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Complex Oligosaccharide Utilization Pathways in *Lactobacillus*

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Abstract

Lactobacillus is the bacterial genus that contains the highest number of characterized probiotics. Lactobacilli in general can utilize a great variety of carbohydrates. This characteristic is an essential trait for their survival in highly competitive environments such as the gastrointestinal tract of animals. In particular, the ability of some strains to utilize complex carbohydrates such as milk oligosaccharides as well as their precursor monosaccharides, confer upon lactobacilli a competitive advantage. For this reason, many of these carbohydrates are considered as prebiotics. Genome sequencing of many lactobacilli strains has revealed a great variety of genes involved in the metabolism of carbohydrates and some of them have already been characterized. In this review, the current knowledge at biochemical and genetic levels on the catabolic pathways of complex carbohydrates utilized by lactobacilli will be summarized.

Introduction

All animals establish symbiotic associations with microbes. Paramount among these is the establishment of complex microbial communities in the gastrointestinal tract. These communities may comprise thousands of species and are particular of each individual (Eckburg *et al.*, 2005; Ley *et al.*, 2006; Turnbaugh *et al.*, 2009). The composition and distribution of microbiota changes along the gastrointestinal tract reflecting the differing physicochemical conditions and epithelial surfaces that the microbes find in the different compartments of the gastrointestinal tract (Gu *et al.*, 2013; Kim and Isaacson, 2015; Stearns *et al.*, 2011; Tropini *et al.*, 2017). For example, *Lactobacillaceae* are predominantly found in the stomach and small intestine of mice whereas anaerobes such as *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* are mainly found in the large intestine and feces (Gu *et al.*, 2013). In the oral cavity of humans, a neutral pH and aerobic conditions prevail although anaerobic niches are also present. The environment in the stomach is acidic and microaerophilic whereas pH increases and oxygen availability decreases along the small intestine and colon. Nutrient availability and immune effectors also vary along the gastrointestinal tract. Furthermore, microbes are not uniformly distributed along the transverse axis of the gut (Tropini *et al.*, 2017). Species such as *Akkermansia muciniphila* and some *Bacteroides* sp. are thought to be predominantly associated with the mucus layer whereas closer to the mucosa, aerotolerant taxa such as *Proteobacteria* and *Actinobacteria* are more abundant due to the radial oxygen gradient established across the intestinal wall (Albenberg *et al.*, 2014).

Numerous studies have demonstrated that gut microbiota has a great influence on manifold host physiology aspects, from metabolism (Li *et al.*, 2008) to behavior (Ezenwa *et al.*, 2012). Perturbation of the gut microbiota may markedly affect the host health (Cho and Blaser, 2012; Claesson *et al.*, 2012) and has been related to a number of diseases such as metabolic disorders (Sonnenburg and Backhed, 2016), inflammatory diseases (Blander *et al.*, 2017), diabetes (Membrez *et al.*, 2008; Vaarala *et al.*, 2008), coeliac disease (Collado *et al.*, 2007), etc. Although the microbiota associated to an individual may display some resilience to perturbation (Lozupone *et al.*, 2012; Sommer *et al.*, 2017), external environmental factors such as food intake (Kohl *et al.*, 2014) and composition (David *et al.*, 2014) can alter it

substantially. This aspect is pivotal in the relationship between microbiota and host nutrition and health as the gastrointestinal microbiota provide important metabolic capabilities, including the ability to obtain energy from indigestible dietary polysaccharides (Goh and Klaenhammer, 2015). The human genome encodes only 17 glycosidases enabling the utilization of a very limited set of polysaccharides (Cantarel *et al.*, 2012; Goh and Klaenhammer, 2015). Therefore, most complex carbohydrates are available to gut microbes able to utilize these compounds.

Carbohydrates, as oligo- and polysaccharides or as glycoconjugates constitute an amazingly diverse group of molecules due to the structural diversity and numerous bonding sites of their constituent monosaccharides that allow their assembly among themselves or to almost any other organic molecule in a wide array of architectures. Because of this structural versatility, carbohydrates fulfill a wide variety of functions in organisms as structural polymers, energy reserve, signaling, etc. Furthermore, as a major component of human diet, carbohydrates also have a determining influence on the interactions between host and associated microbiota (Hooper *et al.*, 2002). In addition to dietary carbohydrates, host-derived glycans constitute a secondary source of carbohydrates for gut microbiota (Hooper *et al.*, 2002).

Although the gut microbiota taken as a whole can utilize a wide variety of glycans, cells belonging to individual taxa can usually metabolize a limited set of them. From this fact stems the concept of prebiotic, defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). Prebiotics were first thought of as a means to selectively enrich probiotic gut microbes, specifically *Lactobacillus* and *Bifidobacterium* (Gibson and Roberfroid, 1995; Goh and Klaenhammer, 2015). Although not limited by the definition, all prebiotics are glycans. The definition of prebiotic has been revised and the specific stimulatory effect of prebiotics on lactobacilli and bifidobacteria has been challenged (Hutkins *et al.*, 2016). Notwithstanding, the original idea gave a strong boost to the study of the glycan catabolic pathways of these organisms.

Lactobacillus is a large genus currently comprising over 200 species that have been isolated from a wide variety of habitats. They are Gram-positive, microaerophilic or anaerobic obligate fermentative organisms that produce lactic acid as the major end product of sugar fermentation. Together with genera *Paralactobacillus*, *Pediococcus* and *Sharpea* they constitute the family *Lactobacillaceae* within the order *Lactobacillales*. *Lactobacillus* strains play a major role in the production of a wide variety of fermented products. Others are naturally associated to mucosal surfaces of humans and animals and have been considered as probiotics (Tannock, 2004). Either as foodstuff fermenters or as probiotics, their capacity to utilize glycans is an important trait for their performance. This review focuses on our current knowledge on the pathways of complex glycan dissimilation identified in species of *Lactobacillus*.

Fructan and fructooligosaccharide catabolic pathways

Structural characteristics of fructans

Fructans are linear or branched fructose polymers and can be broadly divided into inulins (β-2,1-linked) and levans (β-2,6-linked) (Fuchs, 1991) (Figure 1). Fructans are usually synthesized from sucrose by repeated fructosyl transfer so that they have a terminal glucose unit. Levans are produced by many microorganisms, including some lactobacilli (Bello *et al.*, 2001; Tieking *et al.*, 2003; Van Geel-Schutten *et al.*, 1999), and a few plant species (Öner *et al.*, 2016). In contrast, inulins are relatively common in plants, especially *Asteraceae*, but only a few bacterial species produce them, among them, some *Lactobacillus* and *Leuconostoc* species (Anwar *et al.*, 2008; Olivares-Illana *et al.*, 2003; van Hijum *et al.*, 2002). While bacterial fructans have a very high degree of polymerization (DP) up to 10^5 fructose units, the DP of plant-derived fructans does generally not exceed DP 100. High DP of bacterial fructans is possibly related to their function as structural components of biofilms, but they also constitute an extracellular nutrient reservoir (Öner *et al.*, 2016). In plants, fructans serve essentially as reserve carbohydrates.

Synthesis of fructans is catalyzed in bacteria by fructosyltransferases, named inulosucrases (EC 2.4.1.9) when they synthesize inulin, and levansucrases (EC 2.4.1.10) when they produce levan. Levansucrases can also form β-2,1-fructosyl-fructose linkages either to synthesize inulin-type fructooligosaccharides (FOS) or to create branching points that connect the β-2,6-linked chains of the polymer (Öner *et al.*, 2016). In contrast to bacteria, the biosynthesis of fructans in plants is catalysed by

three different classes of enzymes: sucrose:sucrose 1-fructosyltransferase (EC 2.4.1.99) (1-SST), fructan:fructan 1-fructosyltransferase (EC2.4.1.100) (1-FFT) and fructan exohydrolase (EC.3.2.1.153) (1-FEH) (van Arkel *et al.*, 2013). 1-SST primarily catalyses the synthesis of the trisaccharide 1-kestose, from two molecules of sucrose. In this reaction glucose is formed in equimolar amounts to 1-kestose (β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside). 1-FFT catalyses the transfer of fructosyl units from 1-kestose and any other fructan molecule onto 1-kestose and higher DP fructan molecules. 1-FEH, catalyses the degradation of inulin by hydrolysing terminal fructosyl units, which results in the formation of fructose and lower DP inulin (van Arkel *et al.*, 2013).

Metabolic pathways for fructans utilization

The interest on fructans in relation to the intestinal microbiota stemmed from the search of carbohydrate sources that reached the large intestine and were selectively used by beneficial microbes such as bifidobacteria. Several studies noted that fructooligosaccharides derived from inulin hydrolysis (usually referred to as FOSs) were not hydrolyzed by the host endogenous enzymes but efficiently used by bifidobacteria (Hidaka *et al.*, 1986; Yazawa and Tamura, 1982). FOS naturally occur in many kinds of plants but they are commercially produced from the hydrolysis of inulin or synthesized from sucrose by transfructosylation by β-fructofuranosidases or β-D-fructosyltransferases (Goh and Klaenhammer, 2015). The most utilized natural source of inulins is chicory and depending on the method of extraction the product obtained may be almost exclusively FOS of the GF_n type (a glucose monomer linked α-1,2 to two or more β-2,1-linked fructosyl units) or a mixture of GF_n and F_m type (two or more β-2,1-linked fructosyl units) oligomers (Roberfroid *et al.*, 1998).

However, the first studies that reported the utilization of fructans by lactobacilli were focused in

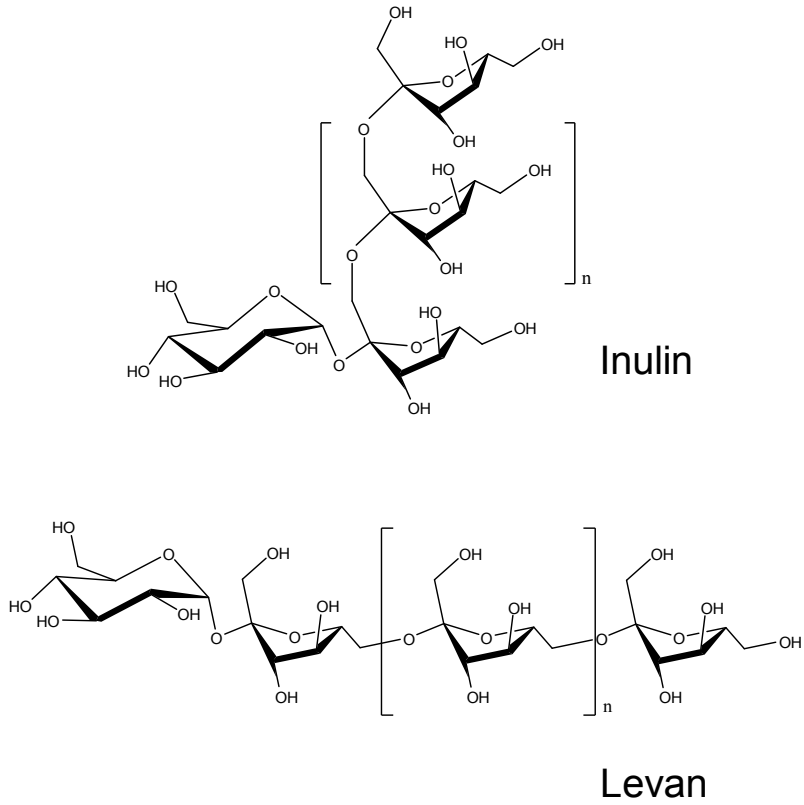

Figure 1. Chemical structures of inuline and levan.

the usage of lactic acid bacteria for ensilage and showed that some lactobacilli could utilize fructans for growth (Kleeberger and Kühbauch, 1976). In a later study, it was shown that sixteen strains out of 712 were able to degrade levan and eight of them could also degrade inulin (Müller and Lier, 1994). Subsequent studies confirmed the ability of some lactobacilli to degrade fructans (Merry *et al.*, 1995; Müller and Steller, 1995; Winters *et al.*, 1998) although the enzymes and pathways involved were not determined. Other studies also established that some lactobacilli could grow on FOSs as carbon sources (Kaplan and Hutkins, 2000; Sghir *et al.*, 1998). The characterization of an extracellular fructan hydrolase from *Lactobacillus paracasei* ssp. *paracasei* P 4134 provided the first clues on the fructan degradative pathways of lactobacilli (Müller and Seyfarth, 1997). The enzyme hydrolized β-2,6 linked fructans more rapidly than β-2,1 linked fructans and the main product of hydrolysis was fructose, suggesting that the enzyme is an exofructanase (Müller and Seyfarth, 1997). Later studies on other *L. casei/paracasei* have confirmed these conclusions (Kuzuwa *et al.*, 2012; Velikova *et al.*, 2017). Subsequently, another extracellular fructanhydrolase purified from *Lactobacillus pentosus* B235 was characterized (Paludan-Müller *et al.*, 2002). The purified enzyme had the highest activity for levan, but also hydrolysed garlic extract, a β-2,1-linked fructan with β-2,6 linked fructosyl sidechains, 1,1,1-kestose, 1,1-kestose, 1-kestose, inulin and sucrose at 60, 45, 39, 12, 9 and 3%, respectively, of the activity observed for levan (Paludan-Müller *et al.*, 2002).

Genome sequencing and transcriptomic analyses paved the way for the identification and characterization of genes involved in the utilization of fructans. The studies carried out so far have characterized three different fructan utilization pathways in *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum* that differ in their transport systems and fructofuranosidase enzymes (Figure 2).

In 2003, Barrangou et al. (Barrangou *et al.*, 2003) identified an operon (*msm*) involved in FOS utilization by *Lactobacillus acidophilus* NCFM. The *msm* operon consisted of genes encoding for a transcriptional regulator of the LacI family (*msmR*), an ABC transport system (*msmEFGK*), a fructosidase (*bfrA*) and a sucrose phosphorylase (*gtfA*; Figure 3) (Barrangou *et al.*, 2003). Similar operons can be detected in a limited number of strains of other species of lactobacilli (Figure 3). In *Lactobacillus delbrueckii*, *Lactobacillus perolens*, *Lactobacillus saniviri* and *Lactobacillus concavus* strains, a gene encoding a putative fructokinase is associated to the *msm* cluster (Figure 3). The transcriptional analysis of the *msm* operon of *L.*

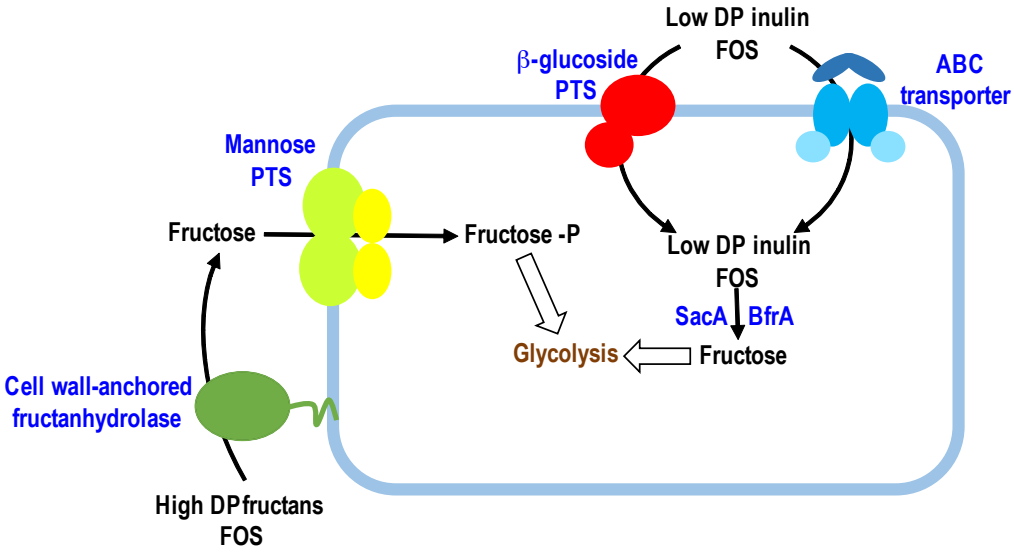

Figure 2. Fructan utilization pathways characterized in LAB.

acidophilus NCFM showed that all genes were transcribed in a single transcriptional unit (Barrangou *et al.*, 2003). Sucrose and oligofructose (both GF_n and F_n types) induced the expression of the operon whereas glucose and fructose did not. The expression of the operon was repressed by glucose suggesting that it is subjected to carbon catabolite repression (CCR). This hypothesis was further supported by the presence of several CRE-like sites in the *msm* promoter region. The functionality of the operon was shown by inactivation *msmE* and *bfrA* that resulted in defective growth on FOS-F_n (Barrangou *et al.*, 2003).

A comparative analysis of *L. ruminis* strains of human and bovine origin led to the identification of an operon possibly involved in FOS utilization consisting of a β-fructan hydrolase and an oligosaccharide H^+ sympoter (O' Donnell *et al.*, 2011) although experimental evidence is still lacking.

In *Lactobacillus plantarum* WCFS1, a gene cluster consisting of a putative fructokinase (*sacK1*), a putative phosphoenolpyruvate-dependent phosphotranferase transport system (PTS) of the β-glucoside family (*pts1BCA*), a β-fructofuranosidase (*sacA*), a LacI family transcriptional regulator (*sacR*) and a putative α-glucosidase (*agl2*) (Figure 3), was induced when this strain was grown in the presence of low molecular weight FOS (Saulnier *et al.*, 2007). The biochemical characterization of the *L. plantarum* ST-III SacA demonstrated that the enzyme has exofructofuranosidase activity with preference for β-2,1 linkages between two fructose moieties in fructans with low DP (Chen *et al.*, 2014). The heterologous expression of SacA in *Lactobacillus rhamnosus* GG, an organism that can utilize fructose but not FOS, enabled this strain to

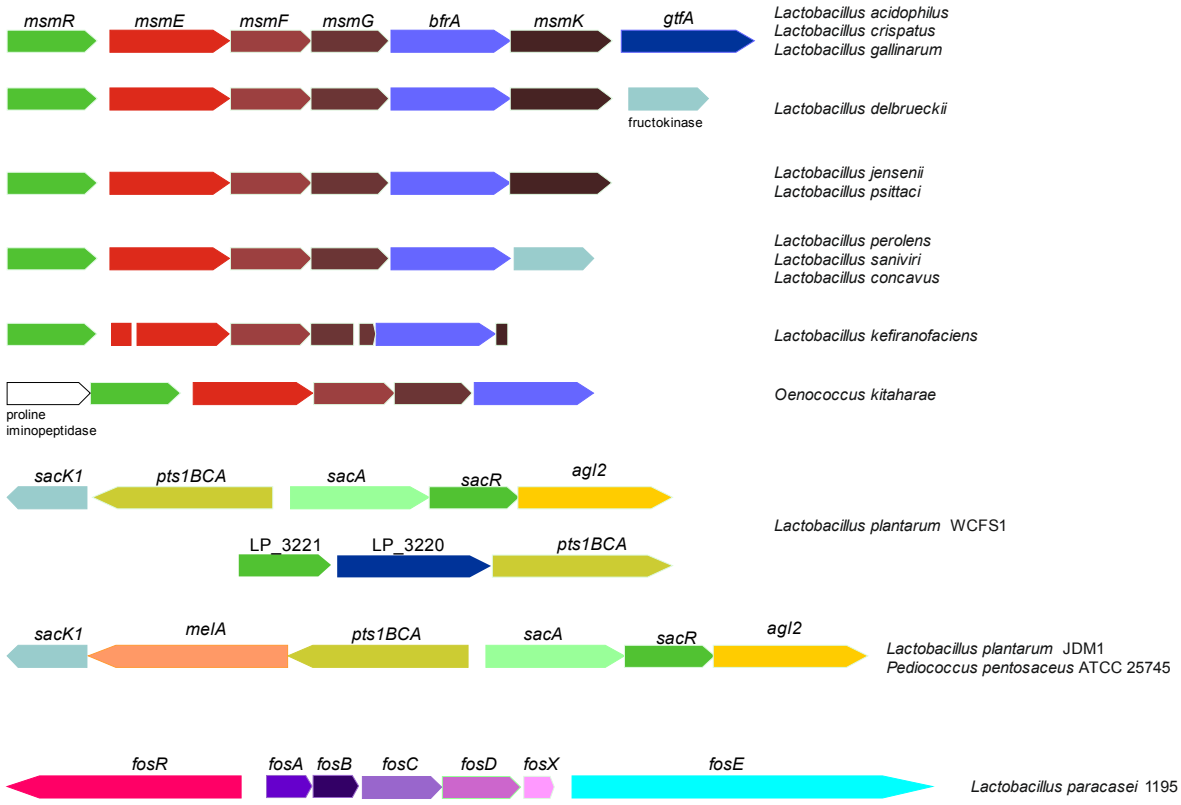

Figure 3. Schematic representation of fructan utilization gene clusters present in selected lactobacilli. Colors indicate homologous genes. Arrows indicate directionality. Interrupted arrows indicate translational frameshifts.

grow on FOSs, thus demonstrating the functional role of this enzyme (Chen *et al.*, 2014). Furthermore, inactivation of *sacA* in *L. plantarum* ST-III severely impaired the growth of this strain on FOSs (Chen *et al.*, 2015). In contrast, a mutant defective in *pts1BCA* still could grow with FOSs although at a lower growth rate than the wild-type strain (Chen *et al.*, 2015). A transcriptomic analysis of this strain had detected a second putative *sac* (*sacPTS26*) gene cluster constituted by a β-glucoside PTS (PTS26), an α-glucosidase (Agl4), and a transcriptional regulator (SacR2), that was also induced in the presence of FOSs (Chen *et al.*, 2015). A double mutant *pts1BCA*/*pts26* was unable to grow on FOS, indicating that both transporters are required for optimal FOS uptake and utilization (Chen *et al.*, 2015). This second *sac* cluster is also present in *L. plantarum* WCFS1 (Figure 3) but it was not detected as upregulated in the presence of FOS in this strain (Saulnier *et al.*, 2007) and its involvement in FOS utilization in WCFS1 remain to be determined (Chen *et al.*, 2015). It is also intriguing the presence of α-glucosidase encoding genes in *sac* clusters. The functional role of these genes remains to be established.

As mentioned above, *L. paracasei* utilizes an extracellular fructanhydrolase. Goh et al. (Goh *et al.*, 2006) determined that the genes required for FOS utilization by *L. paracasei* 1195 are organized in a cluster (*fosRABCDXE*) encoding a putative mannose family PTS transporter (*fosABCDX*), a β-fructosidase (*fosE*) and, divergently transcribed, a transcriptional antiterminator (*fosR*) (Figure 3). Homologous clusters are found in other *L. casei/paracasei* but considerable variability is observed. For example, *L. paracasei* ATCC 334 *fosE* homolog (LSEI_0564) lacks the C-terminal part including the cell-wall anchor motif whereas this gene is absent in strain BL23 (Figure 3). This variability may account for the conflicting observations of extracellular or cell-wall anchored fructanhydrolase activity in different *L. paracasei* strains. Inactivation of *fosE* led to the loss of the ability to grow on sucrose, FOS, oligofructose (FF_n type), inulin and levan, thus demonstrating the functionality of the operon (Goh *et al.*, 2006). Furthermore, introduction of *fosE* into *Lactobacillus rhamnosus* GG enabled this strain to utilize FOS (Goh *et al.*, 2007). The analysis of the FosE encoding sequence revealed an N-terminal signal peptide sequence and an LPQAG cell wall anchor motif at the C-terminal region, suggesting its localization at the cell wall. Cell fractionation assays confirmed this hypothesis as FOS hydrolysis activity was present exclusively in the cell wall extract of *L. paracasei* previously grown on FOS (Goh *et al.*, 2007). In agreement with a previous biochemical characterization of a fructanhydrolase of *L. paracasei* (Müller and Seyfarth, 1997), the analysis of the degradation products of *L. paracasei* 1195 FosE indicated that it is an exofructanhydrolase (Goh *et al.*, 2007). The transcriptional regulation of the *fos* cluster has also been studied. Expression of *fos* genes is induced in the presence of FOS, inulin, and to a lesser extent, sucrose and fructose but repressed by glucose (Goh *et al.*, 2007; Goh *et al.*, 2006). A CRE sequence is present in the *lev* promoter region, suggesting that the operon is subjected to CCR via the P-Ser-HPr/CcpA complex (Goh *et al.*, 2006).

The role of FosR has not been addressed in *L. paracasei* 1195, however the functional role of the homologous LevR of strain BL23 has been studied (Mazé *et al.*, 2004). The intergenic regions of *fosR-fosA* and *levR-levA* in strain BL23 differ only in a single nucleotide and the FosABCD proteins shared more than 99% identity with their BL23 counterparts (Goh *et al.*, 2006). LevR is homologous to the *Bacillus subtilis* LevR transcriptional regulator, which controls the expression of a mannose-class PTS transporter and a levanase involved in levan utilization. *B. subtilis* LevR interacts with the σ^{54} factor and its activity is modulated via phosphorylation by by P-His-HPr and P-EIIB^{Lev} (Martin-Verstraete *et al.*, 1998). In contrast, BL23 strain LevR do not require σ^{54} although the regulation of its activity by phosphorylation still occurs by dual PTS-catalyzed phosphorylation at conserved histidine residues in the EIIA and PRD2 domains of LevR by P-His-HPr and P-His-EIIB^{Lev}, respectively (Mazé *et al.*, 2004). When the PTS^{Lev} transporter is active, P-His-EIIB^{Lev} preferably donates its phosphoryl group to the transported sugar, leading to dephosphorylation of LevR at His-776 by P-His-EIIB^{Lev} and LevR activation and thereby induction of the *lev* PTS. On the other hand, when metabolically preferred PTS sugars, such as glucose, are present, the phosphoryl group of P-His-HPr is used for sugar phosphorylation. Poor phosphorylation at His-488 by P-His-HPr renders LevR less active and downregulates expression of the *lev* PTS.

The different strategies of fructan utilization by lactobacilli possibly determine their abilities to utilize different fructans. Internalization and subsequent hydrolysis possibly limits the ability of fructan utilization to low DP oligosaccharides whereas extracellular degradation would enable the

utilization of high DP fructans. Experimental evidence available supports this view. Makras et al. (Makras *et al.*, 2005) assayed the capacity of ten strains of lactobacilli to degrade inulin-type fructans, observing that *L. acidophilus* only degraded oligofructose whereas *L. paracasei* could also degrade long-chain inulin. *L. plantarum* could utilize short-chain fructooligosaccharides but grew poorly with FOS (Saulnier *et al.*, 2007). It has been proposed that the different strategies of fructan utilization in lactobacilli may respond to different ecological strategies. Internalization and subsequent degradation eliminates cross-feeding while conferring an advantage in a nutrient-competitive environment. On the other hand, extracellular degradation let other organisms profit of the hydrolytic products thereby allowing the establishment of symbiotic relationships with other members of the community (Goh and Klaenhammer, 2015).

Metabolism of glucans and glucooligosaccharides

Structural characteristics of glucans

Lactobacillus species are equipped with the enzymatic machinery to utilize multiple glucan structures, which consist of glucose homopolymers with different linkages and branching types. However, the presence of these capacities is species- and strain-specific and usually linked to particular niche adaptations. Glucans can be classified into α- and β-glucans depending on the type of glycosidic bond present in the molecules (Figure 4). Starch is the main example of α-glucan and it represents the major carbon storage polysaccharide in plants. It is made of linear glucose chains with α-1,4 linkages with high DP (amylose) and shorter chains which in addition to α-1,4 bonds possess around 5% of α-1,6 branching of 18 to 25 glucoses (amylopectine). Other α-glucans can be linear or branched and carry diverse bonds (α-1,2; α-1,3) in addition to α-1,4 and α-1,6. Most of these glucans are produced by bacteria and fungi, such as dextrans (α-1,6 with α-1,3 branching; Figure 4) produced by strains of lactobacilli such as *Leuconostoc mesenteroides* (Chen *et al.*, 2016), *Weisella cibaria* (Malang *et al.*, 2015) or *Lactobacillus sakei* (Nácher-Vázquez *et al.*, 2017), reuteran (α-1,4 with α-1,6 branching), synthesized by *Lactobacillus reuteri* (Chen *et al.*, 2016) or pullulan (maltotriose units linked by α-1,6 bonds; Figure 5), produced by *Aureobasidium pullulans* (Cheng *et al.*, 2011). Isomaltooligosaccharides (IMO) are a type of α-glucans that are gaining interest due to their prebiotic effects (Ketabi *et al.*, 2011; Leemhuis *et al.*, 2014; Yen *et al.*, 2011). They are short α-1,6-linked glucans (e.g. isomaltose [α-D-glucopyranosyl-(1→6)-D-glucopyranoside], panose [α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucopyranoside], isomaltotriose, isomaltotetraose and isomaltopentaose) that are present in some foods or can be commercially prepared from starch. Their prebiotic effect derives from the fact that humans and other monograstric animals generally lack IMO-degrading enzymes. Glycogen is an α-glucan equivalent to starch but present in animals and it is characterized by being more extensively branched. Many bacteria, including lactobacilli (Goh and Klaenhammer, 2013), can synthesize and utilize this molecule as carbon storage.

Cellulose is the most abundant β-glucan present in nature. It is a key component of plant cell walls so that it accounts for the major proportion of fixed carbon in living organisms. Plant as well as fungal cell walls may contain other β-glucans with β-1,3 or multiple alternating β-1,3 and β-1,4 linkages and β-1,6 branching linkages. Similar to α-glucans, some β-glucans can be produced by bacteria, including lactobacilli. Examples of these are the β-glucans produced by *Lactobacillus suebicus* (β-1,3 linked) (Garai-Ibabe *et al.*, 2010) and *L. brevis* (Fraunhofer *et al.*, 2017), where the glycosyl transferases catalyzing the synthetic process have been characterized.

α-glucan metabolic pathways in Lactobacillus

The α-glucans are the substrates of a wide variety of hydrolytic enzymes. The α-amylases cleave α-1,4 linkages at any part of the polymers, whereas β-amylases act at the non-reducing ends liberating maltose (α-D-glucopyranosyl-(1→4)-D-glucopyranoside). Pullulanases and amylopullulanases degrade α-1,6 linkages and α-1,6 as well as α-1,4 linkages, respectively. Among pullulanases, isopullulanases and neopullulanases can be distinguished because they produce isopanose or panose from pullulan, respectively (Figure 5).

Lactobacilli usually encode multiple α-glycosidases in their genomes, mainly from the glycosyl hydrolase (GH) 13 family (CAZy, Carbohydrate Active enZYmes classification; www.cazy.org), the family to which α-amylases belong, although their specificities remain to be investigated in most cases. Among α-glucans, starch degradation by lactobacilli is relatively well characterized. The ability to degrade starch was noticed after the isolation and phenotypic characterization of new

Lactobacillus strains from waste corn fermentations such as *Lactobacillus amylophilus* (Nakamura and Crowell, 1979) and *Lactobacillus amylovorus (*Nakamura, 1981). However, extracellular amylases are not common in this genus (less than 2% of all the GH13 glycosyl hydrolases present in lactobacilli). They are mainly concentrated in five species: *L. acidophilus*, *L. amylovorus*, *Lactobacillus fermentum, L. plantarum* and *Lactobacillus manihotivorans* (Petrova *et al.*, 2013), although some particular strains belonging to different species can also degrade starch (e.g. *Lactobacillus paracasei* B41 (Petrova and Petrov, 2012)). In addition to amylases, pullulanases and amylopullulanases have been found in lactobacilli. A thermostable pullulanase (endo-α-1,6-glucosidase, GH13_14 subfamily) encoded by the LBA_1710 gene from *L. acidophilus* NCFM has been characterized. This enzyme preferentially acts on β-limit dextrins (amylopectins digested by β-amylases) over amylopectin (Møller *et al.*, 2017). The product of LBA_1710 can also act on the linear polymer

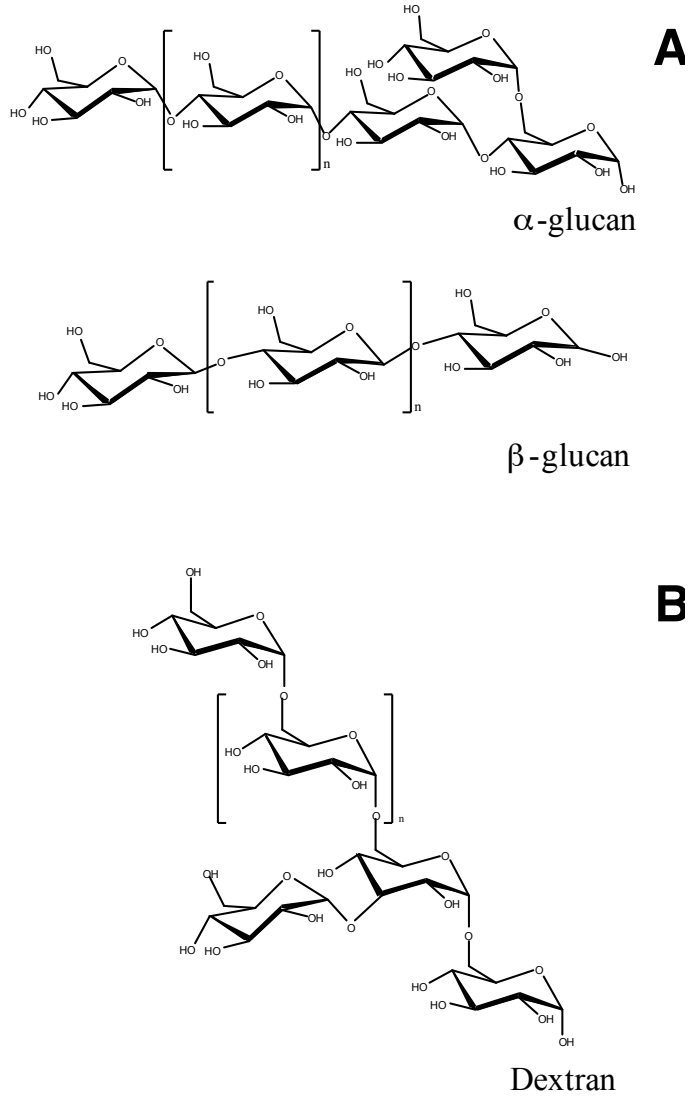

Figure 4. A. Chemical structures of α- and β-glucans. An α-1,6 ramification is represented in the structure of α−glucan. B. Chemical structure of dextran. An α-1,3 ramification is represented.

pullulan [(maltotriose-α1,6-maltotriose)$_n$] and possess a very low K_m and very high specific activity for this polysaccharide. Notwithstanding, pullulan and amylopectine do not support growth of this strain. This would reflect the lack of α-amylase activity and a non-efficient transport system for the resulting oligosaccharides after pullulanase digestion in this strain. The enzyme is secreted due to presence of a signal peptide and possesses two starch binding modules (CBM48), a catalytic domain (GH13_14 subfamily) and an additional surface-layer associated domain (SLAP) which warrants its retention at the cell surface. Homologues of this enzyme are present in many lactobacilli from intestinal origin. The enzyme may act on short-branched α-glucans derived from the degradation of dietary starch and glycogen by the human enzymes, supporting that these debranching enzymes play a role in the adaptation of these lactobacilli to the gut niche (Møller *et al.*, 2017). An extracellular amylopullulanase from *L. plantarum* L137, an isolate from a traditional fermented food containing fish and

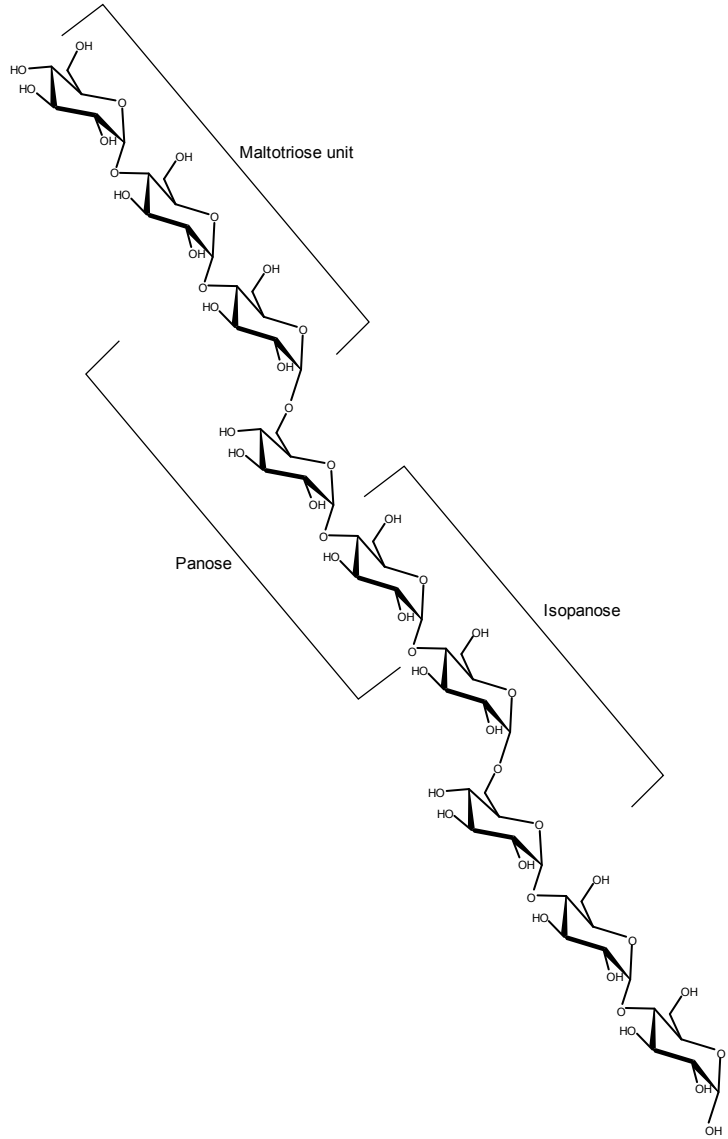

Figure 5. Chemical structure of pullulan. Maltotriose, isopanose and panose moieties are indicated.

rice with high hydrolytic activity towards starch, has also been characterized. The enzyme degrades soluble starch to maltotriose and maltotetraose whereas it produces only maltotriose from pullulan (Kim *et al.*, 2008). The encoding gene (*apuA*) is located in the endogenous plasmid pLTK13 (Kim *et al.*, 2008). The enzyme contains a number of amino acids repeats at the N- and C-terminus that are derived from the same repeated DNA sequence (5'-ACCGACGCAGCCAACTCA-3') but translated in different frames. The C-terminal repeats are similar to mucin-binding domains present in bacterial peptidoglycan-bound proteins and most probably participate in substrate binding (Kim *et al.*, 2008). Domains with amino acid repeats are typically found in carbohydrate degrading enzymes. The amylases from *L. amylovorus*, *L. plantarum* and *L. manihotivorans* carry a C-terminal starch-binding domain (SBD) of almost 500 amino acids consisting of tandem repeat units of 91 amino acids in variable numbers (Morlon-Guyot *et al.*, 2001). SBDs promote attachment to the substrate and allow degradation of non-soluble starch (Rodriguez Sanoja *et al.*, 2000). A neopullulanase has been cloned and characterized from *Lactobacillus mucosae* LM1 (Balolong *et al.*, 2016). A homologous gene had been previously shown to be induced in the amylolytic *L. plantarum* A6 strain during pearl millet fermentation and neopullulanase activity detected (Humblot *et al.*, 2014).

A number of enzymes involved in the degradation of IMOs encoded by lactobacilli have also been characterized. A glucan-α-1,6-glucosidase (GH13_31 subfamily) encoded by the *L. acidophilus* NCFM gene LBA_0264 is involved in the catabolism of IMO (Møller *et al.*, 2012). The participation of glucan-α1,6-glucosidase and maltose phosphorylase (see below) have also been implicated in the catabolism of IMO in *L. brevis* (Hu *et al.*, 2013). The *L. acidophilus* NCFM enzyme is induced after growth in IMO mixtures, displays a high activity on panose and prefers IMO with more than two glucoses, as it acts preferentially on isomaltotriose and isomaltotetraose compared to isomaltose. The LBA_0264 genes and its homologues in other lactobacilli are not clustered with other sugar catabolism or sugar transporter genes except for *Lactobacillus johnsonii* ATCC 33200 and *Lactobacillus gasseri* JV-V03, which belong to the *acidophilus* group and where the IMO-utilizing gene forms part of the maltodextrin operon (Møller *et al.*, 2012), and in other *Lactobacillus* species such as *Lactobacillus casei* (Monedero *et al.*, 2008). Another GH13_13 enzyme has been characterized in *L. plantarum* LL441. It displays activity on isomaltose and isomaltulose [α-D-glucopyranosyl-(1→6)-D-fructofuranoside], but not on panose or isomaltotriose, and its gene is clustered with genes encoding EIIABCD components of a mannose-class PTS (Delgado *et al.*, 2017). Whether the enzyme might be acting on phosphorylated disaccharides needs to be proven.

Metabolism of maltodextrins

Utilization of maltodextrins (linear oligosaccharides derived from starch hydrolysis) is a characteristic more extended in lactobacilli. Species adapted to starch-rich environments (e.g. plant material fermentations where the endogenous plant amylases release maltose and maltodextrins) such as *Lactobacillus sanfranciscensis*, participating in sourdough fermentations during bread making, are particularly efficient in maltose and maltodextrin utilization. The enzymatic machinery for the utilization of these carbohydrates in lactobacilli soon attracted attention and it has been thoroughly investigated. In the 90´s maltose catabolic proteins were partially characterized in *L. sanfranciscensis* DSM20451 (Ehrmann and Vogel, 1998) and *Lactobacillus brevis* ATCC 8287 (Huwel *et al.*, 1997). These microorganisms relied on an intracellular maltose phosphorylase (MapA [EC 2.4.1.8]) non requiring pyridoxal 5'-phosphate and belonging to the GH65 family, which also includes trehalose phosphorylases (EC 2.4.1.64), and kojibiose phosphorylases (EC 2.4.1.230), for catalyzing a phosphorolysis of the disaccharide that involves an inversion of the anomeric configuration of the C-1 atom, giving β-glucose 1-phosphate and glucose. β-glucose 1-phosphate is further converted to glucose 6-phosphate for its incorporation into glycolysis via β-phosphoglucomutase (Pgm), whose gene is co-localized in the chromosome with *mapA* (Figure 6). MapA displays a high specificity for maltose, but it is not active on maltodextrins such as maltotriose or maltotetraose and it cannot phosphorolyse disaccharides with other α-linkage configurations such as isomaltose (α-1,6), nigerose (α-1,3), kojibiose (α-1,2) or trehalose (α-1,1). Furthermore, it differs in sequence from other maltose (maltodextrin) phosphorylases such as that of *E. coli* (GT35 family). MapA is a dimer in solution and its structure has been solved for the *L. brevis* enzyme (Egloff *et al.*, 2001). The structure consists of a β sandwich domain linked to an $(\alpha/\alpha)_6$ barrel catalytic domain, and a C-terminal β sheet domain. The $(\alpha/\alpha)_6$ barrel domain displays striking structural and functional similarities to the catalytic domain of a glucoamylase from *Aspergillus awamori*,

suggesting evolution from a common ancestor (Egloff *et al.*, 2001). In the structures of these enzymes the catalytic conserved Glu residue from the glucoamylase superposes onto a conserved Glu of MapA that likely acts as the acid catalytic residue that promotes the nucleophilic attack of phosphate on the glycosidic bond. Modelling of the *L. acidophilus* MapA protein with different substrates allowed understanding why this enzyme does not accommodate maltotriose or maltotetraose in its active site (Nakai *et al.*, 2009). The protein adopts a configuration where loop His413-Glu421 between α3 and α4 of the $(\alpha/\alpha)_6$ barrel domain blocks the binding of longer malto-oligosaccharides. However, this enzyme has been applied in reverse phosphorolysis reactions for the synthesis of α-1,4-linked disaccharides with β-glucose 1-phosphate as donor and glucose, glucosamine, *N*-acetyl glucosamine, L-fucose, mannose or xylose as acceptors; being unable to use other sugars with axial hydroxyls at C-3 and C-4 positions or disaccharides/trisaccharides (Nakai *et al.*, 2009).

A genome survey of 38 *Lactobacillus* strains revealed that the presence of a set of genes for maltodextrins utilization, including *mapA* and *pgm*,

Figure 6. Comparison of maltose/maltodextrin operons in *Lactobacillus* species. The genetic organization of the operons encoding the enzymes and transporters for maltose/maltodextrin utilization in selected lactobacilli is depicted. The maltose/maltodextrin locus in *Lactobacillus* species carry genes for a specific ABC transporter, maltose phosphorylase and β-phosphoglucomutase together with several α-glycosidases or a MFS permease, maltose phosphorylase, β-phosphoglucomutase and, generally, an aldose 1-epimerase. In *L. plantarum* strains both types of *mal* operons are simultaneously present. Colors indicate homologous genes. Arrows indicate directionality.

is widespread in lactobacilli. These gene clusters usually include different α-glycosidases, although the presence of amylases is scarce (Ganzle and Follador, 2012). The maltodextrin operon which contain the *mapA* and *pgm* genes have been genetically characterized in *L. acidophilus* (Nakai *et al.*, 2009) and *L. casei* (Monedero *et al.*, 2008), showing that in these species maltodextrin transport is carried out by an ABC transporter ($MalEFGK_2$) homologous to that of the well-studied maltose/ maltodextrin transporter of *E. coli* (Figure 6). In *L. casei* BL23, ten *mal* genes are clustered and cotranscribed in a single mRNA whose expression is regulated by MalR, a transcriptional regulator of the LacI/GalR family and repressed by the presence of glucose via the general CCR mechanism mediated by the global regulator CcpA. According to studies conducted with *Streptococcus pneumoniae* MalR, this regulator acts as a transcriptional repressor and maltose strongly inhibits its DNA binding capacity (Puyet *et al.*, 1993). *Cis*-acting sequences recognized by MalR can be identified adjacent to the −10 and −35 promoters of the *L. acidophilus* and *Lactococcus lactis mal* operons (Nakai *et al.*, 2009). In addition to the maltose phosphorylase and phosphoglucomutase genes, three α-glucosidase-encoding genes (GH13) are clustered together with the *L. casei* maltose-catabolic genes: *malL*, encoding a putative oligo-α1,6-glucosidase (putatively acting on short IMO like isomaltose); *malM*, encoding a maltogenic α-amylase (cuts α-1,4 linkages from dextrins yielding maltose) and *dexB*, coding for a second α-1,6 glucosidase (Monedero *et al.*, 2008). This last enzyme belongs to the GH13_31 subfamily (glucan-α-1,6-glucosidase) and, as described before, based on the studies of its *L. acidophilus* counterpart is able to degrade IMO. Therefore, it is postulated that in addition to maltodextrins, the ABC transporter $MalEFGK_2$ would be able to transport IMO. A second operon located in opposite direction encodes an additional ABC transporter with high homology to $MalEFGK_2$ but its function is unknown and mutations in their genes have no phenotypic effects on maltose or maltotriose growth. In other *L. casei* strains, such as ATCC334, maltodextrin utilization is impaired by a large deletion in the *mal* cluster (Monedero *et al.*, 2008).

The *mal* operon of *L. acidophilus* NCFM consists of nine genes and a *malR* regulator (Nakai *et al.*, 2009). In addition to the maltodextrin ABC transporter, MapA and Pgm, *L. acidophilus* encodes two glycosidases: a maltogenic α-amylase (MalM) and an oligo α-1,6-glucosidase (MalL) that are homologous to their *L. casei* counterparts. The *L. acidophilus mal* operon also carries an acetate kinase gene (*ackA*) involved in pyruvate metabolism during glycolysis. Although ABC transporters can be identified as the main maltodextrin transport systems in lactobacilli, in some species that efficiently use maltose as a carbon source a genetic association of a gene encoding a permease of the major facilitator superfamily (MFS) is found with *mapA* and *pgm* genes (Figure 6). This suggests that these species (e.g. *L. sanfranciscensis*, *L. salivarius*, *L. brevis*) make use of a maltose-H^+ symport system for the uptake of the disaccharide. In contrast, no PTS systems for the transport of maltose similar to those described in *Bacillus subtilis* have been identified in lactobacilli. Studies on the maltose uptake in *L. sanfranciscensis* LTH2581, a strain which only ferments maltose and glucose, confirmed the presence of a maltose-H^+ symport system. When maltose is taken up by this strain the intracellularly generated glucose exceeds the metabolic capacity of the cells, which results in glucose expulsion through a glucose uniport system (Neubauer *et al.*, 1994). In lactobacilli utilizing maltose through a MFS permease, a genetic association of the *mal* cluster with a gene encoding an aldose 1-epimerase (EC 5.1.3.3) can be found (Figure 6). This enzyme is involved in the anomeric conversion of D-glucose between the α and β forms, which possibly speeds up the entry of glucose into the glycolytic pathway via its phosphorylation by glucokinase. Strains of *L. plantarum* are remarkable by the fact that they carry two *mapA* and *pgm* genes. One couple is linked to an ABC transporter ($MalEFGK_2$) and α-glucosidases (*mal* cluster 1), whereas the other forms a cluster (*mal* cluster 2) with a MFS permease (Figure 6). Therefore, strains of this species possess the capacity to use maltose and maltodextrins by using two separated sets of genes. Unlike the rest of lactobacilli, the *L. plantarum mal* cluster 1 contains two transcriptional regulators (genes Lp_0172 and Lp_0173) with homology to MalR (Muscariello *et al.*, 2011). Expression of *malE* from this cluster is induced by maltose and repressed by glucose via CcpA. Mutational analysis suggested that only the product of Lp_0173 participated in *malE* regulation. A mutant in this gene showed a glucose-insensitive expression of *malE* together with a lack of induction by maltose (Muscariello *et al.*, 2011). Remarkably, an *in silico* approach for the study of LacI-GalR transcriptional regulators in *L. plantarum* WCFS1 identified five operons putatively controlled by the products of Lp_0172 and Lp_0173 which include the *mal*1 and *mal*2 clusters, an operon for β-glucosides

utilization, an operon carrying the genes for teichoic acid synthesis *tagB1* and *tagB2* and an amino acid permease (Francke *et al.*, 2008).

Transport studies with ^{14}C-maltose has revealed an unusually high K_m for the *L. casei* maltose ABC transporter (around 0.3 mM; K_m for ABC transporters and their substrates are usually in the μM range), which suggested that maltose is not the preferred substrate and points to maltodextrins as the natural oligosaccharides taken up by this transporter (Monedero *et al.*, 2008). This notion is further substantiated by the fact that the three glycosidase enzymes encoded by the *mal* cluster are intracellular. Therefore, in this microorganism maltodextrins are preferentially metabolized over maltose, and they are hydrolyzed in the cytoplasm to render maltose and glucose. This characteristic is probably shared by the rest of lactobacilli harboring maltodextrin clusters with ABC transporters. *In vitro* studies on the binding capacity of the solute binding component of the maltodrextrin ABC transporter from *L. casei* (MalE) revealed that it is able to interact with maltotriose, maltotetraose, maltopentaose and with α, β and γ-cyclodextrins, which carry six, seven and eight glucose molecules, respectively, with K_d values that were in the μM range, albeit showing a preference for linear maltodextrins over cyclodextrins (Homburg *et al.*, 2017). However, contrarily to *E. coli* MalE, the solute binding component of the *L. casei* maltose system does not interact with maltose. The fact that mutants in the *L. casei malK* gene are not able to ferment maltose (Monedero *et al.*, 2008) suggests that other solute binding protein may be responsible to recognize and deliver maltose intracellularly via the MalFG permease component of the ABC system. Alternatively, it cannot be excluded that the MalK ATPase component of the transporter can be shared by an as yet unidentified and incomplete maltose-specific ABC system lacking a cognate ATPase unit (Homburg *et al.*, 2017). Crystallographic data of *L. casei* MalE complexed with maltotriose, maltotetraose and cyclodextrins provided structural clues for its lack of interaction with maltose (Homburg *et al.*, 2017). The globular carbohydrate-binding protein MalE consists of two N- and C-terminal domains which form a ligand-binding pocket situated between them, a characteristic shared by other MalE homologues. However, in *L. casei* MalE three aromatic residues from the C-terminal domain (W234, Y164 and W353) stack against a specific glucose moiety of the bound substrate and create three distinct subpockets, where the position of three glycosidic moieties is fixed with additional hydrogen bonds from the N-terminal MalE domain. Thus, the disaccharide maltose cannot adequately accommodate the three subpockets, preventing MalE to adopt the closed conformation that is achieved after interaction with linear and cyclic dextrins. This observation is also confirmed by the fact that, contrarily to maltotetraose, maltose does not stimulate ATPase activity of the $MalEFGK_2$ complex (Homburg *et al.*, 2017).

Transcriptomic data under laboratory or natural fermentation conditions has shed some light on the regulation of the expression of glucans/starch utilizing enzymes and their concerted action during the degradation of these carbohydrates by lactobacilli. Experiments with *L. acidophilus* NCFM show that this strain induces preferentially the expression of PTS transport systems in the presence of prebiotic glucans such as cellobiose [β-D-glucopyranosyl-(1→4)-D-glucopyranoside], isomaltose, panose or gentiobiose [β-D-glucopyranosyl-(1→6)-D-glucopyranoside], whereas polydextrose (a synthetic glucose polymer consisting of a mixture of different α-glycosidic linkages) induces ABC transporters (Andersen *et al.*, 2012). Transport via the PTS results in intracellular phosphorylated sugars that can be cleaved by different phospho-glucosidases. As expected, MapA was also induced by polydextrose. Of note, MalH, a isomaltose 6-phosphate hydrolase (GH4) encoded by LBA_1689 was induced by isomaltose, isomaltulose, panose and polydextrose. This enzyme participates in the formation of glucose 6-phosphate and glucose from isomaltose 6-phosphate but also glucose 6-phosphate plus fructose from isomaltulose internalized via a PTS encoded by the LBA_0606-LBA_0609 locus (Andersen *et al.*, 2012). MalH has been found in lactobacilli associated to the gastrointestinal tract and it could be a good indicator of prebiotic activity of α-1,6 glucosides such as panose and polydextrose that can in addition be degraded by the activity of the product of LBA_0264, a glucan-α-1,6-glucosidase (GH13_31) which is induced by IMO (Andersen *et al.*, 2012). Expression of genes involved in starch metabolism in *L. plantarum* A6 has been studied during a natural fermentation of pearl millet porridge (Humblot *et al.*, 2014). This highly amylolytic strain expresses α-amylases (intracellular and extracellular), α-glucosidase, neopullulanase, amylopectin phosphorylase and MapA when growing in this natural substrate. The ability of this strain to liquefy the pearl millet gruel compared to other *L. plantarum* strains that do not

grow in this substrate is attributable to the presence of the extracellular α-amylase (*amyA*). Metatranscriptomic analyses during the spontaneous fermentation by natural microbial consortia of different sourdoughs (wheat and spelt) with back-slopping for ten days have been carried out (Weckx *et al.*, 2011). By using a DNA microarray carrying genes from several lactic acid bacteria, a high expression of glycolytic enzymes was observed during these fermentations. However, the expression of *mapA* and maltose/maltodextrins ABC transporter encoding genes was low in both sourdough types and it corresponded mainly to the *L. plantarum* and *Lactococcus lactis* genes, respectively; even though the microarray also carried genes from *L. sakei*, *L. curvatus*, *L. brevis*, and *L. fermentum* (Weckx *et al.*, 2011). These expression levels were related to the concentration of maltose throughout the back-slopping process. The sourdough microbiota was capable of degrading other carbohydrates important in sourdough (e.g. saccharose and fructose) and their metabolism could cause CCR of the utilization of other carbon sources mediated by CcpA, whose gene is highly expressed during sourdough fermentations (Weckx *et al.*, 2011).

Metabolism of glycogen

Lactobacilli carry in their genomes gene clusters for the biosynthesis of the storage polysaccharide glycogen. In some lactobacilli that dwell in specific mucosal niches, such as the vagina, glycogen metabolism has been associated to their proliferation (Miller *et al.*, 2016). Glycogen metabolic genes have been characterized in *L. acidophilus* and it is postulated that they play a role in the persistence of this bacterium in the gut (Goh and Klaenhammer, 2013). The bacterial glycogen synthesis starts by the synthesis of ADP-glucose from glucose 1-phosphate by GlgC or GlgD enzymes. Then the glycogen synthase (GlgA) transfers glucose from ADP-glucose to a chain of α-1,4-glucan, whereas GlgB is involved in the formation of the α-1,6 branching points. For its catabolism, glycogen phosphorylase (GlgP) catalyzes the breakdown of α-1,4 linkages and GlgX participates in the debranching at the α-1,6 bonds in dextrins that cannot be further processed by GlgP. In *L. acidophilus* NCFM the glycogen cluster encompasses 11.7 kb and carry *glgBCDAP* together with two genes coding for a α-amylase and β-phosphoglucomutase (Goh and Klaenhammer, 2013). Similar genetic structures are found in approximately one third of the sequenced *Lactobacillus*, being mainly present in strains associated to the gastrointestinal tract of mammals and other animals. As an example, although the cluster is present in human intestinal isolates of *L. bulgaricus* and *L. helveticus*, it is not found in dairy isolates of these species (Goh and Klaenhammer, 2013). Expression of the *L. acidophilus glg* genes depends on the carbon source and the growth phase, showing maximal expression with raffinose [α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] and repression by glucose. As the enzymes for the synthesis and degradation are produced in parallel, the regulation of intracellular glycogen levels depending on the carbon sources may rely on the carbon fluxes. Mutants impaired in *glgA* or *glgB* present a reduced growth on raffinose and a mutant in *glgB* and in the gene encoding the catabolic glycogen phosphorylase (*glgP*) grows slower in MRS medium (containing glucose) and are less resistant to simulated gastrointestinal conditions (Goh and Klaenhammer, 2013). This suggests that the accumulation of α-glucan polymers with sequestered glucose that cannot be further metabolized results in impaired growth. This highlights the need for co-ordinated glycogen synthesis and degradation for retrieving glucose from glycogen storage under normal and stress conditions. *In vivo* experiments in a germ-free mice model in which wild-type *L. acidophilus* and its *glgA* mutant were delivered to animals by intragastric gavage showed that the wild-type strain was able to compete and to displace the *glgA* mutant in monocolonized mice. This demonstrated the role of glycogen synthesis and utilization in lactobacilli in competitive fitness in the gut (Goh and Klaenhammer, 2014).

β-glucan metabolic pathways in Lactobacillus

Despite the fact that lactobacilli are usually associated with the microbiota of plant decaying material, cellulases (endo-β-1,4-D-glucanases) have not been described for this genus and in general, for the lactic acid bacteria. Notwithstanding, the prebiotic activity of some β-glucans has been established but the metabolism of these polymers by lactobacilli has been rarely demonstrated. Very few examples of growth stimulation effects of β-glucans in lactobacilli are found in the literature, and they have mainly proved by using β-glucan hydrolysates (Dong *et al.*, 2017).

Important efforts have been made to use lactobacilli for the conversion of lignocellulosic material by applying saccharolytic processes prior fermentation or by the use of engineered strains expressing β-D-

glucanases from other microbial sources (Moraïs *et al.*, 2014; Okano *et al.*, 2010; Overbeck *et al.*, 2016). Similarly, lactobacilli expressing β-glucanases from other sources have been engineered for fermentation or health promoting effects (Liu *et al.*, 2005; Wang *et al.*, 2014a).

Metabolism of xylooligosaccharides

Structural characteristics of xylooligosaccharides

Xylooligosaccharides (XOS) are plant-derived oligosaccharides with β-1,4 linkages between xylose (a pentose) monomers that can be decorated with residues of the pentose arabinose. These residues can be linked by α-1,2 or α-1,3 bonds to xylose molecules along the chain (arabinoxylan oligosaccharides) where one or two arabinose residues can be found per xylose. These polymers are abundant in plant cell walls and together with other heteropolysaccharides form the hemicellulose component in plants.

Xylooligosaccharides metabolic pathways in Lactobacillus

In vitro growth assays and studies of the microbiota of humans concluded that these polysaccharides possess a prebiotic effect that stimulates the growth of bifidobacteria (Childs *et al.*, 2014; Lin *et al.*, 2016). This is supported by the characterization of multiple ABC transporters for XOS and XOS-degrading enzymes in species of *Bifidobacterium* (Ejby *et al.*, 2013). *In vitro* growth assays and human trials which explored changes in gut microbiota composition after XOS intake also pointed to XOS as prebiotic polysaccharides that stimulate growth of certain lactobacilli (Lin *et al.*, 2016). Notwithstanding, the capacity to ferment XOS by lactobacilli seems to be limited (Ananieva *et al.*, 2014). In accordance to this, the information about enzymes degrading XOS and arabinoxylans in lactobacilli is scarce. Two different enzymatic activities are needed to completely degrade arabinoxylans: arabinofuranosidase (liberating the arabinose residues that decorate the xylo-oligosaccharide backbone) and β-xylosidase (acting on the β-1,4 linkage between xylose molecules). Enzymes with this activity are classified into GH43 and GH51 glycosyl hydrolase families. *L. brevis* is thus far the only *Lactobacillus* species in which these activities have been studied (Michlmayr *et al.*, 2013; Michlmayr *et al.*, 2011). This species can be found in a wide variety of habitats including fermentations of hemicellulose-rich plant materials. Three GH43 β-xylosidases and two GH51 arabinofuranosidases have been found during the study of the genomes of several strains. The sequences of these enzymes show a high level of amino acid identity to enzymes from typical intestinal bacteria (e.g. bifidobacteria), suggesting events of horizontal gene transfer at the intestinal niche. The GH43 enzymes from *L. brevis* DSM 20054, annotated as β-xylosidases, have been thoroughly characterized (Michlmayr *et al.*, 2013). The β-xylosidase encoded by LVIS_0375 (*xynB1*) gene exhibited activity towards β-1,4-xylobiose and β-1,4-xylotriose. LVIS_2285 (*xynB2*) showed low activity with *p*-nitrophenyl-β-D-xylopyranoside and no activity with β-1,4-xylooligosaccharides, whereas the β-xylosidase encoded by LVIS_1748 (*abf3*) exhibited activity for α−1,5-arabinooligosaccharides. XynB1 and XynB2 are 32% identical and are also present in strains of *Lactobacillus buchneri*, *L. fermentum*, *Lactobacillus hilgardii*, *L. pentosus* and *L. reuteri*. These species belong to the group of heterofermentative lactobacilli so that it has been postulated that the capacity to degrade XOS and arabinoxylans is restricted to this particular group within lactobacilli (Michlmayr *et al.*, 2013). Unlike the arabinofuranosidases Abf1 and Abf2 characterized from *L. brevis* DSM 20054 (GH53) (Michlmayr *et al.*, 2011), Abf3 (and XynB1 and XynB2) cannot release arabinose from arabinoxylans with a composition of 65% α-1,3-linked arabinose, 8% α-1,2-linked arabinose and 26% doubly substituted xylose (two arabinose linkages per xylose monomer) indicating that these enzymes do not act as arabino-furanosidases. Furthermore, Abf1 and Abf2 are selective for α-1,3-linked arabinose residues of monosubstituted xylose (Michlmayr *et al.*, 2011).

The degradation of other hemicelluloses such as xyloglucan (a β-1,4 glucan backbone with xylose residues linked to glucose via α-1,6 bonds) by lactobacilli has received less attention. *L. pentosus* MD353, isolated from cucumber fermentation, carries a xylose operon (*xylAB*) involved in the metabolism of this pentose and encoding a xylose isomerase (*xylA*) and xylulose kinase (*xylB*) (Lokman *et al.*, 1991). These enzymes convert cytoplasmic D-xylose to D-xylulose 5-phosphate, an intermediate of the pentose phosphate pathway. Adjacent to this operon two genes are present, *xylPQ*, which are also induced by xylose via the xylose repressor XylR. XylQ is a GH31 α-xylosidase which has been demonstrated to act on isoprimeverose (α-D-xylopyranosyl-(1→6)-D-glucopyranoside) and it is also able to liberate with very low efficiency small amounts of xylose from xyloglucan oligosaccharides with different linkage configurations (Chaillou *et al.*, 1998). Isoprime-

verose is usually released from xyloglucan by cellulolytic microorganisms producing endoglucanases and it can be taken up by *L. pentosus* via the product of *xylP*. This gene encodes a galactoside-pentoside-hexuronide family transporter that catalyzes the transport of isoprimeverose, but not xylose, through a proton motive force-driven process (Heuberger *et al.*, 2001).

Metabolism of galactooligosaccharides

Structural characteristics of galactooligosaccharides
β-galacto-oligosaccharides (GOS) are non-digestible carbohydrates usually composed of lactose at the reducing end and one to ten galactose units linked by β-1,3, β-1,4 or β-1,6 bonds (Macfarlane *et al.*, 2008). They can be acquired naturally through the diet from the degradation of galactan side chains of the rhamnogalacturonan I fraction of pectin (Jones *et al.*, 1997). In addition, they are also incorporated as prebiotics after their synthesis by the transgalactosylation activity of β-galactosidases on lactose, which acts both as donor and as acceptor of the galactose moiety (Vera *et al.*, 2016). Analysis of some GOS mixtures revealed the presence of oligosaccharides with galactose at the reducing end instead of glucose, and they may also contain disaccharides different from lactose that are considered GOS as well. Indeed, commercial GOS are typically mixed-length galactosylated compounds with a DP ranging from 2 to 12 (Coulier *et al.*, 2009). The type of linkage, mostly β-1,3, β-1,4 and/or β-1,6, and to a lesser extent, β-1,2, is determined by the enzyme source. Commercial enzymes used for GOS synthesis belong to the CAZy glycosyl hydrolase family 2 (GH2) and they are obtained from *Bifidobacterium bifidum* and *Bacillus circulans* for GOS with β-1,3 and β-1,4 bonds, respectively, and from *Kluyveromyces lactis* and *Aspergillus oryzae* to obtain β-1,6 linked GOS (Rodriguez-Colinas *et al.*, 2011). Most of the published studies use the term GOS when referring to β-GOS, but there are also GOS with α-configuration, which are produced by transgalactosylation reactions with α-galactosidases (Wang *et al.*, 2014b). The oligosaccharides 3'-, 4'- and 6'-galactosyllactose have been found in colostrum and human milk, and they constitute the only oligosaccharides contained in common between human milk oligosaccharides (HMOs) and GOS.

GOS are metabolized by specific bacteria in the gastrointestinal tract, and they have been found to modulate the gut microbiota by stimulation of beneficial bacteria such as bifidobacteria and lactobacilli, and inhibition of pathogenic bacteria (Macfarlane *et al.*, 2008; Rastall *et al.*, 2005). The fermentation of GOS in the gastrointestinal tract leads to an increased production of specific short chain fatty acids (SCFA), which are known for their health benefits including reduction of the risk of developing cancer and intestinal disorders (Cardelle-Cobas *et al.*, 2009; Sangwan *et al.*, 2011).

GOS metabolic pathways in Lactobacillus
Lactobacillus species in general can efficiently utilize GOS, although their utilization is a strain-dependent character and it may also vary depending on GOS DP (Endo *et al.*, 2016; Thongaram *et al.*, 2017). A study on GOS utilization by different species of *Lactobacillus* showed that 9 out of 10 tested species metabolized the galactosyllactose fraction (35%) of a GOS mixture to a different degree (Endo *et al.*, 2016). All tested strains of *L. delbrueckii*, *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. johnsoni* and *L. acidophilus* metabolized galactosyllactose whereas only some strains of *L. rhamnosus*, *L. paracasei* and *L. sakei* did it. As with fructans, the availability of genomic sequences allowed the determination of the genetic basis of GOS metabolism by lactobacilli. In this way, transcriptomic analyses of *L. acidophilus* NCFM with whole-genome DNA microarrays, revealed that GOS induce the *lac-gal* gene cluster, which encodes a galactoside-pentose-hexuronide permease (LacS), two β-galactosidases belonging to the GH family 42 (LacA) and GH family 2 (LacLM), and enzymes of the Leloir Pathway (GalM, GalT, GalK and GalE) involved in the metabolism of galactose (Andersen *et al.*, 2011). Inactivation of LacS impaired growth on lactose, lactitol and GOS (Andersen *et al.*, 2011). Phylogenetic analysis showed that *lacS* is mainly found in human gut-associated *Lactobacillus* species, suggesting that transport and catabolism of those carbohydrates could be a significant energy source for lactobacilli in the gut (Andersen *et al.*, 2011). Possibly, GOS are transported into the cells by the LacS permease, hydrolyzed by the β-galactosidases LacA and LacLM into galactose and glucose, which would be directed to the Leloir Pathway and glycolysis, respectively. Interestingly, the *lac-gal* cluster is also induced by bile acids (Barrangou *et al.*, 2006; Pfeiler *et al.*, 2007), suggesting that bile may act as a location signal in the gut environment where GOS and related carbohydrates would be readily available.

L. fermentum is able to utilize α-GOS as carbon source for growth in soymilk (LeBlanc *et al.*, 2004). This capability relies in the expression of the gene *melA*, that encodes an α-galactosidase with activity on α-1,6 linkages (Carrera-Silva *et al.*, 2006). α-GOS such as raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), and stachyose (α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside), are abundant in vegetables and they are substrates for MelA. Other α-galactosidases that hydrolyze those α-galactosides were also identified in *L. plantarum* (Silvestroni *et al.*, 2002) and *L. reuteri* (Tzortzis *et al.*, 2003). The growth of *L. acidophilus* NCFM in the presence of stachyose induced a cluster of genes encoding an ATP-binding cassette (ABC) transporter, a GH36 α-galactosidase and enzymes from the Leloir Pathway. Inactivation of the α-galactosidase-encoding gene resulted in the loss of the ability to grow on raffinose, stachyose and the disaccharide melibiose (α-D-galactopyranosyl-(1→6)-D-glucopyranoside) (Andersen *et al.*, 2012). These results suggested that *L. acidophilus* NCFM transports α-galactosides into the cytoplasm via an ABC system and then, they are hydrolyzed by the action of the GH36 α-galactosidase into galactose and sucrose or galactose and glucose.

Metabolism of human milk oligosaccharides (HMOs)

Structural characteristics and functional properties of HMOs

HMOs constitute a group of non-conjugated and structurally diverse carbohydrates that represent the third largest solid component of human milk after lactose and lipids (Kunz *et al.*, 2000; Thurl *et al.*, 2010). They consist of combinations of five monosaccharides: D-glucose, D-galactose, *N*-acetylglucosamine (GlcNAc), L-fucose and sialic acid (Sia). The only form of Sia found in human milk is *N*-acetylneuraminic acid (Neu5Ac), while oligosaccharides present in milk of other mammals may contain too *N*-glycolylneuraminic acid (Neu5Gc) (Urashima *et al.*, 2013). All HMOs contain a lactose unit (β-D-galactopyranosyl-(1→4)-D-glucopyranoside) at their reducing end, that can be further elongated by the addition of β-1,3-linked lacto-*N*-biose (LNB; β-D-galactopyranosyl-(1→3)-*N*-acetyl-D-glucosamine; type-1 core) and/or β1-3/6-linked *N*-acetyllactosamine (LacNAc; β-D-galactopyranosyl-(1→4)-*N*-acetyl-D-glucosamine; type-2 chain). The basic core structures can be modified by L-Fuc with an α1-2, α1-3 or α1-4 linkage and/or Sia with an α2-3 or α2-6 linkage. HMOs can be simple trisaccharides as 2'/3'-fucosyllactose (2'/3'FL) and 3'/6'-syalyllactose (6'SL) or complex oligosaccharides with several LNB and LacNAc repeat units (Bode, 2012). These disaccharides have also been found in free form in human milk (Balogh *et al.*, 2015). Currently, over a 100 structurally distinct HMOs have been identified, including neutral (non-sialylated) and acidic (sialylated) compounds (Kobata, 2010). Most of the neutral HMOs are fucosylated, with concentrations ranging from 50 to 80%, whereas sialylated HMOs ranged from 10 to 20%. In contrast, only about 1% of the oligosaccharides are fucosylated in bovine milk (Bode, 2012). The overall concentration of HMOs varies during lactation, colostrum contains about 20-25 g/l HMOs and this amount diminishes to approximately 5-20 g/l in mature milk (Bode, 2012; Thurl *et al.*, 2010). In addition, the HMOs composition and quantity is variable among mothers, since their synthesis is correlated with the Secretor and Lewis blood group characteristics, which depend on the expression of FUT2 and FUT3 fucosyltransferases, respectively, within the mammary gland (Kunz *et al.*, 2017; Thurl *et al.*, 2010).

Human milk also contains highly glycosylated proteins, including mucins, that have attached oligosaccharide moieties with structures that resemble those of the free HMOs (Liu and Newburg, 2013). Both HMOs and the glycan moieties of the proteins are synthesized by the same glycosyltransferases. *N*-glycans are linked to an asparagine residue through an GlcNAc, that is elongated by an additional GlcNAc residue through a β-1,4 linkage and three mannose residues. The GlcNAc residue linked to Asn can be modified via α-1,6-fucosylation and the mannose residues with other monosaccharides, including L-fucose and Sia, and it becomes a complex structure (Nwosu *et al.*, 2012). *O*-glycans usually contain an *N*-acetylgalactosamine (GalNAc) linked to a serine or threonine residue. In the type-1 sugar chain found in mucins, the GalNAc is extended with Gal, linked via a β-1,3 bond, forming the disaccharide galacto-*N*-biose (GNB; β-D-galactopyranosyl-(1→3)-*N*-acetyl-D-galactosamine) (Figure 7). GNB is also present in glycosphingolipids and in bioactive sugar structures like the T-antigen disaccharide (Liu and Newburg, 2013; Moran *et al.*, 2011).

In the last years, many studies have suggested that HMOs act as anti-adhesins against pathogens. HMOs are structurally similar to host receptors for

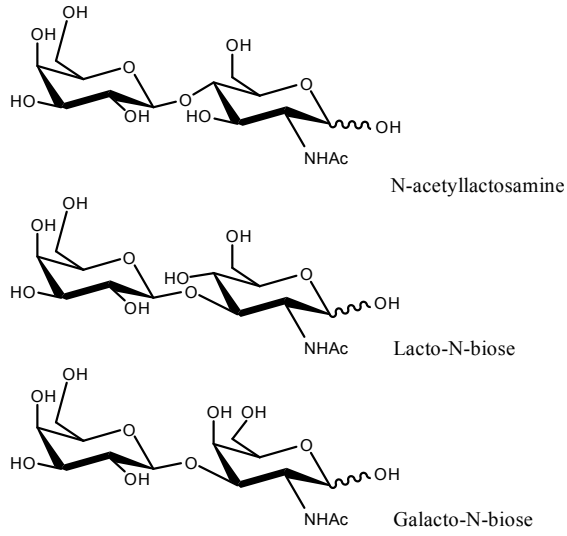

Figure 7. Chemical structures of *N*-acetyllactosamine, lacto-*N*-biose, and galacto-*N*-biose.

pathogens since they are synthesized by the same glycosyltransferases that synthesize cell surface glycoproteins and glycolipids. As soluble receptor analogs, HMOs can act as decoys protecting infants against infections. Some HMOs inhibit the attachment of norovirus and bacterial pathogens such as *Listeria monocytogenes* and pathogenic *E. coli* strains (Newburg *et al.*, 2005), *Campylobacter jejuni* (Ruiz-Palacios *et al.*, 2003), *Helicobacter pylori* (Mysore *et al.*, 1999) and parasites such as *Entamoeba histolytica* (Jantscher-Krenn *et al.*, 2012), explaining the fact that breast-fed infants are at lower risk to acquire *E. histolytica* infections than formula-fed infants (Islam *et al.*, 1988). Two disaccharides, α-L-fucosyl-(1→3)-*N*-acetyl-D-glucosamine (3FN) and α-L-fucosyl-(1→6)-*N*-acetyl-D-glucosamine (6FN) (Rodríguez-Díaz *et al.*, 2013) that form part of the structure of many HMOs, either free or glycoconjugated to proteins, possess anti-adhesive properties against the enteropahogenic *E. coli* (O86). 6FN was also able to block the binding of the enteropathogenic *E. coli* (O127a) to HT29 cells (Becerra *et al.*, 2015a). Recently, it has been shown that HMOs function as antimicrobial and antibiofilm agents against *Streptococcus agalactiae*, an invasive pathogen in both children and adults (Ackerman *et al.*, 2017; Lin *et al.*, 2017). Interestingly, specific neutral HMOs directly inhibit the growth of this bacterium and a mutant impaired in a putative glycosyltransferase is resistant to those HMOs (Lin *et al.*, 2017).

Metabolic pathways for HMOs in Lactobacillus

Convincing evidence supports that HMOs favor the growth of beneficial bacteria present in the gastrointestinal tract of breastfed infants. HMOs were first identified as the prebiotic "bifidus factor" described for human milk. Bifidobacteria constitute a considerable proportion of the intestinal microbiota of infants (Gomez-Llorente *et al.*, 2013), and they are highly adapted to use HMOs as a carbon source (Garrido *et al.*, 2013). Genome analyses have revealed that strains of *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium breve* and *B. bifidum* encode a battery of enzymes involved in HMOs catabolism (Kwak *et al.*, 2016; LoCascio *et al.*, 2010; Zúñiga *et al.*, 2018). Unlike *Bifidobacterium*, species of the genera *Lactobacillus*, that are often isolated from breast-fed infant feces (Albesharat *et al.*, 2011; Martín *et al.*, 2007; Rubio *et al.*, 2014), usually showed a limited capacity for HMOs utilization. The only exception is represented by members of the *L. casei/paracasei/rhamnosus* group, which contain several genes encoding enzymes involved in the hydrolysis of fucosyl-oligosaccharides (Rodríguez-Díaz *et al.*, 2011) and in the metabolism of the type-1 (Bidart *et al.*, 2014) and type-2 core structures from HMOs (Bidart *et al.*, 2018) (Figure 8). Three α-L-fucosidases (AlfA, AlfB and AlfC) encoded in the *L. casei* BL23 genome have been characterized and they were able to hydrolyze *in vitro* fucosylated HMOs (Rodríguez-Díaz *et al.*, 2011). All three enzymes are possibly

intracellularly located as they lack secretion signals, suggesting that *L. casei* must transport the fucosylated substrates into the cytoplasm before their hydrolysis. This notion was demonstrated for the disaccharide 3FN that is transported into the cells by the mannose-class PTS encoded by the genes *alfEFG*, without being phosphorylated: L-fucose is a 6-deoxy-galactose and therefore lacks a phosphorylatable hydroxyl group at the carbon in the sixth position. These genes are divergently oriented from the gene cluster *alfBR*, encoding the α-L-fucosidase AlfB and the transcriptional repressor AlfR (Rodríguez-Díaz *et al.*, 2012). AlfB digested the disaccharide within the cells into L-fucose and GlcNAc. The latter is metabolized by *L. casei*, whereas the L-fucose moiety is excreted to the medium (Figure 8) because, contrarily to *L. rhamnosus* GG (Becerra *et al.*, 2015b), *L. casei* lacks L-fucose catabolic genes. Recently, the α-L-fucosidase AlfC, responsible for 6FN hydrolysis, has been shown to be involved in the use of the core-fucosylated structures of *N*-glycosylated proteins (Becerra *et al.*, 2020). AlfC is encoded in a gene cluster (*alf*-2) that also contains the gene *alfH*, encoding a major facilitator superfamily (MFS) permease, and the divergently oriented *asdA* (aspartate 4-decarboxylase), *alfR2* (transcriptional regulator), *pepV* (peptidase), *asnA2* (glycosylasparaginase), and *sugK* (sugar kinase) genes (Becerra *et al.*, 2020). The pathway involves the uptake of the glycoamino acid fucosyl-α-1,6-*N*-GlcNAc-Asn (6'FN-Asn) by the AlfH permease,

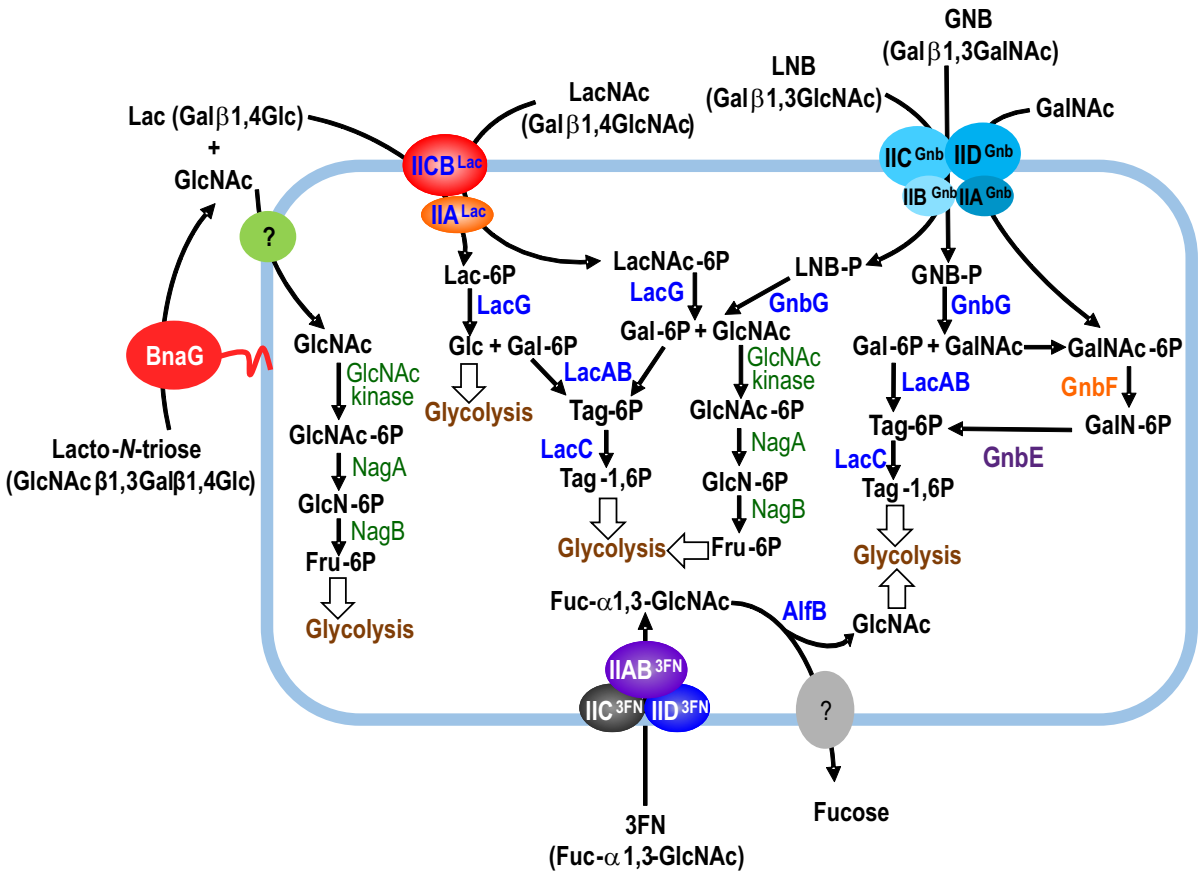

Figure 8. HMOs catabolic pathways identified in *Lactobacillus casei*. LNB, lacto-*N*-biose; GNB, galacto-*N*-biose; LacNAc, *N*-acetyllactosamine; Lac, lactose; GalNAc, *N*-acetylgalactosamine; GalN, galactosamine; Gal, galactose; Glc, glucose, GlcNAc; *N*-acetylglucosamine; GlcN, glucosamine; Tag, tagatose; 3FN, fucosyl-α1,3-*N*-acetylglucosamine; Fuc, fucose; $IICB^{Lac}$ and IIA^{Lac}, lactose-specific domains of the phosphoenolpyruvate: phosphotransferase system (PTS); IIC^{Gnb}, IID^{Gnb}, IIA^{Gnb} and IIB^{Gnb}, LNB/GNB/GalNAc-specific domains of the PTS; IIC^{3FN}, IID^{3FN} and $IIAB^{3FN}$, $3FN^{3FN}$ 3FN-specific domains of the PTS; BnaG, beta-*N*-acetylglucosaminidase; LacG, phospho-β-galactosidase; GnbG, phospho-β-galactosidase; GnbF, *N*-acetylgalactosamine 6-phosphate deacetylase; GnbE, galactosamine 6-phosphate isomerase/deaminase; LacAB, galactose 6-phosphate isomerase; LacC, tagatose 6-phosphate kinase; NagA, *N*-acetylglucosamine 6-phosphate deacetylase; NagB, glucosamine 6-phosphate deaminase.

removal of the α1,6-fucosyl residue by the α-L-fucosidase AlfC, and cleavage of the resulting GlcNAc-Asn to 1-amino-GlcNAc and Asp by the glycosylasparaginase AsnA2 (Figure 9). AlfH and AlfC are also involved in the transport and metabolism of other α1,6-fucosylated structures such as fucosyl-α-1,6-*N,N'*-diacetylchitobiose (N2F *N*-glycan), fucosyl-α-1,6-galactose and fucosyl-α-1,6-glucose (Becerra *et al.*, 2020).

The release of L-fucose and Sia from the non-reducing ends is the first step to degrade the HMOs core structures. *Bifidobacterium* spp. and *Bacteroides* spp. generally are good consumers of fucosylated and sialylated HMOs as they usually possess fucosidase and sialidase activities. The last activity is not a common feature among lactobacilli, although *L. delbrueckii* ATCC7830 showed sialidase activity when cultured in the present of 6'-sialyllactose (Yu *et al.*, 2013). A number of lactobacilli contain genes enabling the use of L-fucose or Sia moieties released from HMOs. *L. rhamnosus* GG can utilize L-fucose since it contains an operon encoding a specific catabolic pathway similar to that of *E. coli* (Becerra *et al.*, 2015b). *L. sakei* 23K contains two gen clusters, *nanTEAR* and *nanKMP* involved in the catabolism of Neu5Ac (Anba-Mondoloni *et al.*, 2013), and some strains of *L. plantarum*, *L. salivarius* (Almagro-Moreno and Boyd, 2009) and *L. paracasei* (Hammer *et al.*, 2017) also contain genes for Sia metabolism. Extracellular sialidase and fucosidase activities have not been described in those species; therefore, the utilization of these carbohydrates as well as the glycan core structures by lactobacilli probably depends on their release from HMOs and mucin by other members of the intestinal microbiota. This is the case of *L. casei*, which has a complete machinery to metabolize HMOs and *O*-glycans core structures such as LNB, GNB (Bidart *et al.*, 2014), LacNAc (Bidart *et al.*, 2018) and lacto-*N*-triose II (LNTII; β-*N*-acetyl-D-glucosamine-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside) (Bidart *et al.*, 2016) (Figure 8). LNB and GNB utilization relies on the *gnb* operon, which contains genes encoding a transcriptional repressor (*gnbR*), a galactosamine 6-phosphate isomerizing deaminase (*gnbE*), a GalNAc 6-phosphate deacetylase (*gnbF*), a phospho-β-galactosidase (*gnbG*) and four genes (*gnbBCDA*) encoding the EIIB, EIIC, EIID and EIIA components of a mannose-class PTS system (PTS^{Gnb}) (Bidart *et al.*, 2014). LNB, GNB and also GalNAc are transported and phosphorylated by the PTS^{Gnb} and then, both disaccharides are hydrolyzed by the specific β-1,3-galactosidase GnbG (GH family 35) into galactose 6-phosphate and the corresponding *N*-acetylhexosamines (GlcNAc and GalNAc). Galactose 6-phosphate is metabolized through the tagatose 6-phosphate pathway, whereas GlcNAc and GalNAc would be phosphorylated by as yet unknown kinases before entering different catabolic routes (Figure 8). GlcNAc 6-phosphate is converted by the NagA deacetylase to glucosamine 6-phosphate, which enters the glycolysis pathway via conversion to fructose 6-phosphate by the NagB deaminase. GalNAc 6-phosphate would be deacetylated and deaminated to tagatose 6-phosphate by the products of the genes *gnbF* and *gnbE*. Therefore, all *gnb* genes would participate in GNB and GalNAc metabolism while LNB utilization by *L. casei* would not require GnbE and GnbF activities. It is worth noting that *gnb* genes are highly induced by GNB and GalNAc which relieve repression by GnbR, whereas the presence of LNB barely induces the *gnb* operon (Bidart *et al.*, 2014). According to this, the *gnb* operon would be primarily adapted to catabolize GNB and GalNAc. The coexistence of these sugars with LNB in environments as the gastrointestinal tract might account for the utilization of LNB by this pathway although this sugar would not induce the expression of *gnb* genes. The *gnb* gene cluster is conserved in the *L. casei/paracasei/rhamnosus/zeae* group, and it has been shown that both GNB and LNB are fermented by several strains of *L. casei*, *L. rhamnosus* and *L. zeae* species (Bidart *et al.*, 2017). Strains belonging to *L. gasseri* and *L. johnsonii* species are also consumers of LNB and GNB, although they do not have a *gnb* operon (Bidart *et al.*, 2017). Therefore, at least another catabolic system for those disaccharides remains to be discovered in lactobacilli.

Lactose metabolism in lactic acid bacteria has been widely studied due to the economic relevance of lactose fermentation in the dairy industry (Cavanagh *et al.*, 2015; de Vos and Vaughan, 1994; Lapierre *et al.*, 2002; Stefanovic *et al.*, 2017). Lactose can be transported by lactose/galactose antiport permeases, proton symport permeases or through a PTS transporter (Alpert and Chassy, 1990; de Vos and Vaughan, 1994; Francl *et al.*, 2012; Gosalbes *et al.*, 1997; Leong-Morgenthaler *et al.*, 1991). Recently, it has been shown that the *lac* operon from *L. casei* is also responsible of the utilization of LacNAc (Bidart *et al.*, 2018). This carbohydrate is transported and phosphorylated by the PTS^{Lac} (Gosalbes *et al.*, 2002; Gosalbes *et al.*, 1997; Gosalbes *et al.*, 1999), and then is hydrolyzed by the phospho-β-galactosidase LacG (GH family 1)

into galactose 6-phosphate and GlcNAc. In fact, the *lac* operon of *L. casei* showed higher induction levels in the presence of LacNAc than with lactose, suggesting that LacNAc may be the preferential substrate of this transporter. Indeed, this carbohydrate is present in the human gastrointestinal tract through all stages of life (Marionneau *et al.*, 2001; Moran *et al.*, 2011) whereas lactose would be only present during the lactating period since the introduction of dairy farming is a very recent event in the evolutionary history of humankind. Genome sequence analyses (http://www.ncbi.nlm.nih.gov/genomes) showed that many species belonging to the genus *Lactobacillus* contain genes encoding PTS transporters homologous to the PTS^{Lac} from *L. casei* BL23, suggesting that lactose-specific PTS transporters are quite common among lactobacilli. Lactose is also the product resulting from the metabolism of LNTII by the action of the exoglycosidase β-*N*-acetylglucosaminidase BnaG (GH family 20) from *L. casei* (Bidart *et al.*, 2016). Unlike the other glycosidases active on HMOs characterized in this species, BnaG is a cell wall-anchored extracellular protein. This enzyme shows high specificity for *N*-acetylhexosaminyl-β-1,3-linked sugars as it releases GlcNAc not only from LNTII but also from β-*N*-acetyl-D-glucosamine-(1→3)-D-mannopyranoside, a disaccharide that forms part of glycoproteins (Garrido *et al.*, 2012). As well, BnaG liberates GalNAc from β-*N*-acetyl-D-galactosamine-(1→3)-D-galactopyranoside, which forms part of globotetraose, a glycan moiety of human glycosphingolipids present at cell surfaces (Schnaar *et al.*, 2009). The oligosaccharide part of these lipids have recently been described as substrates for lacto-*N*-biosidases isolated from *B. longum* subsp. *longum*, which, in contrast to BnaG, are endoglycosidases and release GNB (Gotoh *et al.*, 2015). The possession of cell-wall attached

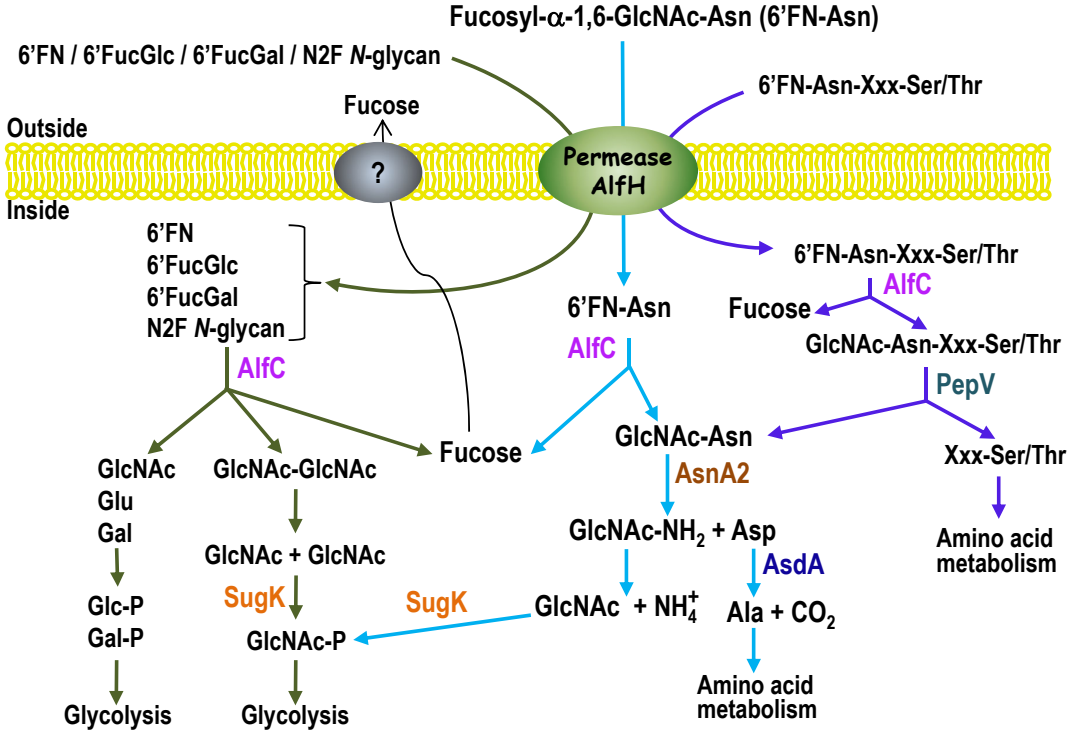

Figure 9. Schematic presentation of the transport and catabolic pathways for fucosyl-α-1,6-*N*-acetylglucosamine-asparagine (6'FN-Asn), fucosylated glycans and fucosylated *N*-glycopeptides in *Lactobacillus casei*. AlfC, α-L-fucosidase AlfC; AsnA2, *N*(4)-(β-*N*-acetylglucosaminyl)-L-asparaginase; AsdA, aspartate 4-decarboxylase; PepV, peptidase V; SugK, sugar kinase; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Gal, galactose; 6'FN, fucosyl-α-1,6-*N*-acetylglucosamine; 6'FucGlc, fucosyl-α-1,6-glucose; 6'FucGal, fucosyl-α-1,6-galactose; N2F, *N*-glycan fucosyl-α-1,6-*N*,*N*'-diacetylchitobiose.

glycosidases may provide a competitive advantage by allowing cleavage and consumption of complex-linked sugars. In addition, there is also evidence suggesting that these enzymes might also modulate the activity of host glycoproteins and pathogen-host receptor interactions through modification of the surface-exposed host glycans structures (Garbe and Collin, 2012; Garrido *et al.*, 2012; Kobata, 2013).

The presence of α-L-fucosidases and catabolic pathways for the utilization of LNB, GNB, LacNAc and LNTII in *L. casei* shows the capacity of this species for the exploitation of human milk and mucosa-associated glycans. This feature probably constitutes an adaptation of these bacteria to survive in the gastrointestinal tract of breastfed infants.

Concluding remarks

Lactobacilli play a major role in the production of many fermented foods and, in human and animal health as components of the microbial communities associated to different mucosal surfaces or as health-improving food supplements. These organisms rely on sugar utilization for growth so that knowing their sugar utilization pathways is a must to understand their role in microbial communities and their performance in food production. Due to their economic relevance, some species of lactobacilli have been extensively studied (e.g. specific strains of *L. plantarum*, *L. casei* or *L. acidophilus*) and the pathways of utilization of many monosaccharides and disaccharides have been elucidated. However, there are still great gaps in our knowledge of the utilization of complex glycans, especially host mucosal glycans, even in well characterized strains of lactobacilli. Also, information is partial or simply lacking for many other species that inhabit mucosal and food/feed niches. Genomic sequencing in lactobacilli has revealed an enormous variety of putative glycan transport systems and glycosyl hydrolases belonging to different GH families. However, the function of most of these transporters and enzymes remains to be elucidated. Furthermore, most studies have been carried out using pure cultures in laboratory conditions. However, utilization of complex glycans in food matrixes or microbial communities is possibly a key factor for growth and survival in these environments. In the natural niches where lactobacilli dwell, complex ecological relationships are found and cross-feeding is established between different microbial groups, where sequential and cooperative degradation of complex glycans probably takes place. Unraveling these associations will require the use of different "omic" technologies that include metagenomics, transcriptomics and metabolomics. This constitutes a future challenge in the study of carbohydrate metabolism in lactobacilli and its functional and ecological relevance.

Acknowledgements

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