interest on their usage as health promoters.

"Dairy or classical" propionibacteria	"Cutaneous" propionibacteria
<i>P. acidipropionici</i>	<i>P. acidifaciens</i>
<i>P. cyclohexanicum</i>	<i>P. acnes</i>
<i>P. freudenreichii</i>	<i>P. australiense</i>
<i>P. jensenii</i>	<i>P. avidum</i>
<i>P. microaerophilum</i>	<i>P. granulosum</i>
<i>P. thoenii</i>	<i>P. humerusii</i>
	<i>P. propionicus</i>

Table 1. Current species of the genus *Propionibacterium*

Isolation and enumeration of propionibacteria can be made by microbial culture and molecular methods [19]. Various agarized media with different degrees of selectivity have been used for detection and enumeration of classical propionibacteria in dairy environments, animal and human fecal samples. Among them it could be mentioned YELA [20], Pal Propiobac® medium, which contains glycerol, lithium lactate and antibiotics [21] or others including lithium chloride and sodium lactate in concentrations high enough to limit the growth of accompanying bacteria [22]. In all cases, incubations are made in anaerobiosis with an atmosphere of 10–20% CO₂. Although these media may be successful for the isolation of classical and cutaneous strains of *Propionibacterium*, they have limitations for selective enumeration of bacteria in very complex ecosystems like intestinal microbiota. Furthermore, plate count methods for propionibacteria are time consuming since long incubation periods for at least 6 days are needed to obtain typical colonies and enumerations may be underestimated due to aggregation of bacteria in the diluents used, and/or growth inhibition by the selective agents used.

Molecular methods are a valuable alternative to plating assays, being far more specific, and unhindered by the presence of non-target microorganisms. Different fingerprinting methods such as SDS-PAGE of whole cell proteins [23], 16S rDNA targeted PCR-RFLP [24], ribotyping [25], 16S-23S ribosomal spacer amplification and restriction [26], Pulsed-Field Gel Electrophoresis [27], Conventional Gel Electrophoresis Restriction Endonuclease Analysis (CGE-REA) and Randomly Amplified Polymorphic DNA-PCR [28] have been used for detection and accurate identification of dairy propionibacteria from various environments like milk, cheese, whey and flour. Genus and species-specific primers targeted to the genes encoding 16S rRNA for PCR-based assays were also designed for detection of dairy propionibacteria [29].

Recently, a multicolor fluorescent *in situ* hybridization (FISH) assay targeting the 16S rRNA [30] or 23S rRNA [31] of *P. acnes* was developed and used to detect this bacterium in blood samples and tissues of patients with prostate cancer, respectively. A FISH protocol and oligonucleotide probes targeting the 16S rRNA of dairy propionibacteria were developed in our laboratory [32] and successfully used for enumeration of *P. acidipropionici* in cecal samples of mice fed with a strain of this species [33].

Finally, a real-time PCR method, based on the transcription of the enzyme transcarboxylase involved in propionic fermentation, was successfully used to detect a strain of *P. freudenreichii* in the intestinal ecosystem [34] and would be a valuable tool for monitoring survival and metabolic activity of propionibacteria in different environments.

2.2. Genotypic characteristic of *Propionibacterium*

The members of the genus *Propionibacterium* possess a circular-shaped chromosome like most bacteria that varies in size between 2.3 and 3.2 Mb depending on the different species [35]. The G+C content in their DNA is in the range of 53-68 mol% and although they generally do not possess plasmids their existence has been reported in strains of *P. acidipropionici*, *P. freudenreichii* and *P. jensenii* [36]. In fact, it has been informed that between 10 and 30% of *P. freudenreichii* strains possess one or two cryptic plasmids [37]. The presence of two types of bacteriophages has also been described for propionibacteria. One of them, the bacteriophage B22, belongs to the Group B1 of Bradley classification, whereas the other, bacteriophage B5, would be the first infectious filamentous virus described in a Gram positive bacterium [38].

Up to few years ago, the only completely sequenced and publicly available genome within the genus *Propionibacterium* was that of the commensal cutaneous species *P. acnes* [39]. However, in the year 2010, the complete genome of a species that belongs to the taxonomic group of dairy propionibacteria was described for the first time.

The genome of the type strain, *P. freudenreichii* subsp. *shermanii* strain CIRM-BIA_{IT}, was sequenced with an 11-fold coverage [40]. It consists of a circular chromosome of 2,616,384 base pairs (bp) with 67% GC content, 2 rRNA operons and 45 tRNAs. The chromosome is predicted to contain 2439 protein-coding genes and also contains 22 different insertion sequences that represent 3.47% (in base pairs) of the genome. Insertion sequences and transposable elements may promote genome plasticity and induce phenotypic changes that contribute to bacterial adaptation to different environments; being particular for propionibacteria the synthesis of capsular EPS and the ability to ferment lactose [40].

P. freudenreichii subsp. *shermanii* CIRM-BIA_{IT} is able to metabolize lactose, although this trait is strain-dependent, since the Lac genes may have been acquired through a horizontal transfer event mediated by phage infection. In this sense it should be emphasized that the presence of the enzyme β -galactosidase should be the only feature that allows these bacteria to adapt to dairy niches like cheeses.

The genome sequence also showed that *P. freudenreichii* possesses a complete enzymatic machinery for de novo biosynthesis of aminoacids and vitamins (except panthotenate and biotin) and genes involved in the metabolism of carbon sources, immunity against phages, chaperones for stress resistance, and storage of inorganic polyphosphate, glycogen and compatible solutes such as trehalose that confer these bacteria a long survival in stationary phase [40]. Although propionibacteria are usually described as anaerobes, all the genes encoding enzymes required for aerobic respiration such as NADH dehydrogenase, succinate dehydrogenase, cytochrome bd complex, ATPase and the complete pathway for heme synthesis have been identified in the genome of *P. freudenreichii* [40].

With respect to technological application in dairy industries, various pathways for formation of cheese flavor compounds were identified in the genome of this strain such as the enzymes involved in the production of propionic acid, volatile branched chain fatty acids from amino acid degradation, and free fatty acids and esters from lipids catabolism.

In relation to probiotic functionality, it has been identified the complete biosynthesis pathway for a bifidogenic compound (DHNA) as well as the sequences corresponding to a high number of surface proteins involved in the interactions with the host (like adhesion and immunomodulation). By comparative genomics with *P. acnes*, no pathogenicity factors were identified in *P. freudenreichii*, which is consistent with the Generally Recognized As Safe and Qualified Presumption of Safety status of this species.

2.3. Main physiological characteristics of *Propionibacterium*

Propionibacteria are heterotrophic microorganisms that mean they need an organic carbon source to grow and posses a fermentative metabolism [41-43]. They degrade carbohydrates like glucose, galactose, lactose, fructose and other sugars; poliols like glycerol; erythritol and others; and organic acids such as lactic and gluconic acids producing propionic, acetic and CO₂ as the main fermentation end-products [1].

The production of propionic acid by these bacteria involves a complex metabolic cycle with several reactions in which substrates are metabolized to pyruvate via glycolysis, pentose phosphate or the Entner-Doudoroff pathways, generating ATP and reduced co-enzymes. Pyruvate is then oxidised to acetate and CO₂ or reduced to propionate. The latter transformation occurs via the Wood-Werkman cycle or transcarboxilase cycle which represents the key component of the central carbon metabolic pathway in propionibacteria [41].

The most important reaction of this cycle is transcarboxylation that transfers a carboxyl group from methylmalonyl-CoA to pyruvate to form oxaloacetate and propionyl-CoA, without ATP consumption. The enzyme catalyzing this reaction is a methylmalonyl-CoA carboxytransferase that has been fully characterized and its structure resolved [34; 40].

Then, oxaloacetate is reduced to succinate, via malate and fumarate in two NADH requiring reactions. Succinate is then converted to propionate via methylmalonyl-CoA intermediates (succinyl-CoA and propionyl-CoA); the carboxyl group removed from methylmalonyl-CoA is transferred to pyruvate to yield oxaloacetate, thus completing one cycle. Methylmalonyl-CoA is also regenerated from succinyl-CoA during propionate production, thus creating the second of the two transcarboxylase cycles, and can react with a new molecule of pyruvate. All the reactions of this cycle are reversible. It must be emphasized that the Wood Werkman cycle used by propionibacteria to produce propionate is coupled to oxidative phosphorylation and yields more ATP than in the other bacteria producing propionic acid [42, 43].

Depending on the strains, the substrate used, and the environmental conditions, propionibacteria modulate the proportions of pyruvate either reduced to propionate, or oxidised to acetate and CO₂, to maintain the redox balance [43]. In this way the oxidation of glucose and lactic acid leads to a molar ratio of propionate:acetate of 2:1 whereas the oxidation of glycerol leads to the formation of propionate only. The co-metabolism of aspartate/asparagine and lactate has also been reported [44]. During lactate fermentation, aspartate is deaminated to fumarate by an aspartate ammonia lyase; fumarate is then converted to succinate, with a concomitant production of NAD and ATP. Cells using this pathway convert less pyruvate to propionate and oxidised more pyruvate to acetate+CO₂.

Figure 1. Propionic acid fermentation in propionibacteria

Propionibacteria are also mesophilic microorganisms, with optimal growth conditions at 30 °C and pH 6.8. However, they grow in a temperature range between 15 a 40 °C and tolerate pH variations between 5.1 and 8.5 [1, 2]. Their nutritional requirements are low and almost the same for all the species. Dairy propionibacteria like *P. freudenreichii* are able to synthesize all amino acids [40]. They can grow in a minimal medium containing ammonium as the sole nitrogen source, but a higher growth is observed in media containing amino acids [45].

Although *P. freudenreichii* subspecies *shermanii* is able to ferment lactose, dairy propionibacteria show poor growth in milk, as they do not possess proteases capable of hydrolyzing milk caseins [46]. Some proteinases have been described for *Propionibacterium*, one cell wall associated and one intracellular or membrane bound but their activities are weak. By contrast, different peptidases such as aminopeptidases, proline iminopeptidase, proline imidopeptidase, X-prolyl-dipeptidyl-amino-peptidase, endopeptidases and carboxypeptidase, have been described. and characterized. Amino acids, especially aspartic acid, alanine, serine and glycine, are degraded by *Propionibacterium*, with variations among species and strain [47]. On the other side, cutaneous propionibacteria, have the ability to hydrolyze different proteins, like gelatin and fibronectin, and to promote damages and inflammation of the host tissues.

Regarding vitamins, all propionibacteria strains require pantothenate (vitamin B5) and biotin (vitamin H). In addition, some strains require thiamine (B1) and p-aminobenzoic acid [40, 41].

2.4. Long term and stress survival of Propionibacteria

It is known that propionibacteria are able to adapt and survive to different stresses like industrial processes and the gastrointestinal transit, as well as to remain active for long periods of time in such adverse environments [43].

In this sense, the manufacture of a swiss type cheese represents for microbial starters successive stresses like acidification of the curd, heating during cooking, osmotic stress due to brining, and low temperature (4 to 12 °C) during cheese ripening. The transit through the digestive tract also suppose stressful conditions for bacteria such as gastric acidity and the presence of other aggressive intestinal fluids like bile and pancreatic enzymes.

Interestingly, the cell machinery involved in general stress adaptation in *P. freudenreichii* was shown to be encoded by multicopy stress-induced genes [40]. The redundancy and inducibility of this chaperone and protease machinery is in agreement with the ability of *P. freudenreichii* to adapt rapidly and efficiently to various unfavorable conditions [48-50].

The stress adaptation proteins were particularly investigated in *P. freudenreichii* and its genome, finding out that they are differentially expressed depending on the strain and the stress [40, 48-50]. Acid and bile stresses, induce the synthesis of the following proteins: pyruvate-flavodoxin oxidoreductase and succinate dehydrogenase which are involved in electron transport and ATP synthesis, as well as glutamate decarboxylase and aspartate ammonia-lyase, which are involved in intracellular pH homeostasis. Bile

also induces oxidative stress so that survival and activity within the gut depend on remediation of oxidative damages. *P. freudenreichii* possesses an arsenal of genes for disulfide-reduction and elimination of reactive oxygen species. Moreover, in response to bile salts, *P. freudenreichii* overexpresses the iron/manganese superoxide dismutase, Glutathione-S-transferase, two cysteine synthases and S-adenosylmethionine synthetase [40]. The occurrence of a sodium/bile acid symporter (PFREUD_14830) reflects adaptation to the gut environment. Other inducible proteins involved in protection and repair of DNA damages include Ssb protein which is involved in DNA recombination and repair, as well as Dps which protects DNA against oxidative stress are stress-induced in *P. freudenreichii* [49].

With respect to thermotolerance, the over-expression of constitutive stress-related molecular chaperones and ATP-dependent proteases as well as the induction of the dihydroxyacetone kinase locus (dhaKL, PFREUD_07980 and PFREUD_07990) by stress and starvation seems to be related to survival to thermal stress by difference to thermosensitive strains [40, 50].

Stress tolerance and cross-protection in strains of *Propionibacterium freudenreichii* were examined after exposure to heat, acid, bile and osmotic stresses. Cross-protection between bile salts and heat adaptation was demonstrated. By contrast, some other heterologous pretreatments (hypothermic and hyperosmotic) had no effect on tolerance to bile salts. Furthermore, acid pretreatment sensitized cells to bile salts challenge and vice versa. Heat and acid responses did not present significant cross-protection and no cross-protection of salt-adapted cells against heat stress was observed for these propionibacteria [48-50].

In addition, long term survival of propionibacteria on adverse environments could be due to the accumulation of storage compounds, compatible solutes, and the induction of a multi-tolerance response under carbon starvation [40]. In contrast to other bacteria that use ATP, *P. freudenreichii* accumulates inorganic polyphosphate (polyP) as energy reserve. Short chains of PolyP are synthesized when bacteria grow on glucose whereas long chains are accumulated when the main carbon source is lactate. The synthesis of PolyP is catalysed by polyphosphate kinase (PPK) that transfers the terminal phosphate of ATP to polyP. It is proposed that PolyPs enable microorganisms to tolerate adverse conditions since ppk mutants are unable to survive during stationary phase [51]. The genes encoding for polyP or pyrophosphate (instead of ATP) using enzymes were found in the genome of *P. freudenreichii* CIRM-BIA1T [40].

Propionibacteria is also able to synthesize glycogen and all the genes related to glycogen metabolism were identified in the genome of the strain *P. freudenreichii* CIRM-BIA1T [40]. Some of these genes were also found in *P. acnes*. These enzymes seem to be involved in intracellular accumulation and hydrolysis of glycogen as neither *P. freudenreichii* nor *P. acnes* are able to ferment extracellular glycogen

It has been reported that propionibacteria are able to withstand osmotic stress by accumulation of compatible solutes like glycine betaine and trehalose [52]. Trehalose is a non-reducing disaccharide that can be used by bacteria as a carbon and energy source and also can be accumulated as a compatible solute. All dairy propionibacteria are able, in a strain dependent manner, to synthesize and accumulate trehalose from glucose and pyruvate [53]. Both processes are enhanced at stationary phase and under oxidative, osmotic, and acid stress conditions [54]. Trehalose is commonly synthesised via the trehalose-6-phosphate synthase/phosphatase (OtsA–OtsB) pathway and catabolised by trehalose synthase (TreS). The genes *otsA*, *otsB*, and *treS* were identified in *P. freudenreichii* by Cardoso et al., 2007 [55] and Falentin et al 2010 [40].

It is also known that dairy propionibacteria survive for many months at room temperature even under conditions of carbon starvation, being the majority of the strains non-lytic [2]. This long-term survival in stationary phase or dormant phase could be the consequence of a multi-tolerance response that involves the synthesis and accumulation of polyP, glycogen, trehalose and the over-expression of molecular protein chaperones. Besides, a gene encoding an Rpf (resuscitation promoting factor) protein which is essential for the growth of dormant cells from actinobacteria has been described in the genome of *P. freudenreichii* and is probably involved in long-term survival of propionibacteria [40].

3. Technological importance of dairy propionibacteria

3.1. Dairy starters for Swiss-type cheeses and other products

The main industrial application of the genus *Propionibacterium* is the usage of “classical propionibacteria” as dairy starters for the manufacture of Swiss type cheeses. This denomination refers to cheese varieties, such as Sbrinz, Emmental, Gruyère, Comté, Appenzeller and others riddled with holes and made with raw or pasteurized milk (depending on the variety).

In these products propionibacteria are responsible for the typical sweet, nutty taste by production of acetic and propionic acids; aminoacids like proline and leucine but mainly for the characteristic “eyes” formation by releasing of CO₂ [56-57]. However, propionibacteria can also be used in the manufacture of various cheeses without eyes just to enhance flavour formation [58].

In swiss type cheeses, propionibacteria may be present either as contaminants of raw milk or as components of starter cultures. The typical starter for this variety includes *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsps. *lactis* or *bulgaricus* and *Propionibacterium freudenreichii*. During manufacture and early stages of ripening, the thermophilic bacteria develop at expense of lactose of milk being responsible for lactic acid production, and also contributing to casein hydrolysis during pressing of the cheese.

Interactions between microbiota and milk throughout ripening lead to biochemical changes that result in the development of the typical texture and flavor. During maturation in the cold room (15 °C) most of lactic starter lyse and release peptidases that produce free amino acids, which are precursors of many flavor compounds. The subsequent period of warm room ripening is characterized by a marked growth of propionibacteria that metabolize the lactate produced by the lactic acid bacteria into propionate, acetate and CO₂. At the end of maturation that ranges from 6 weeks to 12 - 18 months in the hardest varieties, the number of propionibacteria reaches 10⁸ - 10⁹ cfu/g of cheese [41, 57].

P. freudenreichii greatly contributes to Swiss-type cheese flavour by producing compounds from three main pathways: lactate and aspartate fermentation, amino acid catabolism, and fat hydrolysis [59]. As described above, the end-products of propionic fermentation are considered as flavour compounds in cheese whereas the co-metabolism of aspartate leads to additional CO₂ production. However, strains with a high ability to metabolise aspartate can be associated with undesirable slits and cracks [60].

Propionibacteria degrade branched-chain amino acids to branched-chain volatile compounds mainly 2-methylbutanoic acid and 3-methylbutanoic acid, which derive from isoleucine and leucine degradation, respectively [61]. These important flavour compounds are almost entirely produced in cheese by propionibacteria that synthesize them in closely related manner to that of cell membrane fatty acids [62].

P. freudenreichii also contributes in a great manner to cheese lipolysis by releasing free fatty acids from fat during cheese ripening. Two esterases, one extracellular and other surface-exposed seem to be involved in lipolysis of milk glycerides [63, 64]. Furthermore, ten intracellular esterases were found in the *P. freudenreichii* genome that could be involved in the synthesis of the volatile esters associated with the fruity flavor of cheese [65].

In contrast, although it possesses diverse intracellular peptidases, *P. freudenreichii* has a limited role in secondary proteolysis, compared to starter and non-starter lactic acid bacteria (NSLAB), because it does not lyse in cheese [66].

It is important to emphasize that propionibacteria maintain metabolic activity up to the end of ripening, as shown by molecular methods [68] producing flavour compounds during growth in cheeses at 24 °C, and further cold storage [60].

Other dairy products such as yogurt and fermented milks seem to be less appropriated for delivery of propionibacteria due to their weak proteolytic activity, the presence of inhibitory substances and the low pH attained by lactic acid fermentation that do not allow their development. Currently, yogurt is used to deliver probiotic propionibacteria to the host's intestine or to produce nutraceuticals, but in both cases inoculums higher than those used for cheese manufacturing are necessary.

3.2. Antimicrobials production: Propionic acid and bacteriocins

Propionic acid and its salts, as well as *Propionibacterium* spp strains, are widely used as food and grain preservatives due to their antimicrobial activity at low pH. They are commonly incorporated in the food industry to prolong the shelf-life of many products by suppressing the growth of mold and spoilage microorganisms in bread and cakes, on the surface of cheeses, meats, fruits, vegetables, and tobacco.

Most commercial propionic acid is produced by petrochemical processes since biosynthesis by microbial fermentation is limited by low productivity, low conversion efficiency, by-product formation (acetic acid and succinic acid) and end-product inhibition. However, different attempts have been made to improve biological production of propionic acid for industrial applications [68]. In this sense, it has been determined that the most appropriated species for bioproduction of propionic acid from carbohydrate-based feedstock, including glucose and whey lactose, is *P.acidipropionici* [69, 70]. Since the use of glycerol as the principal carbon source enables the production of propionic acid without acetic acid, recent investigations have focused on the optimization of this particular homopropionic fermentation by propionibacteria [71, 72].

Two commercial products that include propionibacteria or their metabolites aimed for controlling spoilage microorganisms are currently available at market. Microgard™ is a food grade biopreservative obtained by fermentation of skim milk with *Propionibacterium shermanii* that is active against some fungi and Gram negative bacteria, but not against Gram positive ones [73]. The other product named BioProfit, contains viable cells of *P. freudenreichii* subsp *shermanii* strain JS and is effective for inhibiting yeasts growth in dairy products, *Bacillus* spp. in sourdough bread [74]; and also used to preserve grain and produce good quality silages [75].

Propionic acid, produced *in vivo* in the gut by viable bacteria, is also a desired healthy metabolite, as it is related to many probiotic properties of propionibacteria (as will be described below). In this respect, it has been demonstrated that SCFA favours the colonic recovery of water and electrolytes counteracting the osmotic diarrhea induced by lactose and/or other unabsorbed carbohydrates [76]. Besides, they exert anticarcinogenic effects by inducing apoptosis of neoplastic cells but not of healthy mucosa [77]. Finally, SCFA may exert hypocholesterolemic effects, since propionate lowers blood glucose and alters lipid metabolism by suppressing cholesterol synthesis in the liver and intestine [78].

Bacteriocins are antimicrobial peptides or proteins encoded by plasmid or chromosomal DNA of a wide range of Gram positive and negative bacteria. They have an antagonistic activity against species genetically related to the producer strain, but many of them exhibit a rather wide spectrum of activity and inhibit the growth of spoilage and pathogenic bacteria belonging to other genera [79].

Both starters and naturally occurring bacteria on food have the ability to produce bacteriocins. Hence, they may have potentially important applications as food biopreservatives or bacteriocin-producer probiotics to inhibit intestinal pathogens [80].

However, only nisin, a bacteriocin produced by *Lactococcus lactis subsp. lactis*, has attained the GRAS status of the FDA for use in certain foods.

Different bacteriocins produced by both dairy and cutaneous propionibacteria have been reported and characterized. Among them it could be mentioned: Propionicin PLG-1 and GBZ-1 produced by *P. thoenii* 127 [81]; Jensenin G isolated from *P. thoenii* P126 [82]; Propionicins SM1 and SM2 produced by *P. jensenii* DF1 [83]; Propionicin T1 synthesized by *P. thoenii* 419 and LMG2792 [84]; Thoenicin 447 isolated from *P. thoenii* 447 [85]; Acnecin produced by a strain of *P. acnes* [86] and several other propionicins [87-89].

These bacteriocins are active against other propionibacteria, lactic acid bacteria (*Lactobacillus*, *Lactococcus* and *Streptococcus*), other Gram positive bacteria (*Clostridium botulinum* types A, B and E), Gram negative bacteria (*Campylobacter jejuni*, *E. coli*, *Ps. fluorescens*, *Ps. aeruginosa*, *Vibrio parahaemolyticus* *Salmonella typhimurium*, *Yersinia enterocolitica*); yeasts (*Saccharomyces*, *Candida y Scopularopsis sp*) and molds (*Aspergillus ventii*, *Apiotrichum curvatum*, *Fusarium tricinctum*, *Phialophora gregata*).

Although the ability of dairy propionibacteria to produce bacteriocins *in situ* in food products or inside the intestine has not been demonstrated yet, they have a potential application as safe biopreservatives. In this respect, some efforts have been made to improve the production processes [90] since the slow growth, late bacteriocin synthesis and low production represent limitations for the practical application of bacteriocin-producer propionibacteria.

Propionibacteria also produce other peptides and organic acids (2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid 3-phenyllactic acid) with antiviral, antiyeasts and antifungal activities [91-93].

3.3. Nutraceuticals production: CLA, vitamins, EPS and trehalose

Propionibacteria are able to produce many biological compounds that enhance the human health so they can be used as “nutraceuticals cell factories” for food enrichment. In this regard, propionibacteria have already been considered as rich sources of conjugated linoleic acid, vitamins, exopolysaccharides and trehalose.

Many health benefits have been attributed to consumption of CLA-containing foods such as anticarcinogenic, antiatherogenic, antidiabetogenic and antioxidative properties, immune system modulation and reduction of body fat gain [94]. CLA-isomers are formed by biohydrogenation of LA in the rumen and through conversion of vaccenic acid by Δ^9 -desaturase in the mammary gland so that ruminant meats and milk-derived products are main dietary sources of CLA. However, some microorganisms like *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *Propionibacterium* possess a linoleic acid isomerase that allow them to form CLA as a detoxification mechanism [95]. In consequence, they have been intended, either as starter or adjunct cultures, to increase the CLA level and nutritional value of some fermented products like yoghurt and cheese.

In this regard, several studies have shown the potential of propionibacteria for producing CLA enriched products. Both growing and resting cells of dairy (*P. freudenreichii*) [96, 97] and cutaneous propionibacteria (*P. acnes*) [98] produce cis-9, trans-11 and trans-10, cis-12, the major isomers with biological activity, on different growth media: culture broths [97], lipid containing plant materials [99], milk and ripening cheese [100].

By varying the source of LA for conjugation and the fermentation conditions it has been observed that *P. freudenreichii* convert free LA to mainly extracellular CLA with a high efficiency (50-90%), being the optimal conditions that favor the accumulation of CLA also determined [97, 101]. Besides, it has been observed that CLA formation and growth of dairy propionibacteria in fermented milks were enhanced in the presence of yogurt microorganisms whereas organoleptic attributes obtained with yogurt starter cultures were not affected by co-cultures with the propionibacteria [100].

Vitamin B12 also called cobalamin, is an essential nutrient for the human body that plays a key role in the normal functioning of the brain and nervous system, the formation of blood and also the metabolism of every cell, especially affecting DNA synthesis and regulation, fatty acid synthesis and energy production. Its deficiency leads to a serious physiological disorder called pernicious anemia.

The pathway of vitamin B12 synthesis in *Propionibacterium freudenreichii* has been completely elucidated [40, 102]. This microorganism synthesizes cobalamin as a cofactor for propionic acid fermentation [41] and is the only bacteria, among B12 producers that possess the GRAS status of the United States Food and Drug Administration.

In consequence dairy propionibacteria are the preferred microorganisms for the industrial production of this vitamin and many efforts have been made to improve the production process by using genetic engineering [102, 103] and other biotechnological strategies like fermentation manipulations [104, 105].

Vitamin B2, also known as riboflavin, is the central component of the cofactors FAD and FMN, and is therefore required by all flavoproteins. As such, vitamin B2 is required for a wide variety of cellular reactions and is involved in vital metabolic processes in the body. It has been reported that *P. freudenreichii* NIZO2336, a mutant strain that produces larger amounts of riboflavin than the parental strain, improved riboflavin content of yogurt and riboflavin status of rats fed with this product [106].

Different studies have shown the possibility to obtain genetically modified strains of *P. freudenreichii* that overproduce B12 vitamin [102, 107], porphyrin [108], and riboflavin (vitamin B2) [107].

Propionibacteria also produce Vitamin B7 (biotin) and Vitamin B9 (folic acid), so that propionibacteria-containing products could be expected to be good sources of B-group vitamins.

Vitamin K (a group of 2-methyl-1,4-naphthoquinone derivatives), is an essential cofactor for the formation of γ -carboxyglutamic acid-containing proteins that bind calcium ions and are involved in blood coagulation and tissue calcification. Its deficiency has been associated with low bone density and increased risk of fractures from osteoporosis and intracranial hemorrhage in newborns [109]. Vitamin K1 or phyloquinone is present in plants, and vitamin K2, also called menaquinone, is produced in animals and bacteria that live in the intestine.

It has been reported that *Propionibacterium freudenreichii* produces large amounts of tetrahydromenaquinone-9 (MK-9 (4H)) and the precursor 1,4-dihydroxy-2-naphthoic acid (DHNA) which is a known bifidogenic factor [110-112]. In order to improve the production of these metabolites, different laboratory culture protocols that could be applied to an industrial scale have been assayed finding out that DHNA production is markedly influenced by carbon source limitation and the oxygen supply. An improvement in DHNA production could be obtained by a cultivation method that combines anaerobic fed-batch and aerobic batch cultures [112, 113].

In another study, Hojo et al. [114] assessed the concentration of MK-9 (4H) in commercial propionibacteria-fermented cheeses finding out a positive correlation between the increase in propionibacteria and the generation of MK-9 (4H) in cheese. Due to their high MK-9 (4H) concentrations (200 to 650 ng/g), Emmental and Jarlsberg cheeses should be a meaningful source of vitamin K and potential protectors against osteoporosis.

Exopolysaccharides-producing bacteria and their secreted EPS are important biological thickeners for food industry. Besides, some health promoting properties such as immunomodulation and cholesterol lowering activities have been ascribed to EPS [115].

In dairy propionibacteria (*P. freudenreichii* subsp. *shermanii*), the single gene *gtf* encoding for a β -D-glucan synthase that is responsible for the synthesis of surface polysaccharide has been identified [40, 116] and the EPS produced was also characterized. Both homopolysaccharide [116, 117] and heteropolymers [118] were described and it has been reported that production of EPS by propionibacteria is a strain-dependent property (due to an IS element in the *gtf* promoting sequence) that is influenced by the medium composition and the fermentation conditions [119, 120]. Further studies are needed to elucidate the role of these polymers and their potential applications.

Trehalose has been proposed as a healthy sugar substitute in foods because of its anticariogenic and dietetic properties. As described in paragraphs above, propionibacteria synthesize trehalose as a reserve compound and as a stress-response metabolite [52-55]. With respect to the production of this sugar in situ in food products, it has been observed that *P. freudenreichii* ssp. *shermanii* NIZO B365 produces high levels of trehalose in skim milk [54].

Technological property	General comments	References
Dairy starter	<i>Propionibacterium freudenreichii</i> is included in the starter of Swiss type cheeses. It contributes to the typical flavor and the development of characteristic “eyes”	[56, 57], [59].
Antimicrobials	<p><i>P. acidipropionici</i> could be considered for biological production or propionic acid to be used as food preservative.</p> <p>Microgard™ and BioProfit are commercial products that include propionibacteria aimed for controlling spoilage microorganisms.</p> <p>Different bacteriocins are produced by both dairy and cutaneous propionibacteria that are active against gram positive and gram negative bacteria. They have a potential application as safe biopreservatives</p>	<p>[68-71].</p> <p>[73-75].</p> <p>[81-89].</p>
CLA	Propionibacteria produce cis-9, trans-11 and trans-10, cis-12, CLA isomers on culture broths; lipid containing plant materials; milk and ripening cheese. They have potential for producing CLA enriched products.	[96-101].
Vitamins	<p><i>Propionibacterium freudenreichii</i> is the only GRAS status producer of Group B vitamins: B2, B7 (biotin), B9 (folic acid) and B12.</p> <p>Genetically modified overproducer strains have been experimentally obtained.</p> <p>Propionibacteria produces vitamin K (MK-9 (4H) and its precursor DHNA with bifidogenic activity.</p>	<p>[103-108].</p> <p>[110, 114].</p>
EPS	Propionibacteria produce homo and heteropolysaccharides that could be used as food thickeners.	[117, 121].
Trehalose	<i>P. freudenreichii</i> synthesizes trehalose that could be used as sugar substitute in foods	[54].

Table 2. Technological relevance of the genus *Propionibacterium*

4. Probiotic application of dairy propionibacteria

Since the last decades, there has been an increasing interest from food and pharmaceutical industries to develop healthy foods and therapeutic alternatives to conventional antibiotic treatments in response to consumers' demands of natural products. Probiotics are "live microorganisms that confer health benefits to the host when administered in adequate amounts" [121]. In this respect, the great bulk of evidence concerning the beneficial effects of microorganisms both in human and animal health refers to lactic acid bacteria and bifidobacteria as they are common inhabitants of the gastrointestinal tract. However, in recent years several potential probiotic properties of propionibacteria have been reported and many studies on this subject have been published. In the following sections, safety aspects and the major health benefits ascribed to dairy propionibacteria are reviewed.

4.1. Safety and persistence in the gut

Strains selected on the basis of their potential beneficial effects by *in vitro* tests, must demonstrate their safety both in humans and animals, before they could be incorporated as probiotics, either in food or pharmaceutical products.

In this sense, dairy propionibacteria have a long history of safe use in human diet and animal feed. *P. freudenreichii* is widespread consumed in Swiss type cheeses in which they are present in concentrations close to 10^9 bacteria/g. Besides, classical propionibacteria have been isolated from soil, silage, vegetables, raw milk, secondary flora of cheese and other naturally fermented food. Therefore, it could be considered that they would arrive to the gut of different organisms, including the man, at least once in their lives.

At present, no cases of sickness or toxicity after the ingestion of dairy propionibacteria have been reported [122] neither for humans (for a review of human trials see [123]) nor for animals [124-126]. In fact, it has been reported that propionibacteria did not translocate to blood, liver or spleen and no adverse effects on body weight gain and general health status was observed after short [124, 127] and long terms [125] administration of strains of *Propionibacterium acidipropionici*, *P. freudenreichii* and *P. jensenii*, respectively.

Most studies have been performed with strains of *P. freudenreichii* since it is the traditional component of cheese starters being this species granted the Generally Recognized As Safe (GRAS) status from the US Food and Drug Administration. Furthermore, *P. freudenreichii* belongs with *P. acidipropionici*, to the list of agents recommended for Qualified Presumption of Safety (QPS) by the European Food Safety Authority [122, 128].

On the other side, most strains isolated from humans and animals belong to the "cutaneous group" [18, 129] and their use as probiotics is discouraged since they are potential pathogens. However, propionibacteria isolated from the intestine of animals and identified by molecular tools as dairy species, were not associated to pathogenesis.

Besides safety, other criteria to take into account in the selection of strains for dietary adjuncts are the absence of antibiotic resistances (due to the risk of spreading any resistance to intestinal microbiota) and virulence factors. Dairy propionibacteria have natural resistance to some antibiotics and this resistance does not appear to be encoded by plasmids or other mobile genetic elements [36, 122, 130]. By comparative genomics, no virulence factors found in *P. acnes* or in other pathogenic species were identified in *P. freudenreichii*, although some *P. thoenii* and *P. jensenii* strains have β -haemolytic activity [40, 122].

In order to exert their beneficial effects in the host, it is generally accepted that ingested microorganisms must survive the hostile environmental conditions of the gastrointestinal tract represented by the low pH of the stomach and intestinal fluids such as bile and pancreatic enzymes. Many studies have demonstrated by *in vitro* assays the ability of dairy propionibacteria to survive and tolerate the gastrointestinal conditions [130-134]. This tolerance could be improved by a pre-adaptation of the microorganisms to the adverse conditions of the gut by a brief exposure to the stressful conditions at a non-lethal level [48, 135].

Both acid and bile tolerance have shown to be strain-dependent properties. In previous studies [131, 132] we observed that dairy propionibacteria developed in a medium containing bile (0 – 0.5%) behaved as “bile-tolerant” and “non bile-tolerant” strains and that there were differences among *P. freudenreichii* and *P. acidipropionici* strains in their tolerance to pancreatic enzymes when subjected to sequential digestion with artificial gastric and intestinal fluids.

It has also been demonstrated that the vehicle used for delivery of probiotics is important for digestive stress tolerance since cells included in food matrices like milk or cheese had better tolerance to acid challenge than free cultures [132]. Similar results were obtained by Huang and Adams [134], by protecting propionibacteria from acid and bile stresses with a soymilk and cereal beverage, and Leverrier et al. [136], who used yoghurt-type fermented milk.

Survival of propionibacteria during gastrointestinal transit has also been reported *in vivo* in rats [125, 126]; mice [124, 137] and humans [127, 130, 133]. Furthermore, Herve et al. [34], demonstrated that propionibacteria remain metabolically active since the *P. freudenreichii*-specific transcarboxylase mRNA was detected in human faeces. In most studies, a high level of propionibacteria was detected in intestinal contents and feces during the feeding period but this concentration gradually declined and returned to the initial levels a few weeks after consumption ceased.

Besides surviving the gastrointestinal digestion, intended probiotics must remain in high levels in the intestine avoiding normal washout by peristaltic contractions of the gut. Therefore, microorganisms with a short generation time and/or the ability to adhere to the mucosa would have an extended survival in the body of the host. Bacterial adhesion to

intestinal cells and mucus is generally considered as the initial step in the colonization of the gut and has been related to many of the health effects of probiotics, as it prolongs the time that beneficial bacteria can influence the gastrointestinal microbiota and immune system [138]. Since propionibacteria grow slowly in natural environments and culture media, adhesion ability becomes an important property in the selection of strains for probiotic purposes.

Dairy propionibacteria have demonstrated to adhere to immobilized mucus [139]; to isolated mouse intestinal epithelial cells [140,141], to human intestinal cell lines [142-144] and *in vivo* to intestinal cells as was assessed by counting the adhering propionibacteria on the mucosa by a plate count method [124, 125, 137, 145].

In previous studies, we have correlated the *in vitro* and *in vivo* abilities of dairy *Propionibacterium* strains to adhere to intestinal epithelial cells and observed by scanning electron microscopy, that *P.acidipropionici* CRL 1198 adheres well to IEC or the mucus layer covering them [141]. Microscopic examination revealed two adhesion patterns in propionibacteria: autoaggregating strains adhere in clusters, with adhesion being mediated by only a few bacteria, whereas nonautoaggregating propionibacteria adhere individually making contact with each epithelial cell with the entire bacterial surface [140].

Besides, the adhesion of propionibacteria of different dairy species such as *P. freudenreichii* subsp. *shermanii* JS, *P. jensenii* 702 and *P. acidipropionici* Q4 to Caco-2, C2BBel and HT29 cells respectively, was clearly stated [142-144].

Interactions with the host gut mucosa are also suggested by the analysis of the genome of *P. freudenreichii* that revealed the presence of genes encoding for a high number of surface proteins involved in adhesion and present in other probiotic bacteria [40].

To date, the ability of dairy propionibacteria (used alone or combined with other microorganisms) to improve the health of humans and animals by being used as dietary microbial adjuncts has been extensively investigated. Their health promoting effects could be attributed to one or more of the following modes of action: *i*) immunomodulation; *ii*) influence on gut microbial composition and exclusion of pathogens; and *iii*) modulation of the metabolic activities of the microbiota and host. Main evidences obtained by *in vitro* and *in vivo* studies supporting the potential of dairy propionibacteria to be used as probiotics are summarized below.

4.2. Propionibacteria for improving animal health

Nowadays, the usage of probiotics as an alternative to antibiotics to enhance the growth and health of domestic animals is a growing practice. With this aim, different bacterial genera have been isolated from the intestine of farm animals and pets and employed as probiotics, such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus* [146].

To date, most animal studies have been performed with ruminants (cows, calves, steers), chicken, pigs, and to a lesser extent with horses and pets. In this sense, it has been reported that dairy propionibacteria administered alone or combined with other microorganisms increase the weight gain, feed efficiency and health of different animals such as laying hens and broilers [147], pigs [148] and calves [149, 150].

Propionibacteria are natural inhabitants of the rumen microbiota. In consequence, they have been used as direct-fed microbial (DFM) feed additives in ruminant nutrition with strain-dependant results on animal performances.

One desired effect for ruminant probiotics is an improvement in propionate production as it is considered the major precursor for hepatic gluconeogenesis that provides substrate for lactose synthesis in lactating dairy cows. Various strains of *Propionibacterium* have increased the molar proportion of ruminal propionate when fed to ruminants [151, 152]. In this respect, many researches have been done with the dairy strain *Propionibacterium acidipropionici* P169. It has been reported that, when administered to beef cattle, this microorganism was able to increase hepatic glucose production via enhanced ruminal propionate production and absorption, whereas directly fed to early lactating dairy cows, it tended to improve milk proteins content and energetic efficiency during early lactation, without affecting the reproductive function [152-154]. In general, these authors concluded that strain P169 might have potential as an effective direct-fed microorganism to increase milk production in dairy cows.

In other studies, the supplementation of lactating dairy cows with a DFM product containing a mixture of *L. acidophilus* and *P. freudenreichii* improved milk and protein yield, and apparent digestibility of crude protein, neutral detergent fiber, and acid detergent fiber, so that it could be used to enhance the performance of cows subject to heat stress during hot weather [155].

With respect to calves, a preparation called Proma, which is a blended culture of lactic acid bacteria plus *P. freudenreichii* and a DFM product containing *P. jensenii* 702 showed to be effective to improve weight gain during pre-weaning and weaning periods [149, 150].

Propionibacteria have also been assayed as health and growth promoters in monogastric animals like pigs, with positive results. Mantere-Alhonen [148] was the first to achieve growth promotion in piglets fed with different species of propionibacteria being *P. freudenreichii* ssp *shermanii* the most effective probiotic among the species tested. When propionibacteria were fed to piglets in a daily concentration of 2×10^9 cfu/g, the weight gain was 9.2-14.5% higher, the fodder demand was 7.2-46.1% lower than the control group and the animals had less diarrhoea. In bigger swine, the effects were even more evident.

Cutaneous propionibacteria have also been used to improve the health of swine. *Propionibacterium avidum* KP-40 showed to be a potent immunomodulator that stimulated granulopoiesis as well as a faster body weight gain in pregnant swine and their offspring [156]. The usefulness of the prophylactic application of this strain, against porcine microbial infections was tested in swine finding out that propionibacteria application caused positive

immunoregulation of porcine innate immune system effectors, non-specific activation of lymphocytes and antibody production that resulted in milder clinical symptoms, faster recovery and a larger body weight gain [157, 158].

In chicken, both undefined and defined “Nurmi Cultures” have been used to establish an intestinal flora that will prevent colonization by pathogenic bacteria in young animals. These formulas have shown to be effective for the protection against species of *Salmonella* and other avian pathogens; for immune system stimulation in newborn chicks, and also had growth promoting effects [159, 160]. The most frequently assayed bacteria as avian probiotics were several species of lactic acid bacteria [146, 159, 160]. Propionibacteria have not been widely studied in this ecological niche. However, some authors demonstrated the presence of this bacterial group in the ileum and cecum of chickens [161], and cecal Nurmi cultures characterized by microbiological and PCR-DGGE techniques, evidenced the presence of *Propionibacterium propionicus* [147].

In recent studies, the occurrence of *Propionibacterium* in different segments of the gastrointestinal tract of laying hens was demonstrated. Bacteria from this genus were evidenced in 27% of the animals sampled. Half of these isolates were identified by genus and species specific PCR as *P. acidipropionici*, belonging the others to the propionibacteria cutaneus group. This report represents the first evidence of dairy propionibacteria as inhabitants of the gastrointestinal tract of chickens. Some preliminary studies on the probiotic properties of these strains, suggest their potential application as probiotic to prevent intestinal infections in poultry [17].

4.3. Probiotic properties for human application

Immunomodulation: One of the most promoted properties of probiotics is their ability to regulate in a positive manner the innate and adaptive responses of the human immune system. It is well-documented that cutaneous propionibacteria are potent immunomodulators, since they have been tested in several assays both in humans and rodents used as animal models [162]. Administration of cutaneous propionibacteria (*P. avidum*, *P. granulosum*, *P. acnes*) have shown to be beneficial in the treatment of neoplastic and infectious diseases [163-165]. Besides, dead *Propionibacterium acnes* or a polysaccharide extracted from its cell wall have proven to be effective in the induction of macrophages with an antitumor effect [166] and in modulating an experimental immunization against *Trypanosoma cruzi* [167].

With respect to the immunomodulatory properties of dairy propionibacteria, many researches have been done *in vitro* and *in vivo* with the strain *P. freudenreichii* subsp. *shermanii* JS. It has been reported that this microorganism stimulated the proliferative activity of B and T lymphocytes depending on doses administration and treatment duration in mice [168]. Regarding to cytokine production, *P. freudenreichii* JS was able to induce TNF- α and IL-10 production in human PBMCs [169] and inhibited the *H. pylori*-induced IL-8 and PGE2 release in human intestinal epithelial cells [170].

Other dairy *P. freudenreichii* strains also showed promising immunomodulatory properties by strongly inducing the synthesis of anti-inflammatory IL-10 by human PBMCs and could be helpful in the treatment of inflammatory conditions or diseases [171].

Further beneficial results with *P. freudenreichii* JS were obtained with different randomised, placebo-controlled, double-blind trials in humans such as: reduction in the serum level of C-reactive protein (an inflammation marker) [172]; induction of IL-4 and IFN-gamma production in PBMCs of infants with cow's milk allergy [173]; prevention of IgE-associated allergy in caesarean-delivered children [174] and increase in the resistance to respiratory infections during the first two years of life [175].

With respect to other dairy species, an increase in the phagocytic activity of peritoneal macrophages and the phagocytic function of the reticuloendothelial system was observed in mice fed with *Propionibacterium acidipropionici* CRL 1198 [124]. In addition, administration of this strain prior to infection of mice with *Salmonella* Typhimurium led to an increase of the anti-*Salmonella* IgA level and the number of IgA producing cells [176].

Dairy propionibacteria may also act as safe adjuvant for development of oral vaccines. Adams et al [177] found that *Propionibacterium jensenii* 702 co-administered orally with soluble *Mycobacterium tuberculosis* antigens to mice stimulate T-cell proliferation of splenic lymphocytes in a significant manner so that the strain PJ702 could act as a potential living vaccine vector to be used against mucosal transmitted diseases.

4.4. Gut microbial modulation

Stimulation of bifidobacteria: It is well-documented that propionibacteria can modulate gut microbiota in a positive manner by enhancing bifidobacterial growth. This property has been demonstrated both *in vitro* [110, 111, 178, 179], and *in vivo* [127, 180-182] and the bifidogenic growth stimulators (BGS) involved in this effect were identified. The active compounds that were present in supernatants of *P. freudenreichii*, *P. jensenii* and *P. acidipropionici* were purified and identified as 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) [110, 178] and 1,4-dihydroxy-2-naphthoic acid (DHNA) a precursor of menaquinone (vitamin K₂) biosynthesis [111]. It has been proposed that these compounds serve as electron transfer mediators for NADP regeneration in bifidobacteria [183], thus favoring growth.

The bifidogenic effect of selected strains of *P. freudenreichii* [127, 180-182] or purified BGS [184] was assessed in independent studies performed on human volunteers. As a general result, increased fecal bifidobacterial populations were observed even after some days after stopping the consumption of propionibacteria. Besides a reduced colonic transit time and a reduction in the numbers of clostridia were evidenced in some studies.

Inhibition of pathogens: There are several reports on the ability of dairy propionibacteria to inhibit exogenous and opportunistic pathogens. *In vitro* studies have demonstrated that *P. freudenreichii* strain JS was able to inhibit, alone or combined with other probiotics the

adhesion of different pathogens including *H. pylori* to intestinal mucus and Caco2 cell line also improving the epithelial barrier function [170, 185]. Other dairy species like *P. acidipropionici* strain Q4 was able to prevent the adhesion of *Salmonella enteritidis* and *Escherichia coli* to HT29 cells [144] whereas *P. acidipropionici* CRL 1198 regulates *in vitro* the growth of *Bacteroides* and *Clostridium* in cecal homogenates of mice supplemented with propionibacteria and/or inulin [33]. Mice consuming this strain delivered in water, milk or cheese showed a decrease in the number of anaerobes and coliforms in the caecal content one week after feeding [124, 137, 145]. *P. acidipropionici* CRL 1198 also prevented tissue colonization by *Salmonella Typhimurium* in mice [176].

In humans, propionibacteria have been used in combination with *Lactobacillus spp.* and *Bifidobacterium spp.* in the treatment of intestinal disorders and regulation of gut flora and motility. It has been demonstrated that the consumption of probiotic mixtures containing *Propionibacterium freudenreichii* JS reduced oral *Candida* in elderly [186] and gastric inflammation of the mucosa caused by *H. pylori* in the host. [187]. Besides, infants and children fed with Propiono-Acido-Bifido (PAB) milk [188] or milk containing *P. freudenreichii* subsp. *shermanii* and *L. acidophilus* [189], showed a reduction in coliforms with an increase in lactobacilli and bifidobacteria population.

Alleviation of IBD: It has been demonstrated that consumption of either isolated BGS or *P. freudenreichii* strains ameliorate experimental colitis in mice and human ulcerative colitis [171, 189-192]. The mechanism proposed for this effect was restoring of microbiota intestinal balance and suppressing inflammatory lymphocyte infiltration. In this respect, it has been proposed that some surface compounds should be involved in immunomodulatory effects of propionibacteria since removal of surface layer proteins decreased the *in vitro* induction of anti-inflammatory cytokines [171]. By their side, Michel et al. [193] demonstrated that colonic infusion with *P. acidipropionici* reduced the severity of TNBS induced colitis in rats whereas Kajander et al [194] reported that the multispecies probiotic mixture containing *Propionibacterium freudenreichii* JS was effective in alleviating irritable bowel syndrome symptoms.

4.5. Modulation of the host and resident microbiota metabolism

Lactose malabsorption: The ability of probiotics to alleviate lactose intolerance by supplying β -galactosidase for the intraintestinal hydrolysis of lactose has been widely reported for LAB and bifidobacteria [196]. However there are no clinical reports on this property for dairy propionibacteria. Several evidences suggest the potential of *Propionibacterium acidipropionici* strains on this subject: they have high β -galactosidase activity that remain unaltered in the conditions of the human's intestine, and cells are permeabilized by bile, which in turn may favour the hydrolysis of lactose within the intestine [131, 132]. Besides, the manufacture conditions of Swiss-type cheese did not decrease the synthesis and activity of the β -galactosidase of these propionibacteria [197]. When mice were fed with *P. acidipropionici* CRL 1198 included in milk or cheese, the β -galactosidase levels in the small bowel and the propionic

acid concentration in the caecum were significantly increased. High SCFA concentration in the colon could counteract diarrhea induced by non-digested carbohydrates [137].

Hypocholesterolemic properties: The reduction of cholesterol has been assessed for many probiotics with conflicting results. Somkuti and Johnson [198] evidenced the ability of *P. freudenreichii* cells to remove by surface adsorption up to 70% of the cholesterol from the medium, whereas Perez Chaia et al [124] demonstrated, in an animal study, that *P. acidipropionici* CRL 1198 was able to reverse the hyperlipemic effect of a diet with a high lipid content. However, the mechanisms underlying this beneficial effect were not determined in this investigation.

Antimutagenic properties: Vorobjeva [199] demonstrated the antimutagenic activity (AM) of *Propionibacterium freudenreichii* against the mutations induced by 4-nitro-quinoline and N-nitro-N-nitrosoguanidine (transition mutations), and by 9-aminoacridine and 2-nitrofluorene (frame-shift mutations). This AM activity was exerted by live and dead cells and by the cultured media. The active compound responsible for this activity was identified as a cysteine synthase which is induced by some stress factors.

Anticarcinogenic properties: Several *in vitro* and *in vivo* studies (mainly in animal models) have suggested the potential of probiotics to prevent colon cancer as evidenced by a decrease in the incidence and magnitude of tumours and preneoplastic lesions [200]. Among the mechanisms involved it could be mentioned: inhibition of enzyme activities that convert procarcinogens into carcinogens, control of harmful bacteria, antigenotoxicity, production of active metabolites and immunomodulation.

Regarding propionibacteria, it has been demonstrated that *P. acidipropionici* CRL1198 fed to mice was able to modulate the metabolism of the resident microbiota as it prevented the induction of azoreductase, nitroreductase and β -glucuronidase activities caused by a cooked red-meat supplemented diet. Furthermore, feeding with propionibacteria resulted in a remarkable reduction of β -glucuronidase activity and slight reductions of azo and nitroreductase activities [201]. In humans, independent researches have shown that consumption of *P. freudenreichii* subsp. *shermanii* JS decreased to different extents fecal azoreductase activity in elderly subjects, β -glucosidase and urease in healthy young men and β -glucuronidase activity of irritable bowel syndrome patients [202, 203].

Other studies have reported that dairy propionibacteria kill human colorectal adenocarcinoma cells *in vitro* through apoptosis via their metabolites, propionate and acetate [204, 205]. In addition, consumption of *P. freudenreichii* TL133 by human microbiota associated rats significantly increased the number of apoptotic cells in the colon of 1,2-dimethylhydrazine treated rats but have no effect on healthy colonic mucosa [77]. The authors suggest that dairy PAB may help in the elimination of damaged cells by apoptosis within the colon epithelium after genotoxic insult. Long term studies assessing the protective role of PAB against colon cancer are still missing.

4.6. A less studied mechanism: *Binding of toxic compounds*

Foods daily ingested by humans and animals may possess besides nutrients, many toxins and antinutritive factors that could be endogenous (i.e., compounds naturally occurring because of the inherent genetic characteristics of the plant or animal used as food) or produced by the action of microorganisms, under the influence of physical factors, or by chemical reactions between food constituents. Among these deleterious compounds it could be mentioned: trypsin inhibitors, lectins, biogenic amines, mycotoxins, etc. In this respect, several studies have focused, in recent years, on the ability of safe bacteria to bind and remove toxic compounds from different environments such as the gut and food.

Numerous findings have shown that intestinal microorganisms and lactic acid bacteria ingested with food, including probiotics, play a role in detoxification of various classes of DNA-reactive carcinogens such as heterocyclic aromatic amines (HAs), pyrolysis products of amino acids contained in meat and fish products [206-209].

Most studies have ascribed this effect to the physical binding of the mutagenic compounds to the bacteria rather than their metabolism. The binding of the HAs (Trp-P-2, PhIP, IQ and MeIQx) to bacteria is generally measured by HPLC and/or the decrease in mutagenicity in bacterial assays (mainly in *Salmonella* frameshift tester strains) and genotoxicity by comet assay. In attempts to elucidate the mechanisms involved in the binding of Tryptophan pyrolysates it was found that the structure of the cell wall plays a role in the inactivation and that the effect may involve cation exchange processes. Although gram-positive strains were more effective than gram-negative to remove HAs, these compounds bound both to peptidoglycan and outer membrane. Sreekumar and Hosono [209] studied the binding of Trp-P-1 to *Lactobacillus gasseri*, and postulated that the binding receptors of the HAs are the carbohydrate moieties of the cell walls and that glucose molecules play a key role in the binding reaction. By comparing, the effects of heat inactivated cells with those of living cells, it was suggested that living bacteria may also produce metabolites or catalyze reactions which lead to the detoxification of the amines [208]. However there are no reports on the ability of propionibacteria to detoxify HAs.

Another detoxification property proposed for probiotics is their ability to remove mycotoxins. These fungal metabolites are carcinogens that unavoidable contaminate cereals and grains destined for human consumption. Mycotoxins are also forage contaminants, which impair animal performances and health. Several probiotic bacteria, commonly used in food products, have been shown to bind Aflatoxin B1 and the toxins produced by *Fusarium* sp such as zearalenone, fumonisins B1 and B2 and trichothecenes, like deoxynivalenol (DON), nivalenol (NIV) and T-2 toxin (T-2) preventing their absorption in the gastrointestinal tracts of animals and humans [210-214].

The capacity of *Propionibacterium freudenreichii* strain JS used alone and combined with lactobacilli (*L. rhamnosus* GG or LC705) to remove mycotoxins has been studied by *in vitro* [210-212], *ex vivo* [211] and *in vivo* assays [213-214]. It has been determined that both viable and heat-killed forms of propionibacteria are able to remove efficiently aflatoxin B1,

fumonisin and trichothecenes from liquid media. Binding, not biodegradation appeared to be the mode of action, as no toxin derivatives were observed and removal was not impaired in nonviable bacteria. Kinetics of adsorption and desorption of Aflatoxin B1 by viable and no viable bacteria have also been determined [215]. Tested *ex vivo* in the intestinal lumen of chicks, there was a 63% reduction in the uptake of AFB1 by the intestinal tissue in the presence of *P. freudenreichii* JS and its binding ability seems to be even better than *in vitro* results [211]. When combined with *L. rhamnosus* LC-705, 57-66% of AFB1 was removed by the probiotic mixture *in vitro* whereas 25% of AFB1 was bound by bacteria in *ex vivo* experiments being tissue uptake of AFB1 also reduced when probiotic bacteria were present in the duodenal loop [211].

Intestinal mucus significantly reduced AFB1 binding by the probiotic mixture and vice-versa (preincubation with AFB1 reduced mucus binding) [216]. However, similar binding sites are unlikely to be involved, since heat-treated bacteria lost their ability to bind intestinal mucus, whereas AFB1 binding was found to be enhanced by heat treatment. It has been proposed that proteins must be involved in the binding of mucus, whereas carbohydrates must bind AFB1 [217, 218]. Other mechanisms, such as steric hindrance, may cause interference in AFB1 and mucus binding by bacteria. These findings have relevance, since probiotics adhering to the intestinal wall are less likely to bind and consequently accumulate AFB1 in the host. On the other hand, probiotics with AFB1 bound to their surfaces are less likely to adhere to the intestinal wall and prolong exposure to dietary AFB1. Specific probiotics may be significant and safe means to reduce absorption and increase excretion of dietary AFB1 from the body.

On clinical trials it has been observed that the consumption of a probiotic preparation containing both *P. freudenreichii* JS and *L. rhamnosus* LC-705 reduced in a significant manner the levels AFB₁ in fecal samples [213] and the concentration of urinary AFB-N⁷-guanine [214] of healthy volunteers during treatment and even after several days after probiotic consumption ceased. These results suggest that the probiotic bacteria used in these trials could block the intestinal absorption of aflatoxin B1.

Dietary exposure to heavy metals and cyanotoxins may have detrimental effects on human and animal health, even at low concentrations. Specific probiotic bacteria may have properties that enable them to bind these toxins from food and water. In this respect, it has been reported that *P. freudenreichii* spp. *shermanii* JS alone and combined with other probiotics have the ability to remove microcystin-LR [219] and also cadmium and lead from aqueous solution [219, 220] and could be considered a promising microorganism for decontamination in food and intestinal models.

Lectins are proteins which interact selectively and reversibly with specific residues of carbohydrates present in glycoconjugates [221]. Although their biological relevance as recognition molecules is well-known their physiological role and impact on health is controversial since both beneficial and deleterious effects have been ascribed to different lectins [222, 223]. Plant lectins are widespread in the human diet, in food items such as

vegetables, fruits, cereals, legumes, etc, so their ingestion could be significant [224]. They are also present in other members of the *Leguminosae* and *Gramineae* Families that are used as farm feeds.

Most plant lectins are highly resistant to degradation by cooking and by digestive processes, so after consumption, they reach the intestinal lumen in a bioactive state and bind specifically to carbohydrate moieties expressed on the glycocalix of enterocytes affecting cellular physiology [221]. In general, lectins from the *Leguminosae* Family are considered as antinutritive or toxic substances since they lead to deleterious morphological and physiological changes after binding to the intestinal mucosa. Those changes include the thinning of the mucus lining, reduction of the absorptive function and nutrient utilization, genotoxic effects like single strand breaks in the DNA and stimulation of cellular proliferation and turnover that could lead to tumors development [225-229]. Some of these alterations could be initially unnoticed but lead to important nutritional deficiencies in the long term, being their impact on health of significant relevance.

Different alternatives have been proposed in order to prevent or counteract the deleterious effects of toxic or antinutritional dietary compounds on the GIT (Figure 2), being of particular interest those that focus on a suitable complementary diet. Regarding lectins, it has been proposed that a high dietary intake of carbohydrate-containing foods, complementary to most toxic lectin expected in the diet, would offer protection by binding free lectin in the colonic lumen (Figure 2a). In this sense, it has been reported that the consumption of sucrose may reduce the toxic effects of legume lectins such as red kidney beans by protecting barrier function, bacterial overgrowth and bacterial translocation [230]. In the same way, it has been proposed, that a high consumption of galactose-containing carbohydrates, such as galactose-containing vegetable fiber, would offer protection against binding and proliferative effects of galactose-N-acetylgalactosamine-binding dietary lectins (such as PNA) on colonic neoplastic epithelium [229, 231].

The same role could be played by bacteria with suitable sugar residues on their surface, that would reduce the interaction between dietary lectins and cells by competing for the sites where these molecules bind (Figure 2b), by capturing and removing free lectins (Figure 2c) or by binding to different receptors and blocking lectin access to their receptors (Figure 2d).

With this concept in mind, it could be proposed that probiotic microorganisms with the appropriate surface glycosidic moieties could be consumed as a part of human or animal diets to interfere with the cell-lectin recognition process preventing some toxic effects. In consequence, in recent years we have initiated a research line aimed to assess the capacity of dairy propionibacteria to protect the intestinal mucosa from the deleterious effects of dietary lectins.

Figure 2. Mechanisms proposed to counteract the interaction lectin-intestinal cell. A) Dietary carbohydrates complimentary to free lectin in the intestinal lumen; B) Bacterial binding analogous to lectin binding; C) Microorganisms that bind free lectins; D) Microorganisms that adhere to the epithelium blocking the binding of lectins to intestinal receptors.

In a recent study [232], we have assessed *in vitro* the citotoxic effects of three plant lectins: concanavalin A (Con A), peanut agglutinin (PNA) and jacalin (AIL) on intestinal epithelial cells (IEC) of mice finding out that the three lectins used in the study induced cells death in a different extent. The effect was remarkable only with Con A and AIL since they reduced the percentage of viable cells from $88 \pm 12\%$ to $63 \pm 10\%$ and $64 \pm 12\%$ respectively after 120 min of contact as determined by Trypan Blue dye exclusion.

Then we evaluated the ability of different dairy propionibacteria to bind those lectins decreasing their citotoxic effects and the relation between bacterial adhesion to epithelial cells and protection against lectins. Two bacterial strains, with and without the property of adhesion to IEC, were studied for their ability to remove lectins from the reaction mixture. Both *Propionibacterium acidipropionici* (adh+) and *P. freudenreichii* (adh-) were able to remove 60–70% of Con A and AIL as determined by the free protein detected in the interaction supernatants. Removal was due to binding with specific sugar moieties on the bacterial surfaces, as was evidenced by inhibition in the presence of sugars specific for each lectin. It is known that dairy propionibacteria possess residues of glucose, mannose and galactose in

their cell walls depending on the species [233] that would allow their interactions with ConA and AIL. Besides, no growth or production of SCFA was observed in a synthetic medium supplemented with ConA or AIL as sole carbon and energy sources confirming the binding hypothesis.

When the supernatants of the interactions bacteria-lectin reaction mixtures were assayed for their toxic effect against IEC a great reduction on the percentages of necrotic cells was observed for both lectins (Table 3)

Conditions	Percentage of cells		
	Viable	Necrotic	Apoptotic
Control	85 ± 6	10 ± 7	5 ± 2
Con A	58 ± 3	35 ± 5	7 ± 5
<i>P. acidipropionici</i> + Con A	82 ± 4	9 ± 1	11 ± 4
<i>P. freudenreichii</i> + Con A	89 ± 2	5 ± 4	6 ± 2
AIL	62 ± 13	36 ± 5	2 ± 3
<i>P. acidipropionici</i> + AIL	78 ± 9	8 ± 2	13 ± 5
<i>P. freudenreichii</i> + AIL	75 ± 5	15 ± 2	10 ± 1

Table 3. Cytotoxic effects of lectins, and protection of colonic cells by lectin removal by propionibacteria. *Control*: Cells exposed to PBS. *Con A* and *AIL*: Cells exposed to 100 µg/mL of lectins; *Propionibacteria+lectins*: Supernatant of interactions bacteria-lectins after removal of bacteria. Viability was assessed by counting cells under the fluorescence microscope after propidium iodide/fluorescein diacetate/Hoescht staining. Adapted from Zárate and Pérez Chaia, J. Appl. Microbiol (2009)106: 1050-1058 [232].

Since the cellular damage was almost completely abolished when lectin solutions were preincubated with bacteria it is evident that microorganisms remove these compounds from the media avoiding their deleterious effects on cells.

Both strains were subjected to chemical and enzymatic treatments used to remove surface structures previous to their interaction with Con-A, and then were assayed for their ability to bind this lectin and to adhere to IEC. As shown in the Figure 3 different components are involved in the Con A-bacteria interaction depending on the strain studied.

In adherent *P. acidipropionici* both carbohydrates and proteins seemed to be involved in Con A removal since high cytotoxic effects of interaction supernatants was observed when these surface structures were removed. In contrast, the lectin removal by a nonadherent strain of *P. freudenreichii* only depended on cell wall carbohydrates as periodate treatment of bacterial cells was the only responsible for the loss of protective effect on IEC of this strain (Figure 3a, right). Besides, in adherent *P. acidipropionici* the lectin receptors on the bacterial surface and the adhesion determinants seem to be related,

since both the abilities to adhere to IEC and to remove Con A were lost after treatments with periodate and pronase E (Fig. 3a left and 3b). Con A bound to *P. acidipropionici*, reduced but not abolished adhesion of *P. acidipropionici* to IEC suggesting that carbohydrates other than glucose and mannose on the bacterial surface are also involved in the bacteria-IEC interaction (Fig. 3b)

Figure 3. Influence of bacterial surface components on lectins removal (a) and adhesion property (b). (a) Viability of IEC exposed to the interaction supernatants of Con A and propionibacteria treated with chemical agents in order to remove cell surface structures. (b) Adhesion ability (%) of treated propionibacteria after incubation with Con A. **wL**: propionibacteria without lectin interaction, **wb**: lectin without bacteria; **a**: Non-treated bacteria; **b**: protease treatment (cell wall proteins remotion); **c**: LiCl treatment (S-layer); **d**: periodate treatment (polysaccharides); **e**: phenylmethylsulfonylfluoride treatment (lectin-like adhesins). Reproduced from: Zárate and Perez Chaia, Journal of Applied Microbiology (2009) 106: 1050–1057 [232].

Although Con A is not a regular component of human diets, it is a good model to study the behaviour of members of the mannose binding lectins family, which include, among others, lectins found in lentils and kidney beans. However, Con-A and other lectins like WGA (from wheat) and SBA (from soy) could be found in feed formulations for broilers leading to epithelial damages and growth depression of BB chicks. In consequence, probiotic bacteria could be considered also by avian industry to avoid the undesirable effects of lectins on animal's health by capturing them or by blocking their ligands in the mucosa. In this respect, it has been observed that some LAB and *P. acidipropionici* isolated from the chicken gut were able to bind Con A and WGA (Babot et al 2012 unpublished results) so that further studies are actually ongoing in order to develop a lectin-protector probiotic for broilers.

Since the removal *in vitro* of Con A and AIL by dairy propionibacteria was an effective way to avoid the toxic effects against intestinal cells, we assessed *in vivo* the effects of Con A on some morphological and physiological parameters related to intestinal functionality such as small bowel architecture, main microflora components and disaccharidase activities of Balb/c mice after long term feeding with this lectin alone (8 mg/kg/day of Con A for 3 weeks) or with the simultaneous consumption of *Propionibacterium acidipropionici* CRL 1198 (5×10^8 CFU/mice/day) [145].

Long-term inoculation of adult Balb/c mice with Concanavalin A resulted in a less food efficiency since food consumption was not affected but animals gained less weights during this treatment, suggesting an alteration of the digestion/absorption function of the intestine in the presence of lectin. Other deleterious effects observed during Con A feeding include a significant increase of the stomach size and transient enlargement of other organs such as liver, small bowel and cecum; and histomorphological and physiological alterations. In fact, an increased intestinal epithelial cell proliferation, evidenced by the higher cellularity of the epithelium lining the villus and the disarrangement and stratification of nuclei was observed at the optical microscopic level. At the ultrastructural level, a marked shortening and shedding of microvilli were evidenced in the lectin treated group as could be seen in Fig. 4(a) and (b). Similar results were reported previously by Lorenzsonn and Olsen [225] who observed in the jejunum of normal rats, an increased shedding of brush border membranes, acceleration of cell loss and shortening of villi as acute effects after an intraluminal injection of Con A. or WGA.

The histomorphological modifications induced by Con A were greatly prevented by consumption of propionibacteria at the same time than Con A (Fig. 4c and 4d). By their side, mice that consumed *P. acidipropionici* CRL 1198 showed no remarkable differences with respect to the control animals.

Intestinal microbial populations were also modified by lectin feeding. Mice fed Con A showed increased enterobacteria and enterococci populations whereas lactobacilli, bifidobacteria and propionibacteria were not affected. Inclusion of *P. acidipropionici* CRL 1198 in the diet prevented these microbial modifications induced by Con A.

Figure 4. Transmission electron microscopy photomicrographs of the microvillous surface of the small bowel of mice fed with Con A (Group 2) (panels a-b) and those that consumed lectin plus propionibacteria (Group 4) (Panels c-d). Reproduced from Zárate and Perez Chaia, Food Research International (2012), 47(1): 13-22 [145].

With respect to physiological effects, since lectins interact in the intestine with the mucosa membrane; it could be expected that the processes that take place at this level, such as hydrolysis of dietary components and nutrients transport may be affected leading to a low nutritional status. Besides, structural alterations could also contribute to physiological changes. The four disaccharidases assessed in this study were affected by Con A to some extent. Daily Con-A feeding led to a significant decrease of lactase, sucrase, and trehalase activities whereas maltase seemed to be less affected. One week after treatments were finished sucrase and trehalase were still below control values. In general, consumption of propionibacteria with Con A resulted in activities similar to those of untreated animals and those fed propionibacteria alone (Figure 5).

From the results obtained up to now it could be suggested that consumption of foods containing these propionibacteria would be a valuable tool for protecting the intestinal mucosa of humans and animals from the undesirable interactions with antinutritional lectins.

Figure 5. Effect of Concanavalin A, *P.acidipropionici* CRL 1198 and lectin plus propionibacteria feeding on the disaccharidase activities of intestinal mucosa homogenates of Balb/c mice. G1: Control; G2: Con A, G3: *P. acidipropionici* CRL 1198, G4: Con A+ CRL 1198. Values are means $\pm$ SD. The asterisk indicates significant differences with the control group (G1) ($P<0.05$). Reproduced from Zárate and Perez Chaia, Food Research International (2012), 47(1): 13-22 [145].

Although probiotic microorganisms are considered a promising alternative to physico-chemical methods to be used as biological sequestering agents of toxins, further in vivo studies are needed in order to confirm that the inclusion of such microorganisms in the diet may reduce the absorption of deleterious compounds in the gastrointestinal tract.

5. Concluding remarks

From the extensive data reviewed in the present article it can be concluded that dairy propionibacteria are valuable microorganisms for both technological applications and health promotion. Although many studies have been made and the current knowledge of the genus has increased in different and well-defined fields further studies are needed in order to select the best strains and their most appropriate delivery vehicles. In this sense the

unique nature of the genus *Propionibacterium* (such as the resistance to stress and particular technological and probiotic properties) turns it, and particularly dairy species, as promising microorganisms to be incorporated in new types of food products. However, randomized, placebo-controlled, double blind human trials that confirm the properties of individual propionibacteria are still lacking. It could be expected that in the near future this void will be filled and new possible applications for propionibacteria will be discovered on the basis of newly available genome sequence and the recent development of molecular tools.

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