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Genetically Engineered Lactobacilli for Technological and Functional Food Applications

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1. Introduction

Lactic acid bacteria (LAB) are Gram-positive microorganisms that produce lactic acid as a major product of their metabolism. Among them the genus *Lactobacillus* comprises a large heterogeneous group of low G+C DNA content, anaerobic and nonsporulating bacteria, that includes species widely used in the food industry. They play key roles in fermented dairy, meats and vegetables products. Due to their claimed health-promoting properties certain lactobacilli species are used as probiotics and they are commonly applied to dairy and functional foods products. Lactobacilli have a relatively simple fermentative metabolism focused to rapidly convert carbohydrates into lactic acid, and exhibited a limited biosynthetic capacity. In addition, several tools and strategies to manipulate them genetically are available. All those characteristics make lactobacilli specially suited for genetic engineering aimed to increase existing compounds or to produce novel metabolites of interest for the food industry. Regarding probiotic lactobacilli, through genetic manipulation, the health attributes of probiotic strains can be enhanced or new probiotic activities can be developed and additionally, an understanding of the underlying molecular mechanisms can be obtained. Here, we review metabolic engineering strategies in lactobacilli that have successfully been used to efficiently reroute sugar metabolism to compounds such as L-lactic acid, aroma compounds (acetoin, diacetyl), low-calorie sugars (mannitol, sorbitol) and exopolysaccharides. We will also describe strains of probiotic lactobacilli that have been developed to exploit their adherence and immunomodulatory properties, and to delivery proteins at the intestinal mucosa.

1.1 Metabolic potential of lactobacilli

Because of their fermentative metabolism and global utilization in food fermentations, LAB are specially suited for rerouting sugar metabolism to produce industrially important food compounds. During fermentation, monosaccharides are catabolized through glycolysis (Embden-Meyerhoff pathway) and related pathways (Figure 1). Glycolytic catabolism of sugars involves phosphorylation reactions that drive hexoses to fructose-1,6-bisP, which is then hydrolysed to glyceraldehyde-3P (GADH-3P) and dihydroxyacetone-P. Then, GADH-

3P undertakes dephosphorylation and oxidation, which yields 2 pyruvate, 2 ATP and the reduction of 2 NAD^+ to 2 NADH per glucose. Under normal metabolic conditions NADH is used mainly to reduce pyruvate. Some LAB exclusively produce lactate from pyruvate (homofermentative), while other species (heterofermentative) produce lactate, acetate, ethanol and CO_2. There are other differences in heterofermentative species, since they can shift sugar catabolism towards the so called pentose phosphate or phosphoketolase pathway that renders GADH-3P and acetyl-P. Then, GADH-3P enters the lower part of the glycolysis. In this pathway two additional NADH molecules are oxidised by means of alcohol dehydrogenase that produces ethanol from acetyl-CoA. Therefore, the global balance of the heterofermentation of one mol of glucose is one mol of lactate, one mol of ethanol and one mol of CO_2, with a net energy yield of one mol of ATP.

An important strategy frequently used during metabolic engineering consists in blocking the formation of natural proton sinks, such as lactate or ethanol in the final steps of glycolysis. However, dissipation of the H^+ pool has such a great relevance that LAB normally has several isoenzymes of L-lactate dehydrogenase (L-LDH) as showed by the analysis of different genomes such as *Lactobacillus plantarum* and *Lactobacillus casei* (Kleerebezem *et al.*, 2003; Rico *et al.*, 2008), and alternative dehydrogenases yielding D-lactate have been found in most LAB genomes. An additional difficulty is imposed by the fact that glycolysis is subject to a strict allosteric regulation by its own intermediate and final metabolites, as well as by Pi, fructose-1,6bisP, phospho*enol*pyruvate (PEP), ADP, ATP and NADH/NAD^+ ratio. Its robustness and flexibility would assure an efficient bacterial growth, so that its activity rate would always respond to the cell's energy demand. It has also been observed that there are three enzyme activities especially sensitive to allosteric modulation, which are most relevant in the pathway's regulation and they are: phosphofructokinase (PFK), GADH-3P dehydrogenase and pyruvate kinase. PFK is strongly inhibited by PEP and pyruvate kinase activity is inhibited by Pi in *Lactococcus lactis* and this enzyme is stimulated by ADP and fructose-1,6bisP in *Lactobacillus bulgaricus* (Branny *et al.*, 1998). Furthermore, in *L. lactis*, GADH-3P dehydrogenase has a remarkable role in the modulation of the carbon flux, which is regulated by the NADH/NAD^+ ratio, as it happens with LDH (Garrigues *et al.*, 1997).

LAB suitability as starter cultures in dairy fermentations highly depends on their ability to produce small concentrations of volatile compounds derived from the alternative metabolism of pyruvate. The production of diacetyl and acetoin is quite common in LAB. These compounds are produced through decarboxylation of α-acetolactate obtained from pyruvate by the enzyme α-acetolactate syntase (Figure 2). Additionally, under substrate limitation and anaerobiosis, the enzyme pyruvate-formate lyase produces acetyl-CoA and formate from pyruvate and CoA. Acetyl-CoA is an important metabolite, as it can be used as electron acceptor to oxidise NADH or as energy compound to obtain ATP. The enzymatic complex of pyruvate dehydrogenase also produces acetyl-CoA, CO_2 and NADH from pyruvate, coenzyme A and NAD^+. Under aerobic conditions, this is an anabolic enzyme producing acetyl-CoA used for lipid synthesis, but under aerobic conditions, it also has a catabolic function where NADH oxidases can regenerate the excess of NADH produced. Pyruvate oxidase mediates onversion of pyruvate to acetyl-P, CO_2 and H_2O_2. This activity allows to obtain ATP when carbon sources are limiting, by substrate level phosphorylation of acetyl-P.

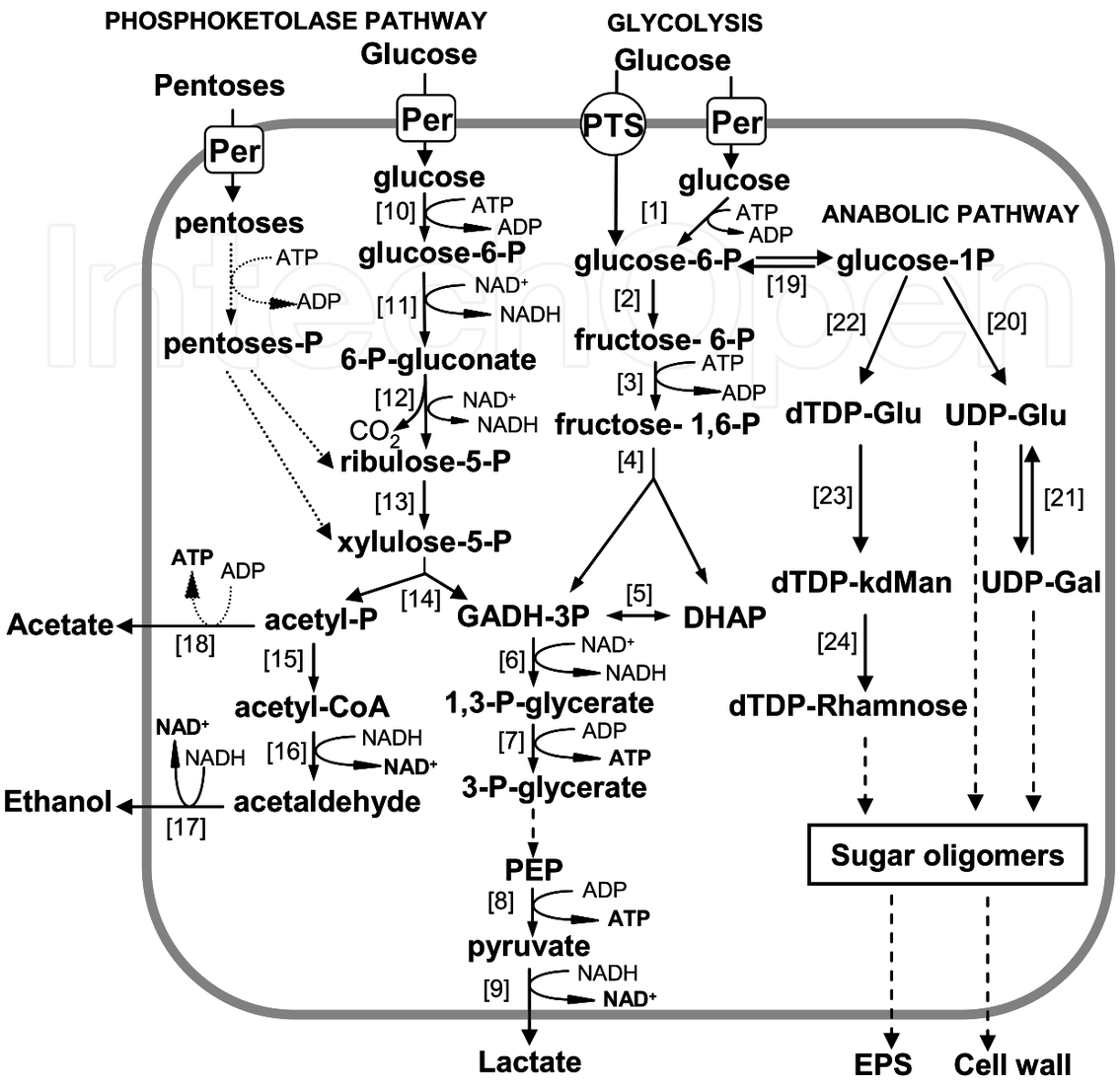

Fig. 1. Scheme of glycolysis, phosphoketolase pathway and anabolic pathway of UDP-sugars in LAB. [1] glucokinase, [2] phosphoglucose isomerase, [3] phosphofructokinase, [4] fructose 1,6-bisP aldolase, [5] triose-phosphate isomerase, [6] glyceraldehyde-3P dehydrogenase, [7] phosphoglycerate kinase, [8] pyruvate kinase, [9] lactate dehydrogenase, [10] hexokinase, [11] glucose-6P dehydrogenase, [12] 6-phosphogluconate dehydrogenase, [13] ribulose-5P 3-epimerase, [14] xylulose-5P phosphoketolase, [15] phosphotransacetylase, [16] acetaldehyde dehydrogenase, [17] alcohol dehydrogenase, [18] acetate kinase, [19] α-phosphoglucomutase, [20] UDP-glucose pyrophosphorylase, [21] UDP-galactose 4-epimerase, [22] deoxyTDP-glucose pyrophosphorylase, [23] deoxyTDP-glucose 4,6-dehydratase, [24] deoxyTDP-rhamnose synthetic enzyme system. PTS, PEP phosphotransferase system; Per: permease; CoA, coenzyme A; DHAP, dihydroxiacetone phosphate; GADH-3P, glyceraldehyde-3-phosphate; UDP-Glu, UDP-glucose; UDP-Gal, UDP-galactose; dTDP-Glu, deoxyTDP-glucose; dTDP-kdMan, deoxyTDP-4-keto-6-deoxymannose; EPS, exopolysaccharide; PEP, phospho*enol*pyruvate.

1.2 Functional properties of lactobacilli

Bacterial populations in the gut of vertebrates have evolved through millions of years to render interdependent functions. They have been studied for long time using classical culturing techniques and recently through molecular approaches and it soon became evident that in humans, the gut's microbioma is formed by numerous bacterial species whose proportions change between individuals (Eckburg *et al.*, 2005). The most abundant genera are *Bacteroides, Faecalibacterium* or *Bifidobacterium*, however, although lactobacilli are not as abundant, they have been proved to play a remarkable role sustaining the global population balance and interact at different levels with the intestinal mucosa. In this environment some strains exerted beneficial health effects and they are considered probiotics. These are defined as live microorganisms that, when administered in adequate amounts, confer a beneficial effect on the health of the host (FAO/WHO, 2001). In addition to probiotics, functional food ingredients also include prebiotics, which are define as selectively fermented ingredients that allow specific changes in the composition and/or activity of the gastrointestinal microbiota that confer benefits upon host wellbeing and health (Roberfroid, 2007). Several beneficial effects of lactobacilli on human host have been reported. Strains of *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *L. bulgaricus* alone or in combination are effectives in reduce the risk of acute infectious diarrhoea and prevent antibiotic-associated diarrhoea (Sazawal *et al.*, 2006). A mixture of probiotics including lactobacilli seems effective in the maintenance of remission of intestinal bowel diseases such as chronic pouchitis and ulcerative colitis, and to decrease symptoms in patient with irritable bowel syndrome (Haller *et al.*, 2010). A synbiotic food composed of the prebiotic oligofructose-enriched inulin, *L. rhamnosus* GG and *Bifidobacterium lactic* Bb12 was able to alter favourably several colorectal cancer markers in patients with cancer of colon (Rafter *et al.*, 2007). Besides to gastrointestinal disorders, lactobacilli have also showed positive effects in other pathologies, such as in the treatment and prevention of bacterial vaginosis (Falagas *et al.*, 2007), the prevention of atopic eczema (Tang *et al.*, 2010) and prevention of dental caries (Stamatova & Meurman, 2009). The health promoting effects of probiotic bacteria are mediated mainly by three mechanisms, (i) microbe-microbe interactions; (ii) beneficial interactions with gut epithelium and (iii) immunomodulatory interactions (Lebeer *et al.*, 2008). Regarding the first mechanism, probiotics can have a beneficial effect on the host by modifying the microbiota trough competition and cooperation for nutrients, production of antimicrobial compounds (lactic acid, bacteriocins, H_2O_2), competition with pathogens for attachment sites to the host mucosal surface and by bacterial cell-host cell communication. With respect to the beneficial interactions of probiotics with gut epithelium, this constitutes the main target tissue of probiotic action, and *Lactobacillus* molecules can modify it by affecting the metabolic and barrier functions of the epithelial cells. The preservation of the epithelial barrier by probiotic lactobacilli has been attributed to induction of mucin secretion, enhancement of tight-junction functioning, upregulation of cytoprotective heat-shock proteins and prevention of apoptosis of ephitelial cells.

The gut mucosal surface is continuously exposed to pathogens, beneficial mutualistic and commensal bacteria, and it is armoured with the largest part of the immune system in the organism, with lymphocytes scattered in the lamina propria or in organized gut-associated lymphoid tissues (GALT) such as the Peyer's patches of the small intestine and mesenteric lymph nodes (MLNs). Those immune cells can discriminate pathogens from harmless antigens, preventing an inappropriate immune response to harmless bacteria, thorough regulatory mechanisms known as "oral tolerance", which is an still incompletely

understood *active nonresponse* to dietary and commensal enteric bacteria or food derived antigens administered orally, also related to the maintenance of homeostasis in the gut (Murphy *et al.*, 2007). The subepithelial dendritic cells (DCs), B cells and T cells, in the lamina propria and GALT express a wide range of pattern-recognition receptors (PRRs), surface Toll like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), to acquire antigens from the intestinal lumen. Then, secreted cytokines and chemokines from DCs will determine *tolerance* and *active immune responses* against a particular antigen, and whether lymphocyte differentiation, innate, adaptive or allergy immune responses will be displayed (Hart *et al.*, 2004). Also intestinal epithelial cells (IECs) at the mucosal surface express PRRs and can also secrete cytokines and regulatory molecules, therefore, they participate actively in the discrimination of both pathogenic and commensal bacteria (Artis, 2008). In the gut, certain *Lactobacillus* strains have been proved to play a remarkable role sustaining the global population balance through their ability to synthesize antagonistic compounds that restrain the proliferation of a number of pathogens. However, their mutualistic behaviour with the host involves different levels of interaction with the intestinal mucosa, resulting in an anti-inflammatory effect and restoration of the mucosal homeostasis (Haller *et al.*, 2010). The proinflammatory cytokine profiles occasionally induced by some lactobacilli in model systems (Dong *et al.*, 2010) be related to the moderate degree of inflammation (*physiological inflammation*) elicited by some commensals and moderate pathogens, and it has been conceived as a beneficial feature that creates a state of awareness for a rapid immune defence response against possible infective aggressions, while preserving homeostasis (Sansonetti & Medzhitov, 2009).

1.3 Tools and strategies for genetic manipulation of lactobacilli

1.3.1 Gene cloning vectors, genetic markers and promoters

Several constitutive or inducible gene expression systems have been developed for lactobacilli (Fang & O'Toole, 2009). The vectors have different parameters such as copy-number and host-range, they are derivatives of the rolling-circle plasmids pWVO1 or pSH71 from *L. lactis* or the theta-type plasmids pAMβ1 from *Enterococcus faecalis* (Perez-Arellano *et al.*, 2001) and pRV500 from *Lactobacillus sakei* (Crutz-Le Coq & Zagorec, 2008). Vectors for controlled expression are mainly based on genes and promoters involved in bacteriocin production, sugar utilization genes and stress resistance. In addition, following the production of proteins by LAB using a specific expression vector they should be properly folded, targeted and sometimes recovered. Several vectors included secretion cassettes, such as those based on the secretion signal of the lactococcal Usp45 protein (Schotte *et al.*, 2000), the expression and secretion signals of S-layer proteins (Savijoki *et al.*, 1997), the PrtP signal sequence (Kajikawa *et al.*, 2010) or the M6 carboxy-terminal domain to anchor proteins to the cell wall (Reveneau *et al.*, 2002).

The nisin-controlled expression (NICE) system, based on the autoregulation mechanism of the bacteriocin nisin, is a very effective expression vector for production of heterologous proteins in LAB (Mierau & Kleerebezem, 2005). The NICE system contains the promoter *nisA* conducting gene expression under the control of the transcriptional regulator NisR, which is modulated by phosphorylation due to the histidine-protein kinase NisK immersed in the cytoplasmic membrane. The expression of the genes placed behind the P_{nisA} is induced by the addition of subinhibitory concentrations of nisin into the culture medium, in such a way that increasing amounts of nisin resulted in a linear dose-response curve. Optimization

of the NICE system includes the incorporation in the vectors of the nisin immunity gene *nisI*, which resulted in better tolerance of the cells to high amounts of the inducer nisin (Oddone *et al.*, 2009). The NICE system was created for expression of genes in *L. lactis* but it has been adapted to other low-GC Gram-positive bacteria including *Lactobacillus helveticus* (Kleerebezem *et al.*, 1997), *L. plantarum* (Pavan *et al.*, 2000), *Lactobacillus brevis* (Avall-Jaaskelainen *et al.*, 2002), *L. casei* (Hazebrouck *et al.*, 2007), *L. salivarius* (Sheehan *et al.*, 2006) and *L. reuteri* (Wu *et al.*, 2006). In these species different strategies have been used to express the *nisRK* genes: on a different plasmid in relation to the *nisA* promoter with the target gene, both on the same plasmid or with the *nisRK* genes inserted into the chromosome. Similar to the NICE system, in *L. plantarum* (Mathiesen *et al.*, 2004) and *L. sakei* (Axelsson *et al.*, 2003) vectors have been developed using a pheromone-regulated bacteriocin promoter and the regulatory system of sakacin A production, respectively. The pSIP vector series, based on the genes and promoters involved in sakacin A and P, used erythromycin as selection marker (Sorvig *et al.*, 2005). In order to developed a potential food-grade expression system the erythromycin gene in the pSIP vectors has been replaced by the *alr* gene, which encodes the alanine racemase enzyme that is essential for cell wall biosynthesis (Nguyen *et al.*, 2011). In *L. casei* an integrative vector, pIlac, has been constructed that allowed stable gene insertion in the chromosomal lactose operon. The vector is based on the nonreplicative plasmid pRV300 and it contains the 3' end of *lacG* and the complete *lacF* gene (Gosalbes *et al.*, 2000). Both vectors, pSIP and pIlac, are based on the complementation host/marker system, a gene in the host is mutated or deleted, and a wild copy is inserted into the vector. Other potential food-grade vectors are based on a selection marker that confers a new ability to the host strain. In this sense a vector has been recently developed that contains a bile salt hydrolase gene from *L. plantarum* and which allows the host to grow in media containing bile salts (Yin *et al.*, 2011). Bioluminiscence markers have also been used in lactobacilli and they are based on genes encoding enzymes that produce light as *lux*, which encodes bacterial luciferase, and *gfp* that encodes green fluorescence protein (Chang *et al.*, 2003; Perez-Arellano & Perez-Martinez, 2003).

1.3.2 DNA mutagenesis systems: integration and insertion systems, and random mutagenesis systems

There are two principal methods to generate mutations in lactobacilli: (i) integration, which is a rec-dependent recombination of cloned DNA with an homologous locus; (ii) rec-independent, which involves transposons and insertion elements (Fang & O'Toole, 2009). The integration procedures mostly used in LAB are based on vectors able to integrate by homologous recombination with known chromosomal genes, causing their disruption by inserting foreign genes. The integrative vectors developed for lactobacilli are either based in temperature-sensitive replicons such as pG+host, pIP501, pTNI and pGID or non-replicative plasmid such as pUC18/19 and pBlueScript SK-. As well, a two plasmids system have also been used to direct integration into *Lactobacillus* chomosomes via homologous recombination (Russell & Klaenhammer, 2001). This system utilizes pOWV01-derived vectors from which the *repA* gene has been removed. The *repA* is supplied in trans in a temperature-sensitive helper vector. A subsequent temperature shift selects for loss of the helper plasmid and integration of the pOWV01-derived vector. In addition, there are other mutagenesis systems as that of the Cre-*lox*-based system used in *L. plantarum* (Lambert *et al.*,

2007) and site-specific integrative vectors based on prophage fragments (Martin *et al.*, 2000). Other important genetic tool used to study chromosomal genes and their regulation in lactobacilli is random transposon mutagenesis. The insertional sequence ISS1, combined with the thermosensitive pG+ replicon, was used to inactive genes involved in the regulation of phenolic acid metabolism in *L. plantarum* (Gury *et al.*, 2004) and several genes in *L. salivarius* (Mason *et al.*, 2005). Tn5 transposome system was also efficiently used to generate a library of transposon insertion mutants in *L. casei* (Ito *et al.*, 2010). As well, factors affecting the reduction of serum cholesterol by *L. acidophillus* were identified by random transposon mutagenesis (Lee *et al.*, 2010).

2. Lactic acid production

Lactic acid produced by many LAB is a racemic mixture of L-lactate and D-lactate isomers. D-lactate is not metabolized by humans, then L-lactate is the most important isomer for food biotechnological applications, and also for pharmaceutical and biopolymers industries. Many efforts have been made to construct LAB strains affected in one or several of the identified *ldh* genes, as they can be used in the production through fermentation of non-racemic, optically active lactic acid. In *L. casei* BL23, a strain that has been widely used for genetic, physiological and biochemical studies, five genes encoding proteins with LDH activity have been described (Rico *et al.*, 2008). Mutant strains for those genes demonstrated the involvement of each *ldh* gene in L- and D-lactate formation in this bacterium. Gene *ldh1* codes for an L-LDH responsible for the main synthesis of L-lactate, whilst *hicD* encodes a D-hydroxyisocaproate dehydrogenase which renders D-lactate. However, an *L. casei* BL23 *ldh1* mutant still produced substantial amounts of L-lactate and an increase in the production of D-lactate was observed (Viana *et al.*, 2005). D-lactate was probably synthesized via the activity of HicD, since it was abolished in a *Δldh1 hicD* double mutant. *ldh2*, *ldh3* or *ldh4* single mutations or combined with an *ldh1* deletion (*Δldh1 ldh2*, *Δldh1 ldh3*, *Δldh1 ldh4*) had a low impact on L-lactate synthesis showing that *ldh2*, *ldh3* and *ldh4* genes play a minor role in lactate synthesis (Rico *et al.*, 2008). Comparable behaviour has been reported for many LAB where *ldh*s have been deleted. In this sense, mutation of the genes encoding L- and D-LDHs from *L. plantarum*, an organism which produces a mixture of 50% D- and 50% L-lactate, never resulted in a complete lack of lactate production (Ferain *et al.*, 1996). An *ldhL* mutation in *L. sakei*, a lactic acid bacterium which lacks D-lactate dehydrogenase activity, rendered a strain with strongly reduced L- and D- lactate production (the D isomer was a consequence of the presence of a racemase activity able to transform L- into D-lactate), but small amounts of lactate were still produced (Malleret *et al.*, 1998). Recombinant strategies have also been used in *Lactobacillus* strains to produce lactic acid from sugars others than glucose and from biomass such as starch and cellulose. In an *L. plantarum* Δ*ldhL1* strain, that only produced D-lactate from glucose, the phosphoketolase gene was substituted by a transketolase gene from *L. lactis*, and the resulting *L. plantarum* Δ*ldhL1-xpk1::tkt* strain produced 38.6 g/l of D-lactate from 50 g/l of arabinose (Okano *et al.*, 2009). The production of D-lactate from xylose was also achieved in *L. plantarum* by disrupting a phosphoketolase 2 gene in the *L. plantarum* Δ*ldhL1-xpk1::tkt* strain and transforming it with a plasmid that contains the genes *xylAB*. The *L. plantarum* Δ*ldhL1* strain was transformed with plasmids expressing amylolytic or cellulolytic enzymes, and the resulted strains were able to produce D-lactate from raw corn starch or cellulosic compounds, respectively (Okano *et al.*, 2010).

In addition to rational methods of metabolic engineering, lactic acid production has also been enhanced by a combination of classical strain improvement methods (nitrosoguanidine and ultraviolet mutagenesis) with whole-genome shuffling by recursive protoplast fusion. In this way, shuffled strains derived from an industrial strain of *Lactobacillus* have been isolated, and they produce threefold more lactic acid than the wild type at pH 4.0 (Patnaik *et al*., 2002). Shuffled *L. rhamnosus* strains with improved tolerance to glucose and enhanced L-lactate production has also been obtained (Yasuda *et al*., 2008). In the same way, a fusant derived from *Lactobacillus delbueckii* able of growing at low pH and utilizing starch from cassava bagasse was obtained and it produced large amounts of L-lactic (John *et al*., 2008).

3. Diacetyl and acetoin production

Diacetyl and acetoin are important compounds of buttery flavor in fermented foods and are used as additives in the food industry. Both compounds are derived from pyruvate, which is converted to α-acetolactate by the action of α-acetolactate synthase or acetohydroxyacid synthase. Then, acetoin is formed by the activity of α-acetolactate decarboxylase on α-acetolactate and diacetyl results from a non-enzymatic oxidative decarboxilation of α-acetolactate (Figure 2). Most metabolic engineering approaches to produce diacetyl/acetoin by fermentation have been developed in the model LAB *L. lactis*, in which strains that divert an important part of pyruvate flux towards the production of α-acetolactate have been constructed (Hugenholtz *et al*., 2000; Lopez de Felipe *et al*., 1998). *ilvBN* genes, encoding acetohydroxyacid synthase from *L. lactis*, have been expressed from the lactose operon in *L. casei*, an organism which shows marginal production of diacetyl/acetoin, resulting in increased diacetyl formation (Gosalbes *et al*., 2000). In addition, to enhance diacetyl/acetoin production, the amount of pyruvate available for IlvBN was increased by blocking pyruvate alternative pathways in *L. casei*. Thus, the *L. casei* strain that expresses the *ilvBN* genes was mutated in the *ldh* gene and in *pdhC*, encoding the E2 subunit of the pyruvate dehydrogenase enzyme. The introduction of these mutations resulted in an increased capacity to synthesize diacetyl/acetoin from lactose fermentation in whey permeate (1400 mg/l at pH 5.5) (Nadal *et al*., 2009).

4. Mannitol and sorbitol production

Sugar alcohols are hydrogenated carbohydrates widely used in the food industry as sugar replacers. Mannitol and sorbitol are used as food additives due to their sweetening effect (about half as sweet as sucrose) and low calorie content. They are also used in the food and pharmaceutical industries due to their technological properties, such as texturing agents, humectants, softeners and color stabilizers. In nature, mannitol is found in some plants, algae and mushrooms, and sorbitol is found in many fruits and vegetables. Those polyols are also produced by fungi, yeast and bacteria, where they play several roles in carbon storage and protection during osmotic and oxidative stresses. Industrial production of most sugar alcohols is performed by catalytic reduction of sugars with hydrogen gas and nickel at high temperature and pressure, for which highly pure sugar substrates and costlychromatographic purification steps are required. Regardless the limitations of this chemical method, it is until now the only process able to assume the high market demand of sorbitol and mannitol, estimated to be thousands of tons per year. However, processes using

bacteria and yeasts have demonstrated that biotechnological production may represent an efficient and cost-effective alternative to the chemical production.

The production of polyols by using genetically engineered LAB has been recently reviewed (Monedero *et al.*, 2010). Mannitol is a natural fermentation product in heterofermentative LAB, in which the NADH generated during sugar metabolism is regenerated by the

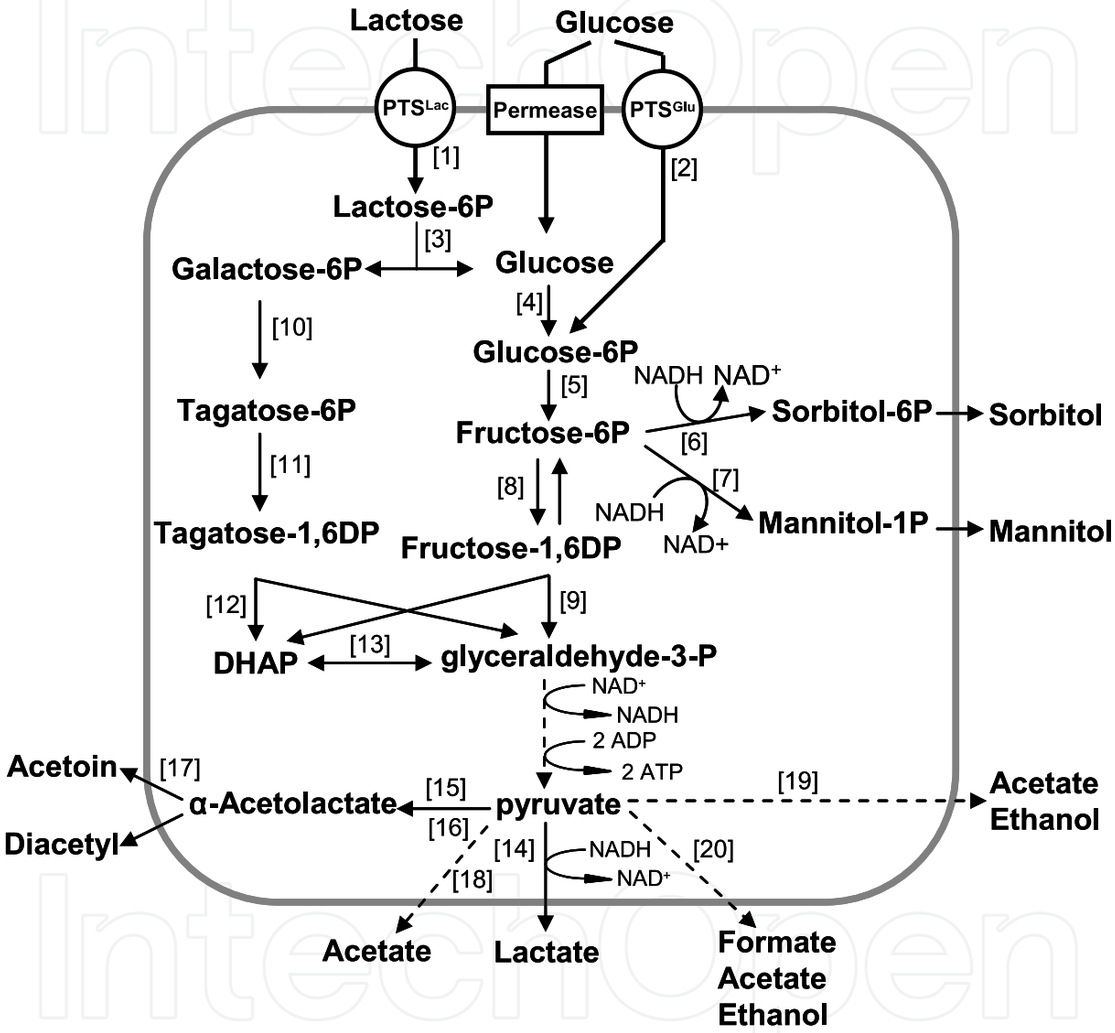

Fig. 2. Proposed pathways for sorbitol, mannitol, acetoin and diacetyl production by engineered lactic acid bacteria. [1] phosphoenolpyruvate: lactose phosphotransferase system, [2] phosphoenolpyruvate: glucose phosphotransferase system, [3] β-phosphogalactosidase, [4] glucokinase, [5] phosphoglucose isomerase, [6] sorbitol-6P dehydrogenase, [7] mannitol-1P dehydrogenase, [8] phosphofructokinase, [9] fructose 1,6-bisP aldolase, [10] galactose-6P isomerase, [11] tagatose-6P kinase, [12] tagatose-1,6DP aldolase, [13] triose-phosphate isomerase, [14] lactate dehydrogenase, [15] α-acetolactate synthase, [16] acetohydroxyacid synthase , [17] α-acetolactate decarboxylase, [18] pyruvate oxidase, [19] pyruvate dehydrogenase complex, [20] pyruvate-formate lyase. DHAP, dihydroxiacetone phosphate.

production of lactate and ethanol. However, in the presence of fructose, a mannitol dehydrogenase activity (MDH) can account for NADH recycling with the concomitant production of mannitol. Homofermentative LAB, which use the glycolytic pathway for sugars fermentation and lack MDH, are also able to produce mannitol under special circumstances (Figure 2). Mutants of *L. plantarum* and *L. casei* impaired in NADH regeneration by the lactate dehydrogenase were able to produce small amounts of mannitol from glucose due to a mannitol-1-P dehydrogenase (M1PDH) activity on fructose-6P (Ferain *et al.,* 1996; Viana *et al.,* 2005). M1PDH can recycle NADH rendering mannitol-1P that can be dephosphorylated to mannitol and excreted from the bacterial cell. M1PDH activity is generally low, because its gene (*mtlD*) is only induced by the presence of mannitol. Furthermore, mannitol is also a common carbon and energy source that can be fermented. Therefore, subsequent re-uptake and metabolism of the produced mannitol should be avoided. In bacteria, mannitol is usually taken up by a mannitol-specific phospho*enol*pyruvate: sugar phosphotransferase system (PTS^{Mtl}) which catalizes the simultaneous mannitol uptake and phosphorylation to mannitol-1P. *L. lactis ldh* mutants have been constructed which were deleted in *mtlA* and *mtlF*, encoding the $EIICB^{Mtl}$ and $EIIA^{Mtl}$ components of the PTS^{Mtl}, respectively, involved in mannitol uptake (Gaspar *et al.*, 2004). This resulted in strains unable to utilize mannitol which converted 33% of the fermented glucose into mannitol. In another approach, the M1PDH encoding gene from *L. plantarum* and a gene encoding a mannitol-1P phosphatase from the protozoan parasite *Eimeria tenella* were overexpressed by using the NICE system in an *L. lactis ldh* mutant (Wisselink *et al.*, 2005). This strategy avoided the main bottleneck in mannitol production: most mannitol was accumulated inside the cell as mannitol-1P, which could reach concentrations up to 76 mM in high density non-growing cells of an *L. lactis ldh* mutant (Neves *et al.*, 2000). In this new strain 50% of the glucose was converted to mannitol (maximum theoretical yield of 67%). Other alternatives comprise the expression of MDH genes from heterofermenters. The *mdh* gene from *L. brevis* was expressed in a *L. plantarum* strain deficient in both *ldhL* and *ldhD* genes, and resulted in an increase in mannitol synthesis from glucose (Liu *et al.*, 2005).

The sorbitol (*gut*) operon of *L. casei* contained the genes *gutCBA*, encoding the EII component of the sorbitol-specific PTS involved in sorbitol transport and phosphorylation, two regulatory genes, *gutR* and *gutM*, and the gene *gutF*, encoding a sorbitol-6P dehydrogenase (S6PDH) (Alcantara *et al.*, 2008). A recombinant strain of *L. casei* with the *gutF* gene integrated in the chromosome at the lactose operon produces sorbitol from fructose-6P by reversing the sorbitol catabolic pathway (Nissen *et al.*, 2005) (Figure 2). Resting cells of this strain synthesized small amounts of sorbitol from glucose, with a conversion rate of 2.4 %. Subsequent inactivation of *ldh1* gene, encoding the main LDH (Rico *et al.*, 2008) promoted an increment in the conversion rate (4.3 %), suggesting that the engineered route provides an alternative pathway for NAD^+ regeneration. Once glucose was depleted, reutilization of the produced sorbitol by *L. casei* recombinant strains was avoided by deleting *gutB* gene that encodes the IIBC component of the PTS^{Gut} (De Boeck *et al.*, 2010). *L. casei* recombinant strains produced mannitol in addition to sorbitol and this polyol mixture was avoided by inactivation of the *mtlD* gene that encodes a M1PDH. The engineered *L. casei* strain (*lac::gutF* Δ*ldh1* Δ*gutB mtlD*) produced sorbitol from lactose, the milk sugar, in non-growing cells or in growing cells under pH control. Fed-batch

fermentations using whey permeate, a waste product from the dairy industry with high concentration of lactose, resulted in a conversion rate of 9.4% of lactose into sorbitol (De Boeck *et al.*, 2010). *L. plantarum* has also been metabolically engineered to produce sorbitol by constitutive overexpression of either *srlD1* or *srlD2* genes that encode S6PDH activities in a mutant strain deficient in LDH activity (Ladero *et al.*, 2007). Using non-growing or growing cells under pH control resulted in a very efficiency conversion rate of about 65% and 25%, respectively, of sugar into sorbitol. The different efficiencies were suggested to be the result of a higher ATP demand for biomass production in growing cells.

5. Exopolysaccharides (EPS) production

Some LAB produced EPS, which are extracellular polysaccharides, with important characteristics for the dairy industry. They are used to improve the rheological and textural properties of fermented foods. EPS have also potential as food additives and functional food ingredients. In this sense they are claimed to act as prebiotics in the intestine (Bello *et al.*, 2001) and to stimulate the immune system (Vinderola *et al.*, 2006). The synthesis of EPS in LAB starts at the glycolytic intermediate glucose-6P, which connects the anabolic pathways of biosynthesis of sugar nucleotides, the precursors of the EPS, and the catabolic pathways for obtaining energy through the glycolysis (Figure 1). Glucose-6P is converted to glucose-1P by the α-phospoglucomutase (α-Pgm) activity, and this sugar phosphate is further metabolized to UDP-glucose and UDP-galactose by the consecutively action of enzymes UDP-glucose pyrophosphorylase (GalU) and UDP-galactose 4-epimerase (GalE) (Boels *et al.*, 2001). Glucose-1P is also substrate for dTDP- glucose pyrophosphorylase to produce dTDP-glucose, which will be further metabolized to dTDP-rhanmnose. Glucose, galactose and rhamnose are the principal sugars found in the EPS produced by LAB. The subsequent steps in the synthesis of EPS is the assembly of the monosaccharide repeating unit by specific glycosyltransferases, the polymerization of the repeating units and the secretion from the cell (Welman *et al.*, 2006). The enzymes that participate in these stages are encoded by genes that form part of *eps* gene clusters. Genetic engineering strategies could be applied to one or more of those stages involved in the EPS biosynthesis in order to increase the EPS production or to modify its composition, however, until now only strains of *L. lactis* and *Streptococcus thermophilus* have been genetically modified to enhance EPS biosynthesis. In *S. thermophilus* the modification of the levels of the GalU, PgmA and the Leloir route enzymes resulted in increased levels of EPS (Levander *et al.*, 2002). Homologous overexpression of a complete *eps* operon in *L. lactis* resulted in about fourfold increase in EPS production (Boels *et al.*, 2003). In *Lactobacillus* species there are no examples of metabolic engineering strategies aimed to produce EPS. In this species the synthesis of EPS has been improved by modifying the culture conditions, such as carbon source and pH. As well, chemically induced mutants of *Lactobacillus* species that produce higher amounts of EPS than the parental strain have been isolated. The synthesis of EPS by *L. casei* strain CRL 87 was improved by using galactose as carbon source at a controlled pH of 5.0, and the high EPS production was correlated with high activity level of the enzymes involve in the synthesis of UDP-sugars (Mozzi *et al.*, 2003). Similar approaches were applied for *L. helveticus* strain ATCC 15807, which produces a higher amount of EPS from lactose at pH 4.5 than at pH 6.2, which was correlated with higher levels of α-Pgm activity (Torino *et al.*, 2005). A *L. delbrueckii* subesp. *bulgaricus* mutant with improved EPS production has been isolated, and it showed higher

amounts of GalU activity, glucose-6P and ATP than the parent strain. These characteristics suggest that GalU and α-Pgm enzymes play important roles in the synthesis of high EPS production. The elevated concentration of ATP in the mutant indicated that the glycolysis influence the anabolic route of EPS biosynthesis (Welman *et al.*, 2006). A metabolic engineering strategy aimed to direct the carbon flux towards UDP-glucose and UDP-galactose biosynthesis was successfully applied in *L. casei*. The *galU* gene coding for GalU enzyme in *L. casei* strain BL23 was cloned under control of the inducible *nisA* promoter, and the resulting strain showed about an 80-fold increase in GalU activity, a 9-fold increase of UDP-glucose and a 4-fold increase of UDP-galactose (Rodriguez-Diaz & Yebra, 2011). *L. casei* strain BL23 does not produce EPS, hence it would be an adequate host for the production of heterologous EPS.

6. Improvement of probiotic properties

6.1 Adhesion to intestinal epithelial cells

Adhesion of probiotic bacteria has been employed as a criterion for strain selection and, although it is not indispensable for some probiotic traits, it has positive effects on strain persistence at the gastrointestinal tract and in pathogen inhibition by displacement and competition for adhesion sites. Also, it has been suggested that the capacity to adhere to mucosal surfaces influences the cross-talk established between probiotic bacteria and host cells (Sanchez *et al.*, 2008; Velez *et al.*, 2007).

Probiotic strains have shown the ability to bind to intestinal epithelial cultured cells (e.g. Caco-2, HT-29), to mucus components and to proteins of the extracellular matrix (ECM) such as collagen, fibronectin or laminin. Although theses late molecules are not commonly found at the mucosal surface, they may be shed into the mucus or may be exposed in case of trauma or inflammation. They are common targets for pathogen adhesion during the process of infection and adhesion to them by probiotic bacteria can compete with pathogen binding. In contrast to the knowledge about adhesive factors in bacteria causing infectious diseases in humans and animals, the knowledge about adhesion mechanisms in probiotics is very limited. Some molecules from probiotics have been identified as responsible for adhesion, including lipoteichoic acid and exopolysaccharides. However, surface proteins are the major responsible for adherence. Typical surface adhesins from pathogenic bacteria with binding capacity to cultured cells and ECM components are not found in probiotics although it is hypothesized that they may share similar mechanisms for attachment. Similar to some pathogens, probiotic lactobacilli display on their surface *moonlighting* proteins which are in most cases of cytoplasmic location and are exported and retained on bacterial surfaces by yet unknown mechanisms. These include glycolytic enzymes such as enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), molecular chaperones (DnaK, GroEL) and translational elongation factors (EF-Tu, EF-G) (Sanchez *et al.*, 2008; Velez *et al.*, 2007). These proteins have demonstrated binding ability to ECM proteins and epithelial cells and in some cases interfere with host pathways (plasminogen activation mediated by enolase from *Lactobacillus crispatus* and *Lactobacillus johnsonii* (Antikainen *et al.*, 2007) or immunoregulation by GroEL from *L. johnsonii* (Bergonzelli *et al.*, 2006). Other surface proteins with identified roles in adhesion are surface layer (S-layer) proteins or Slp. S-layers from lactobacilli such as *L. acidophilus* or *L. brevis* are formed by small basic proteins which form a crystalline matrix on the bacterial surface. The real function of the S-layer is uncertain

but is has been demonstrated that some Slp proteins display binding capacity to ECM proteins and possess immunoregulatory capabilities. In general the adhesive properties of the above extracellular proteins did not show a strict specificity for substrate binding and it is postulated that they may possess lectin-like characteristics that allow their binding to highly glycosylated ECM proteins and mucosal surfaces (Velez *et al.*, 2007).

6.1.1 Specific proteins implicated in mucus binding

Species of lactobacilli from intestinal origin (*L. plantarum*, *L. reuteri*, *Lactobacillus gasseri, L. acidophilus, L. johnsonii*) express surface proteins covalently anchored to the cell wall by a sortase-dependent mechanism with mucus binding ability (Msa and Mub proteins) (Boekhorst *et al.*, 2006). They are large multidomain proteins containing up to fifteen tandem copies of a mucin-binding domain (MucBP) and act as mannose-dependent adhesins with the capacity to aggregate *Saccharomyces cerevisiae* cells. The presence of these proteins in certain *L. reuteri* isolates correlates with the binding ability to mucus. Similarly, the presence in *L. plantarum* of *msa* genes is the sole requisite for mucus binding in this species, but domain composition and subtle amino acids changes in each specific Msa protein account for the diverse adhesion properties reported in different strains. In *L. rhamnosus* GG (LGG), a well characterized probiotic with established mucus adhesion properties, the product of the LGG_02337 gene is the only protein encoded in the genome which contains four MucBP domains about 50 amino acids shorter in length compared to the large mucus binding proteins Msa and Mub. This protein is anchored at the bacterial surface and possesses *in vitro* binding activity to mucus (von Ossowski *et al.*, 2011).

A genome analysis of several *L. rhamnosus* strains identified the *spaCBA* cluster as another trait responsible for mucus adherence in LGG. This cluster codes for the three components of pili structures similar to pili described in Gram-positive pathogens that can be identified by immune electron microscopy at the LGG surface (Kankainen *et al.*, 2009). SpaA is the major pilin protein that forms the pilus shaft, while SpaC and SpaB are ancillary pilus proteins which are present at the pilus tip or along the pilus structure and possess adhesive properties. Adhesion experiments with purified proteins, specific antibodies, and mutant construction have demonstrated that SpaC and SpaB are responsible for the mucus binding activity displayed by LGG. This is the first example of the presence of pili adhesive structures in a probiotic strain and exemplifies the adaptation of these bacteria to persist in host tissues.

6.1.2 Engineered lactic acid bacteria with enhanced adhesion

Some of the adhesion factors characterized in probiotic bacteria may be targets for strain engineering aimed to enhance bacterial adhesion. In addition, heterologous expression of well characterized adhesins from different sources can be envisaged. This can be useful to increase residence times at the gastrointestinal tract, enhance interactions with the mucosal immune system and promote competitive exclusion of pathogens by probiotics. Some probiotic strains like *L. casei* Shirota have been engineered to express a fibronectin binding domain from the Sfb protein of *Streptococcus pyogenes*, allowing this strain, which barely binds fibronectin, to bind this ECM substrate, fibrinogen and human fibroblasts (Kushiro *et al.*, 2001). However, to date most genetic engineering strategies aimed to increase lactic acid bacteria adhesion have been carried out in the model *L. lactis* species. This bacterium is not a

normal inhabitant of the gastrointestinal tract but it has been used as a food grade delivery vehicle for presenting bioactive molecules to mucosal surfaces, including antigens, cytokines or enzymes. Expression of a protein containing a chitin-binding domain from *L. plantarum* on the surface of *L. lactis* resulted in enhanced capacity to attach to natural compounds carrying polymers of *N*-acetylglucosamine, such as human mucins (Sanchez *et al.*, 2011). The recombinant strain also showed increased attachment to epithelial Caco-2 cells. In another approach, the receptor binding domain of FedD adhesin from *E. coli* F18 fimbriae was expressed and anchored to the bacterial surface by creating a fusion with the surface anchoring domain of the *L. lactis* autolysin AcmA. This fusion protein promoted the binding of *L. lactis* to porcine intestinal epithelial cells (Lindholm *et al.*, 2004). Finally, expression in *L. lactis* of either a fibronectin binding protein from *Staphylococcus aureus* or Internalin A from *Listeria monocytogenes* promoted its binding to human epithelial cells and bacterial internalization, providing a tool for DNA delivery into eukaryotic cells (Innocentin *et al.*, 2009).

6.2 Inmunomodulation of colonic mucosa

In the case of functional properties of lactobacilli, due to legal restrictions and public opinion attitudes against the use of genetically modified microorganisms, the most general strategy has been the selection of naturally competent probiotic strains, nevertheless, some examples of mutants and genetically engineered strains can be found with specific and improved properties (see below). The molecular mechanisms underlying this process are still unknown, however, intervention studies using probiotics in controlled placebo double blind clinical assays are very abundant and different meta-analysis confirmed that several specific beneficial effects of probiotics pass very stringent examination criteria (Williams, 2010), however, they are costly in time and resources and cannot be used to test a great number of strains. Hence, this review will initially describe the general features that characterise the recognition of bacteria by the mucosa and, then, it will focus on the characterisation of the mechanisms of action and the understanding of the effect of probiotics on model systems, as a mean efficiently select funtional strains.

As described in the introduction, the mucosal surface is continuously exposed to both potential pathogens and beneficial commensal microorganisms. This creates a requirement for a homeostatic balance between tolerance and immunity that represents a unique regulatory challenge to the mucosal immune system. Dendritic cells (DCs) in the lamina propria efficiently recognise microbial components from the intestinal lumen through PRRs, TLRs and NLRs. Then, DC migrate to draining lymph nodes, where they have the unique ability to activate and influence functional differentiation of naïve T cells. Signals from DC can determine whether tolerance or active immune responses occur to a particular antigen and furthermore influence whether a T helper (T_h) cells of the type T_h1 (innate immune response), T_h2 (adaptive immune response and allergy), T_h17 or T_{reg} (lymphocyte differentiation) predominates. The DC subtype, whether CD11c+ (myeloid) or CD11c- (plasmacytoid), maturation status, and cytokine production contribute to the type of T cell response. For example, when DCs upregulate the coestimulatory molecules CD80 and CD86, produce IL-12 which contributes a Th1 response, but if DCs produce IL-10 and IL-4, they will promote the generation of a Th2 or regulatory T cells (Hart *et al.*, 2004).

Furthermore, intestinal epithelial cells (IECs) are not just a simple physical barrier. They express TLRs as well as intracellular NLRs and they can secrete cytokines and regulatory molecules (TSLP, TGFβ, IL-10, etc) that regulate cytokine secretion by DCs and

macrophages. Therefore, EICs actively participate in the discrimination of both pathogenic and commensal bacteria, they are crucial in triggering lymphocyte differentiation, maintaining intestinal immune homeostasis and mechanisms of innate defense (Artis, 2008). As consequence, commensal bacteria and pathogens are detected at different levels, in IECs, DC and macrophages. Different receptors recognise different bacterial ligands, so that the mucosa would integrate the information to recognise the microorganisms approaching the mucosa. PRRs in IECs and DC binding to bacterial molecular patterns (PAMPs) are expressed at the cell surface (TLR2, TLR4, CD14, TLR5) or in specialised endosomes (TLR3, TLR7, TLR8, TLR9). They can recognise single bacterial ligands or act synergistically to recognise others. As a quick overview, TLR3 recognises double stranded viral RNA, TLR9 hypomethylated CpG bacterial DNA, TLR7 and TLR8 were reported to recognize small imidazoquinoline compounds and TLR4, with the aid of CD14, recognises lipopolysaccharides (LPS) and lipoarabinomannans. Peptidoglycan (PGN) of different grampositive bacteria have been shown to interact with TLR2 (Iwaki *et al.*, 2002), however, TLR2 recognises other PAMPs (PGN, lipoteicoic acid, mycobacterial cell walls, protozoan parasite GPI anchors, lipoproteins, glycoproteins, glycolipids, LPS, etc). Furthermore, the complex TLR2/TLR6 recognises dipalmitoylated mycoplasma lipoprotein (MALP2), phenol soluble modulin from *Staphylococcus epidermidis* and fungal zymosan, and also, TLR2 associated to TLR1 recognises triacylated lipoproteins such as *Borrelia burgdorferi* OspA (for review see (Qureshi & Medzhitov, 2003)). In addition, cell wall components in lactobacilli and firmicutes are recognized by intracellular pattern-recognition molecules members of the nucleotide-binding oligomerization domain (NOD) family. The NLR family (also called Nod-leucine-rich repeats (LRRs)) are responsible for the signalling response induced by bacterial PGN and bacterial surface components, for instance, Nod1/CARD4 receptor in macrophages recognises Meso-diaminopimelic acid (meso-DAP), Nod2/CARD15 recognises muramyl dipeptide (MDP), Nod2 acting as a general sensor for bacteria, Ipaf/CLAN/CARD12 recognises intracellular flagellin (independently of TLR5, which senses extracellular flagellin) and cryopyrin/PYPAF1/NALP3 recognises bacterial RNA (and endogenous danger signals) and among others (for review, see (Franchi *et al.*, 2006). In particular, Nod2 recognizes a PGN motif present on both Gram-positive and Gram-negative bacteria.

6.2.1 Mechanisms of immunomodulation and probiotic factors involved

Nuclear factor κB (NF-κB) is a transcriptional regulator, or rather a regulator family, that controls the expression of hundreds of genes related to different cellular processes, including innate and adaptive immune responses. NF-κB signalling is the major pro-inflammatory pathway controlling the expression of cytokines, chemokines, enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis. It is activated by numerous proteins, among them pathogen-associated molecular patterns (PAMPs). Various lactobacilli have been described to inhibit NF-κB activity (Kim *et al.*, 2008; Petrof *et al.*, 2004). Although the initial steps of the process are not known, it was determined that *L. casei* DN-114.001 can maintain intestinal homeostasis after an inflammatory stimulus through a process that controls the ubiquitin/proteasome pathway upstream of I-kBα resulting in the stabilization of it, therefore blocking NF-κB for nuclear translocation (Tien *et al.*, 2006). Such effects possibly occur through targeting of multiple effectors and, in some cases, through complementary pathways such as NF-κB and p38 MAPK signaling pathways as shown in *L. casei* and *L. reuteri* where they could play important roles in the augmentation of innate

immunity (Iyer *et al*., 2008; Kim *et al*., 2006). Two proteins of *L. rhamnosus* GG (p40 and p75) (Yan *et al*., 2007) have been suggested to preserve the tight junction (TJ) integrity, an effect mediated by the PKC and MAPK pathways. They display antiapoptotic activity, prevent epithelial barrier damage caused by several agents and show *in vivo* effect by decreasing the susceptibility to dextran sulphate sodium (DSS)-induced colon epithelial injury (Yan *et al*., 2007). Interestingly, recent studies (Bäuerl *et al*., 2010) showed that these proteins are active cell wall hydrolases present in the seven genome sequences available for strains in the *L. casei-paracasei/rhamnosus* group. PGNs are cell wall components that interact with the intracellular receptor Nod2. Their *in vivo* effect changes with their composition composition, which also varies between strains. In the case of *Lactobacillus salivarius* Ls33 the muropeptide, M-tri-Lys, acts as ligand that protected mice from colitis in a NOD2-dependent but MyD88-independent manner (Macho Fernandez *et al*., 2011).

Two strains of *L. reuteri* and *L. casei*, but not *L. plantarum*, had the ability to prime DC to drive the development of Treg cells which produced increased levels of the anti-inflammatory cytokine IL-10 (Smits *et al*., 2005). This ability was mediated by binding to the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Targeting of DC-SIGN by certain probiotic bacteria could explain their beneficial effect in the treatment of several immune diseases, including atopic dermatitis and Crohn's disease. However, a DC-SIGN ligand capacity has been found in an extracellular protein (LP_2145) in *L. plantarum* WCFS1(patent application WO/2009/035330) and in the surface-layer protein A (SlpA) from *L. acidophilus* NCFM, inducing IL-10, IL-4 and decreasing IL12p70 synthesis (proinflammatory) (Konstantinov *et al*., 2008).

There are also experiments that reported the *in vivo* effect on gene transcription in human volunteers where biopsies were analysed by microarrays. Strains of *L. rhamosus* GG (Di Caro *et al*., 2005) and *L. plantarum* WCFS1 (van Baarlen *et al*., 2011) were used administrated and a differential expression of analysed, noticing that expression affected mainly to genes involved in the immune and inflammatory response, as well as to genes related to apoptosis, growth and cell differentiation, signalling, adhesion, etc. Remarkably, striking differences were found in the modulation of NF-κB related pathways.

6.2.2 Engineered lactic acid bacteria

An efficient expression of IL-10 was achieved in *L. lactis* , that showed a striking effect in the remediation of DSS (dextran sodium sulphate) induced colitis in IL-10$^{-/-}$ mice (Steidler *et al*., 2000). Then, the genetic manipulation strategy was improved and the human IL-10 encoding gene used to replace the thymidylate synthase gene (*thy*A) in the bacterial chromosome, which achieved a stable genetic construction and a self contained recombinant strain requiring thymidine to proliferate. This strain was used in a double blind assay on Crohn's disease patients (phase I), proving that the intake of this strain significantly decreased the disease activity (Braat *et al*., 2006). Other anti-inflammatory and epithelium repairing peptide, such as the trefoil factor, was similarly successful in mice (Vandenbroucke *et al*., 2004).

Mutants obtained by site directed mutagenesis, holding genetic changes precisely introduced, have been tested for improved health features. Teichoic acids (TA) activate NF-κB through TLR-2 binding and they are one of the main immunostimulatory components of pathogenic grampositive bacteria. This effect was also observed in *L. plantarum* NCIMB8826, however, a mutant (*dlt*) deficient in D-alanylation of TA was much more anti-inflammatory than the parental strain on peripheral blood mononuclear cells and mice (Grangette *et al*., 2005).

In other cases mutants have been very useful to demonstrate the functional effect of certain cell components. In *L. casei* Shirota (LcS), a mutant strain was very useful to prove that inhibition of the pro-inflammatory cytokine IL-6, through Nod2/ NF-κB, was due to a cell wall-derived polysaccharide– PGN complex (PSPG) (Matsumoto *et al.*, 2009).

6.3 Engineered probiotics to delivery bioactive proteins

The ability of lactobacilli to survive on the mucosal surfaces of humans and animals has been utilized for the delivery and presentation of bioactive molecules at these surfaces. These bacteria have several advantages, including their recognised GRAS/QPS status, their capacity to interact with the host at several levels and their public acceptance. Studies of lactobacilli as delivery vehicles have mainly focused on the development of mucosal vaccines. In addition, interleukins have been also co-expressed with antigens in lactobacilli to enhance the immune response. Other applications of *Lactobacillus* species as delivery systems include anti-infectives, therapies for allergic diseases and therapies for gastrointestinal diseases. The ability of lactobacilli and other LAB to express these antigens/bioactive molecules at mucosal surfaces have been widely reviewed (Monedero & Pérez-Martínez, 2008; Wells & Mercenier, 2008).

Some recent examples include the use of *Lactobacillus jensenii* strains isolated from human vaginal mucosa for the delivery vehicle of a surface-anchored two-domain CD4 (2D CD4) molecule for the mitigation of heterosexual transmission of HIV (Liu *et al.*, 2008). *L. casei* was the host to express the viral proteins from porcine rotavirus and porcine parvovirus fused the heat-labile toxin B subunit from *Escherichia coli*. The results showed that mice responded producing increased levels of anti-viral antibodies (Liu *et al.*, 2011). *L. casei* Zhang was engineered to stably express the p23 immunodominant surface protein of *Cryptosporidium parvum* sporozoites. Recombinant *L. casei* Zhang-p23 was able to activate the mucosal immune system and to elicit specific serum immunoglobulin G (IgG) and mucosal IgA in mice. The expression of cytokines such as IL4, IL6, and IFN-gamma was detected in splenocytes of mice by real-time PCR after oral immunization with this strain (Geriletu *et al.*, 2011). A recombinant *L. casei* strain secreting biologically active murine interleukin-1β has been constructed. This strain was able to induce IL8 secretion in Caco2 cells and IL6 in vivo using a ligated-intestinal-loop assay in mice after oral administration. The increased adjuvant properties of this strain were confirmed after intragastric immunization with heat-killed *Salmonella enterica* serovar *Enteritidis* (Kajikawa *et al.*, 2010).

Another application of lactobacilli has been their use to express molecules to deliver passive immunity against pathogens, such as single-chain antibodies (lactobodies). Martin and co-workers have been able to construct a series of expression cassettes stably integrated in the chromosome to mediate the secretion or surface display of antibody fragments in *Lactobacillus paracasei*. These new constructed strains, producing surface-anchored variable domain of llama heavy chain (VHH) (ARP1) directed against rotavirus, showed efficient binding to rotavirus and protection in the mouse model of rotavirus infection (Martin *et al.*, 2011).

Lactobacilli can also be engineered to produce an increased immune response against cancer cells. *L. rhamnosus* GG (LGG) has been used to successfully induce tumor regression in an orthotopic model of bladder cancer. The potential of LGG to induce a directed anti-tumor response was evaluated with modified LGG secreting the prostate specific antigen (PSA) or IL15 and PSA (IL-15-PSA). Recombinant LGG activated neutrophils, induced dendritic cells maturation, T cell proliferation and PSA specific cytotoxic T lymphocytes activity leading to the tumor regression (Kandasamy *et al.*, 2011).

In spite of the dozens of reports describing the capacity of LAB to deliver bioactive molecules to the host mucosa, important issues, such as potential side-effects should be always addressed when working with recombinant LAB. The expression of the *Salmonella* OmpC in a recombinant *L. casei* was fortuitously found to reduce the secretion of tumor necrosis factor alpha (TNF-α) from murine macrophages (Kajikawa & Igimi, 2009). Another non desired effect of a recombinant *Lactobacillus* was found when *L. acidophilus* was engineered to express the interferon-β to study the local delivery of this molecule in a colitis mouse model. Surprisingly the administration of the recombinant bacteria secreting IFN-β has an immunological effect that resulted in the exacerbation of colitis in this model (McFarland *et al*., 2011).

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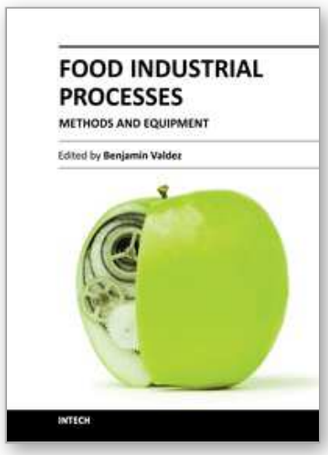

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The global food industry has the largest number of demanding and knowledgeable consumers: the world population of seven billion inhabitants, since every person eats! This population requires food products that fulfill the high quality standards established by the food industry organizations. Food shortages threaten human health and are aggravated by the disastrous, extreme climatic events such as floods, droughts, fires, storms connected to climate change, global warming and greenhouse gas emissions that modify the environment and, consequently, the production of foods in the agriculture and husbandry sectors. This collection of articles is a timely contribution to issues relating to the food industry. They were selected for use as a primer, an investigation guide and documentation based on modern, scientific and technical references. This volume is therefore appropriate for use by university researchers and practicing food developers and producers. The control of food processing and production is not only discussed in scientific terms; engineering, economic and financial aspects are also considered for the advantage of food industry managers.

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