Biotechnology of Lactic Acid Bacteria

Novel Applications
Chapter 1

Updates in the Metabolism of Lactic Acid Bacteria

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Lactic acid bacteria (LAB) are fermentative bacteria naturally dwelling in or intentionally added to nutrient-rich environments where carbohydrates and proteins are usually abundant. The efficient use of nutrients and the concomitant production of lactic acid during growth endow LAB with remarkable selective advantages in the diverse ecological niches they inhabit. Besides lactic acid, LAB metabolism produces a variety of compounds, such as diacetyl, acetoin and 2,3-butanediol from the utilization of citrate, and a vast array of volatile compounds and bioactive peptides from the catabolism of amino acids. The enzymatic reactions of LAB metabolism further modify the organoleptic, rheological, and nutritive properties of the raw materials, giving rise to final fermented products. Last decade witnessed an impressive amount of data on several aspects of LAB physiology and genetics. The latest knowledge was gathered through sequencing and analysis of LAB genomes, and the subsequent use of post-genomic techniques, such as proteomics, comparative genome hybridization, transcriptomics, and metabolomics. Manipulation of the metabolic pathways of LAB to improve their efficiency in various industrial applications (as starters, adjunct cultures, and probiotics) was undertaken soon after the development of early engineering tools. The availability of complete genome sequences of different LAB species and strains has expanded our ability to further study LAB metabolism from a global perspective, strengthening a full exploitation of LAB’s metabolic potential.

1.1. Introduction

Lactic acid bacteria (LAB) encompass a heterogeneous group of microorganisms having as a common metabolic property the production of lactic acid as the majority end-product from the fermentation of carbohydrates (Carr et al. 2002). LAB are Gram (+), non-sporulating, catalase-negative, acid-tolerant, facultative anaerobic organisms. Except for a few species, LAB members are nonpathogenic organisms with a reputed Generally Recognized as Safe status. Typical LAB species belong to the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Oenococcus*, *Enterococcus*, and *Leuconostoc*. Under a biochemical perspective LAB include both homofermenters, producing mainly lactic acid, and heterofermenters, which, apart from lactic acid, yield a large variety of fermentation products such as acetic acid, ethanol, carbon dioxide, and formic acid (Kleerebezem and Hugenholtz 2003). LAB are found in a large variety of nutrient-rich environments, including milk and dairy products, vegetable and plants, cereals, and meat and meat products. Many species are used for the manufacture and preservation of fermented feed and foods from raw agricultural materials in which they are either present as contaminants or deliberately added as starters in order to control the fermentations. Enzymatic activities of LAB contribute to the final organoleptic, rheological, and nutritional properties of fermented products (Leroy and de Vuyst 2004). LAB species are also commonly found
among the resident microbiota of the gastrointestinal tract and genitourinary tract of humans and animals (Eckburg et al. 2005; Marchesi and Shanahan 2007). In these environments LAB are considered essential components, playing a large variety of health-promoting functions, such as immunomodulation, intestinal integrity, and pathogen resistance (Vaughan et al. 2005). For such reasons strains of some species have traditionally been used as probiotics and added as functional bacteria in various food commodities (Ljungh and Wadström 2006). The commercial exploitation of LAB as starter and probiotic cultures is economically very significant. Consequently, research on their genetics, physiology, and applications has bloomed in the last 25 years (Wood and Warner 2003; Gasson and de Vos 2004).

This chapter addresses recent data on LAB metabolism related to their efficient utilization of niche- and product-related nutrients and the concomitant generation of desirable compounds for their industrial application. Modification of metabolic routes aimed to increase the efficacy of LAB in traditional fermentations, and their contribution to health and well-being will further be addressed. However, though important in dairy products, lipid metabolism, which has been only marginally studied in LAB (Collins et al. 2003; Hickey et al. 2006), will not be covered in this review. Additionally, the metabolism of nucleotides, obligatory metabolites for RNA and DNA synthesis and energy donors in many cellular processes, will not be discussed as well. In this last topic, readers are given an overview of the excellent recent work by Kilstrup et al. (2005).

### 1.2. Sugar Metabolism

Sugars are primary carbon and energy sources for LAB grown on substrates used for fermented food and feed production as well as in laboratory media. Many different transport systems involved in the uptake of carbohydrates, including PhosphoTransferase Systems (PTS), ATP-binding cassette (ABC), and Glycoside-Pentoside-Hexuronide transporters, exist in LAB (this book, Chapter 4). Polysaccharides before uptake have to be hydrolyzed; for example, starch is hydrolyzed either by α-amylase into dextrins, which are subsequently hydrolyzed by an extracellular activity to maltose (Calderon Santoyo et al. 2003), or by debranching enzymes (Doman-Pytkula et al. 2004). Monosaccharides entering the cell or liberated in the cytoplasm by hydrolysis of disaccharides enter glycolysis at the level of glucose-6P (G6P) or are processed by the Leloir pathway (Fig. 1.1D). In many strains of *Streptococcus thermophilus* only the glucose moiety of lactose is fermented, while the galactose moiety is excreted into the medium as a result of the weak transcription from gal promoters or mutations in the Leloir genes (Vaughan et al. 2001; de Vin et al. 2005; Fig. 1.1D). In *Lactococcus lactis* lactose transported by the PTS system is hydrolyzed and the galactose-6P moiety is transformed by the tagatose pathway, entering glycolysis at the level of triose phosphate. The initial metabolism of glucose, galactose, and lactose in *L. lactis* has been described by Coign-Bousquet et al. (2002) and Neves et al. (2005). Recently, it was postulated that in *L. lactis* IL1403 the metabolism of β-glucosides (cellobiose) and β-galactosides

**Figure 1.1.** Pathways of glucose metabolism. A. Homofermentative pathway; B. Mixed-acid metabolism; C. Heterofermentative pathway; D. Leloir pathway. Reactions are catalyzed by the following enzymes: 1, glucokinase (GLK); 2, glucose-phosphate isomerase (GPI); 3, phosphofructokinase (PFK); 4, fructose-bisphosphate aldolase (FBPA); 5, triose-phosphate isomerase (TPI); 6, glyceraldehyde-phosphate dehydrogenase (GAPDH); 7, phosphoglycerate kinase (PGK); 8, phosphoglycerate mutase (PMG); 9, enolase (ENO); 10, pyruvate kinase (PK); 11, lactate dehydrogenase (LDH); 12, pyruvate dehydrogenase (PDH); 13, pyruvate formate lyase (PFL); 14, acetaldehyde dehydrogenase (ACDH); 15, alcohol dehydrogenase (ADHE); 16, phosphotransacetylase (PTA); 17, acetate kinase (ACK); 18, α-acetolactate synthase (ALS); 19, α-acetolactate decarboxylase (ALD); 20, 2,3-butanediol dehydrogenase (BDH); 21, diacetyl reductase (DR); 22, glucose-6-P dehydrogenase (G6PDH); 23, 6-P-glucanate dehydrogenase (6PGDH); 24, ribulose-5-P-3-epimerase (RPPE); 25, D-xylulose-5P phosphoketolase (XPK); 26, galactokinase (GK); 27, galactose-1-P-uridylyltransferase (GPUDLT); 28, UDP-galactose-1-epimerase (UDPE); 29, phosphoglucomutase (PGM).
(lactose) are interconnected (Aleksandrzak-Piekarczyk et al. 2005; Kowalczyk et al. 2008) and, interestingly, lactose-P is hydrolyzed by BglS, a P-\(\beta\)-glucosidase exhibiting P-\(\beta\)-galactosidase activity (Aleksandrzak-Piekarczyk et al. 2005).

Phosphorylated trehalose in \(L.\ lactis\) is split by the inorganic phosphate-dependent trehalose-6P phosphorylase (TrePP). TrePP catalyzes the conversion of trehalose-6P to \(\beta\)-glucose-1P (\(\beta\)-G1P) and G6P (Andersson et al. 2001). \(\beta\)-G1P is then reversibly converted into G6P by \(\beta\)-phosphoglucomutase (\(\beta\)-PGM), which has been found to be essential in the catabolism of both trehalose and maltose (Levander et al. 2001; Andersson and Rådström 2002a). This enzyme together with Pi-dependent maltose phosphorylase is involved in the degradation of maltose in \(L.\ lactis\) (Andersson and Rådström 2002b). Hydrolysis of melibiose and raffinose by \(\alpha\)-galactosidase leads to the production of \(\alpha\)-galactose subunits, which are subsequently degraded by the enzymes of the tagatose-6P and Leloir pathways. Characterization and transcriptional analysis of the \(aga\-galKT\) operon in \(Lactococcus raffini\) has been presented by Boucher et al. (2003).

1.2.1. Homofermentation and Mixed-Acid Fermentation

Homofermentative LAB (\(Lactococcus\), \(Streptococcus\), \(Pediococcus\), \(Enterococcus\), and some species of \(Lactobacillus\)) ferment sugars by the Embden-Meyerhoff-Parnas (EMP) pathway to pyruvate, which is converted into lactic acid by lactate dehydrogenase (LDH; Fig. 1.1A). Two types of lactate isomers, L and D, can be produced by stereospecific NAD-dependent enzymes, L-LDH and D-LDH. Both enzymes have been found to be active in most lactobacilli, for example, in \(Lactobacillus plantarum\) (Ferain et al. 1996) and \(Lactobacillus casei\) (Viana et al. 2005a), whereas the single L-LDH in \(Lactobacillus sakei\) and \(L.\ lactis\) is complemented by a racemase or the protein product of the cryptic \(ldhB\) gene, respectively (Bongers et al. 2003; Gaspar et al. 2007).

Under certain conditions (carbon limitation, carbon excess of slowly metabolized sugars) the homolactic metabolism can be shifted to a mixed-acid metabolism (Fig. 1.1B). This type of homofermentation is characterized by the production of formate, acetate, ethanol, and/or \(CO_2\) in addition to lactate. There are several possible pathways for acetate production. Pyruvate can be metabolized anaerobically into acetate by pyruvate formate lyase (PFL), phosphotransacetylase (PTA), and acetate kinase (ACK) or aerobically by the pyruvate dehydrogenase complex, PTA, and ACK. In \(Lact.\ plantarum\), the maximal acetate production is under aerobic conditions with glucose limitation. In this pathway acetate originates from lactate through the action of LDHs, pyruvate oxidase (POX), and ACK. Recently, the role of NAD-dependent LDHs (LdhD and LdhL), instead of the previously proposed NAD-independent LDHs (LoxD and LoxL), was confirmed during the stationary phase of aerobic growth in \(Lact.\ plantarum\) (Goffin et al. 2004). Two genes coding for PoxB and PoxF out of four \(pox\) genes in the genome are also involved in the conversion of lactate to acetate in \(Lact.\ plantarum\) (Goffin et al. 2006). Enzyme kinetic data from literature were used for construction of a detailed glycolytic model of \(L.\ lactis\) during glucose run-out experiments (Hoefnagel et al. 2002).

1.2.2. Control of the Glycolytic Flux

Despite intensive research and a wealth of metabolic information for \(L.\ lactis\), the mechanisms controlling glycolytic flux in this and other LAB species remain unclear. This topic has been extensively discussed in excellent recent reviews (Cocaign-Bousquet et al. 2002; Neves et al. 2005; Kowalczyk and Bardowski 2007).

Several enzymes have been found to participate in the control of catabolic flux in \(L.\ lactis\); however, it should be stressed that their action can be strain- and/or condition-dependent. During homolactic fermentation in \(L.\ lactis\) subsp. \(lactis\) NCDO 2118, the control has been attributed to glyceraldehyde-phosphate dehydrogenase (GAPDH), which is modulated by the NADH/NAD+ ratio (Even et al. 1999). According to other results the key glycolytic enzymes phosphofructokinase (PFK), pyruvate
kinase (PK), and LDH or GAPDH exert, at normal activity levels, virtually no control on the glycolytic flux in *L. lactis* (Andersen et al. 2001; Koebmann et al. 2005). However, at reduced activities both PFK and PK were found to exert a strong positive control on glycolysis (Koebmann et al. 2005). Depending on culture conditions (e.g., oxygen and/or glucose concentration), the overall flux control seems to be distributed over many glycolytic steps and may also reside outside of the pathway (such as ATP-consuming reactions and sugar transport). Furthermore, the end-product demand might also be essential in flux control (Koebmann et al. 2002a, 2002b; Papagianni et al. 2007).

**Shift to mixed-acid fermentation.** Despite extensive studies the exact mechanisms underlying shift in the mode of fermentation remain elusive. Originally, the regulation of the switch was mainly attributed to the modulation of the intracellular concentration of fructose-1,6-bisphosphate (FBP), which allosterically activates both LDH and PK, and of glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP) inhibiting the PFL. Inorganic phosphate has been shown to be a severe inhibitor of LDH and PFL (for a review, Cocaign-Bousquet et al. 2002; Neves et al. 2005).

A metabolic model involving modulation of GAPDH and LDH activities via the NADH/NAD⁺ ratio (redox state) in *L. lactis* subsp. *lactis* NCDO 2118 has been postulated as a main factor regulating the shift in metabolism (Even et al. 1999). However, in other strains such as *L. lactis* subsp. *cremoris* MG1363 and *L. lactis* subsp. *lactis* IL1403, this hypothesis has not been confirmed. In these strains the glycolytic flux is not controlled by GAPDH (Even et al. 2001; Solem et al. 2003). Another study revealed that PK exhibits strong positive control on formate and acetate production and thus plays a role in the metabolic shift from homolactic to mixed-acid fermentation (Koebmann et al. 2005). In *L. lactis* subsp. *lactis* IL1403 a high transcriptional level of the genes coding for PFL and alcohol dehydrogenase (ADH) was noted, while the activity of the enzymes was low, suggesting a post-transcriptional control (Even et al. 2001). Moreover, it has been demonstrated that LDH may be co-regulated by at least two mechanisms (the FBP/inorganic phosphate [Pi] pool and the NADH/NAD⁺ ratio), and the manner of regulation is strain-dependent (van Niel et al. 2004). Several studies suggest that in addition to an allosteric control, pyruvate metabolism may further be controlled at the transcription level of central catabolic pathways (Melchiorsen et al. 2000; Even et al. 2001; Palmfeldt et al. 2004). Apart from the biochemical shift, regulation by allosteric changes of enzymes, metabolite levels, transcript, and protein levels, pyruvate metabolism is influenced by environmental factors such as oxygen, temperature, and pH (for a review, Neves et al. 2005; Kowalczyk and Bardowski 2007).

1.2.3. **Heterofermentation**

Heterofermentative LAB such as *Leuconostoc*, *Oenococcus*, and certain *Lactobacillus* species ferment sugars generally by the phosphoketolase pathway (PKP). Fermentation of pentoses (xylose, ribose) leads to the formation of pyruvate and acetyl-P and their subsequent conversion to lactate and acetate, respectively. Hexoses (glucose, fructose, mannose) in these bacteria can be converted to lactate, CO₂, and ethanol (Fig. 1.1C). CO₂ is a product of 6-P-gluconate degradation, which occurs during conversion of hexoses to pentoses. The specific enzyme of the heterofermentative pathway, D-xylulose-5P phosphoketolase, catalyzes the conversion of xylulose-5P to GAP and acetyl-P. The first metabolite, GAP, enters the EMP pathway leading to the production of lactate, whereas the second metabolite, acetyl-P, is converted into ethanol. The low activity of the ethanol pathway in the reoxidation of the NAD(P)H due to a low acetaldehyde dehydrogenase activity limits heterofermentative growth on glucose (Maicas et al. 2002). Heterofermentative LAB bypass the limiting ethanol pathway by using alternative pathways for NAD(P)H reoxidation. Much higher growth rate on hexoses is observed when O₂, pyruvate, citrate, or fructose is used as external electron acceptors (Zaummüller et al. 2006; Arsköld et al. 2008). A part of the acetyl-P is then converted to acetate instead of
ethanol, making the PKP as efficient as the EMP pathway.

Recently, the simultaneous use of PKP and EMP pathways was reported in *Oenococcus oeni* and *Lactobacillus reuteri* ATCC 55730 (Richter et al. 2003; Arsköld et al. 2008). The use of two different glycolytic pathways has so far only been genetically supported in homofermentative lactobacilli, including *Lact. plantarum* (Pieterse et al. 2005), and *Lactobacillus salivarius* (Claesson et al. 2006). In both, EMP pathway and PKP, the growth phase-dependent expression of glycolytic pathways was demonstrated. Higher expression levels of the catabolic enzymes, such as PK, phosphoglycerate kinase, and GAPDH, during initiation of growth and log phase were reported in *L. lactis* subsp. *lactis* (Larsen et al. 2006). Similarly, proteins preferentially expressed in the early-exponential phase in *Lact. plantarum* were also associated with EMP and PKP pathways (Koistinen et al. 2007). This fact could be expected due to the positive effect of these enzymes on sugar consumption and biomass increase.

### 1.2.4. Control of Sugar Metabolism in LAB

#### The pivotal role of the HPr protein.

HPr is a part of the phosphoenolpyruvate (PEP)-PTS system, and in low-GC Gram (+) bacteria seems to be involved in different activities depending on its phosphorylation state. PEP-dependent phosphorylation of HPr by Enzyme I yields His-P-HPr, which serves as a phosphoryl carrier within the PTS phosphorylation cascade during transport of carbohydrates. However, His-P-HPr phosphorylates not only Enzyme II (EIIAs) but also the key regulators of carbohydrate metabolism. For example, it phosphorylates histidyl residues in non-PTS proteins such as glycerol kinase, antiterminators, transcriptional activators, and non-PTS transporters. As a result of the metabolism of PTS substrates, the concerted action of FBP and an ATP-dependent protein kinase/phosphorylase (HprK/P) generates Ser-P-HPr. This form of HPr plays a crucial role in an inducer-exclusion mechanism and, together with the catabolic control protein A (CcpA), in carbon catabolite repression (CCR; for a review see Deutscher et al. 2006).

#### Characteristics of CcpA-dependent regulation.

The subject of CcpA-dependent regulation has been extensively described and discussed by several authors (Titgemeyer and Hillen 2002; Deutscher et al. 2006; Kowalczyk and Bardowski 2007). The CcpA protein from *L. lactis* IL1403 was successfully crystallized showing a potential cavity for a corepressor (Loll et al. 2007). The CcpA protein, together with its corepressor Ser-P-HPr, binds to 14 nucleotide cis-acting DNA target sites, called catabolite responsive elements (*cre*). Depending on the position of the *cre* sequence, CcpA can act as an activator or a repressor promoting carbon catabolite activation (CCA) or repression (CCR), respectively. *Cre* sites can either be located within the promoter, overlap the transcription start site, or lay between the transcription start site and the initiation codon or within the 5′ part of catabolite-regulated genes. Binding of CcpA to *cre* sites overlapping the promoter region activates or represses the initiation of transcription, while binding to a *cre* situated downstream of the promoter leads to abortion of transcription. Most transcription units subjected to CCA contain *cre* sites in front of their promoter region. In the *L. lactis* IL1403 genome thousands of putative *cre* sequences have been detected (Andersson et al. 2005), although a majority may have no biological significance because of its location. Gene expression analyses using DNA microarrays have shown that CcpA regulates an impressive number of genes involved in carbohydrate transport and metabolism in *L. lactis* MG1363 (Zomer et al. 2007). Several genes containing functional *cre* sites, which are subjected to regulation by the Ser-P-HPr/CcpA complex have been recently described in a number of LAB (Titgemeyer and Hillen 2002; Deutscher et al. 2006; Francke et al. 2008). In *L. lactis* strains, CcpA has been shown to repress different β-glucosides, fructose, galactose, or lactose assimilation genes, but it activates the *las* operon (pfk, pyk, and ldh genes; Aleksandrzak-Piekarczyk et al. 2005; Barriere et al. 2005; Kowalczyk et al. 2008). Interestingly, in *L. lactis* CcpA coordinates the metabolic switch from fermentative to aerobic growth, thus preventing oxidative damage (Gaudu et al. 2003). Non-PTS sugars can also trigger CcpA-
dependent carbon regulation, as is the case of lactose in *Strep. thermophilus* (van den Bogaard et al. 2000). In the genus *Lactobacillus* CcpA has been demonstrated to repress several sugar catabolic genes (Monedero et al. 1997; Chaillou et al. 2001; Muscariello et al. 2001; Marasco et al. 2002). Moreover, in *Lact. casei* and *Lactobacillus pentosus* CcpA has been shown to regulate glycolytic genes (*pfk* and *pyk*) and the central metabolic PKP, respectively (Posthuma et al. 2002; Viana et al. 2005b). In some LAB *ccpA* is preceded by a cre, and its expression is subjected to autoregulation (Morel et al. 2001; Zomer et al. 2007).

**Secondary regulators.** In addition to global regulators such as CcpA, carbon catabolism might also be controlled by specific, local regulators belonging to different protein families such as LacI, LysR, AraC, GntR, DeoR, or BglG, which are widely distributed among LAB. Regulators from these families have been proven to control genes for α-galactosides, fructose, lactose, maltose, salicine, sorbose, and xylose assimilation in lactococci (Boucher et al. 2003; Kowalczyk and Bardowski 2007), *Strep. thermophilus* (Vaughan et al. 2001), or lactobacilli (Yebra et al. 2000; Fortina et al. 2003). For *L. lactis* and *Lact. plantarum* it has been shown that genes encoding secondary regulators are located in direct proximity and in a divergent transcriptional direction to the sugar uptake operon. As is the case for *ccpA*, most secondary regulators seem to undergo autoregulation (Francke et al. 2008).

**Inducer exclusion/expulsion.** These two protein-mediated mechanisms of carbon regulation supplement the above-mentioned mechanisms of transcriptional control (Barriere et al. 2005; Monedero et al. 2008). Based on *in vivo* analysis, Ser-P-Hpr has been proposed to drive the exclusion of the inducer in *Lact. casei*, *L. lactis*, and *Lactobacillus brevis*, but not the expulsion of the inducer (Monedero et al. 2008).

### 1.2.5. Exopolysaccharides (EPS)

LAB are able to direct some part of the sugar pool toward biosynthesis of EPS. These long-chain saccharides can be loosely attached to the cell surface forming some kind of capsules or are secreted to the environment. EPS represent molecules with different structures, sizes, and sugar composition (reviewed by Laws et al. 2001; Broadbent et al. 2003; Ruas-Madiedo et al. 2008). They are classified into two groups: homopolysaccharides consisting of one type of monosaccharide (α-D-glucans, β-D-glucans, fructans, and others represented by polygalactan) and heteropolysaccharides composed of different types of monosaccharides, mainly D-glucose, D-galactose, L-rhamnose, and their derivatives. In the last few years several works presented a diversity of biopolymers produced by cereal-associated and intestinal LAB (Tieking et al. 2003; van der Meulen et al. 2007), *Strep. thermophilus* (Vanengelgem et al. 2004) or other LAB strains isolated from dairy products (Mozzi et al. 2006; van...
der Meulen et al. 2007). During these studies the production of glucan by *L. lactis*, an EPS-containing (N-acetyl) glucosamine by *Strep. thermophilus* (Vanigelgem et al. 2004), a polygalactan synthesized by two strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Mozzi et al. 2006), and a heteropolysaccharide by *Lactobacillus curvatus* (van der Meulen et al. 2007), has been reported for the first time. Pioneering research describing the detection of EPS-producing strains in the human intestinal ecosystem showed that 17% of *Lactobacillus* and *Bifidobacterium* strains could be EPS producers (Ruas-Madiedo et al. 2007).

### Biosynthesis of EPS

Homopolysaccharides are synthesized extracellularly by highly specific glycosyltransferase enzymes, glucan or fructan sucrases (van Hijum et al. 2006). This process requires sucrose as a specific substrate and the energy that comes from its hydrolysis. Recently, a negative effect of glycosyltransferases on growth on sucrose of some *Lact. reuteri* strains has been observed (Schwab et al. 2007). EPS belonging to heteropolysaccharides are synthesized from glucose, galactose, or other monosaccharides by a combined action of different types of glycosyltransferase enzymes. Four major consecutive steps of EPS biosynthesis in LAB involve sugar transport into the cytoplasm, synthesis of sugar-1P, polymerization of repeating unit precursors, and finally EPS export outside the cell (reviewed by Laws et al. 2001; Welman and Maddox 2003).

### Regulation of EPS production

It is postulated that regulation of EPS production can be possible at each of the different steps during biosynthesis. It has been observed that the overexpression of the fructose biphosphate (*fbp*) gene led to an increase of the EPS yield in *L. lactis* cells growing on fructose. The role of enzymes, such as glucokinase, PFK, and PGM controlling the carbon distribution between catabolic (glycolysis) and anabolic reactions (EPS precursor biosynthesis) has recently been studied in *L. lactis* (Boels et al. 2003). It has been shown that overproduction of PGM resulted in increased levels of both UDP-glucose and UDP-galactose; however, did not significantly affect EPS production. In *L. lactis*, EPS production is controlled by a phospho-regulatory system and EPS biosynthesis correlates with an unphosphorylated protein (EpsB) (Nierop Groot and Kleerebezem 2007).

### 1.2.6. The Post-genomic Era of Sugar Metabolism and Regulation in LAB

At the time of writing complete genome sequences have been presented for more than 20 fermentative and commensal LAB species from the order of *Lactobacillales* and more genome projects are ongoing (Mayo et al. 2008). Most genes involved in sugar metabolism and energy conversion are uniformly represented among LAB species with completed genome sequences (Makarova et al. 2006). Moreover, it seems that genes encoding enzymes involved in glycolysis, pyruvate metabolism, and/or regulators such as CcpA, HPr, HprK/P, and EI are highly expressed in *L. lactis* and *Lactobacillus acidophilus* (Guillot et al. 2003; Karlin et al. 2004; Barrangou et al. 2006).

Genome analyses have also shown that lactobacilli, streptococci, and lactococci, probably due to the diversity of environments they inhabit, possess wide saccharolytic abilities. The ability to ferment many different kinds of carbohydrates is a specific characteristic of plant-associated strains (Nomura et al. 2006). Analysis of the genome of different *Lact. plantarum* strains has revealed the existence of many genes involved in sugar transport and metabolism located in the so-called “lifestyle adaptation island,” a 213 kb region of high plasticity (Kleerebezem et al. 2003; Molenaar et al. 2005). Furthermore, unique genes and clusters for xylan, glucan, and fructan/mannan breakdown have been identified in the genome of nondairy *L. lactis* subsp. *lactis* strains isolated from plant material (Siezen et al. 2008). On the other hand, in milk-specialized bacteria, for example *Strep. thermophilus*, the capacity to degrade starch or glucans has been shown to be severely reduced (Salzano et al. 2007).
1.2.7. Practical Aspects of Sugar Catabolism

The ability of LAB to ferment sugars has been widely utilized in various food production processes. Recently, great attention has been directed to optimization of lactate production from natural substrates like starch or cellulose derived from waste products (for a review see, John et al. 2007; Reddy et al. 2008). Probiotic effects related to lactose catabolism have been the subject of several studies. Of particular interest is the selection of potential probiotic bacteria exhibiting high lactose hydrolysis (Drouault et al. 2002; Honda et al. 2007). The elucidation of the metabolic adaptation of bacteria to lactose in the digestive tract is also important (Roy et al. 2008). Some probiotic LAB can utilize prebiotic compounds such as non-digestible fructooligosaccharides (FOS) or inulin-type fructans, which stimulate the growth of beneficial commensals in the gastrointestinal tract. Lactic acid has been shown to be the main metabolic end-product of oligofructose-enriched inulin (OEI) fermentation by Lactobacillus paracasei subsp. paracasei 8700:2, although significant amounts of acetic acid, formic acid, and ethanol were also produced when long-chain inulin or OEI was used as the sole energy source (Makras et al. 2005). Lact. acidophilus hydrolyzes FOS by a fructosidase, which renders fructose and sucrose; the latter sugar is subsequently hydrolyzed into fructose and glucose-1P by a sucrose phosphorylase (Barrangou et al. 2003; Goh et al. 2006). In Lact. plantarum a sucrose PTS, a β-fructofuranosidase, and a fructokinase were found to be involved in the degradation of short-chain FOS (Saulnier et al. 2007). As already mentioned, LAB can direct part of the sugar pool toward biosynthesis of EPS. These microbial polysaccharides have been used in the food industry as emulsifiers, thickeners, viscosifiers, and stabilizers. Important studies from a practical point of view concern the kinetics of EPS formation and factors affecting EPS production, such as nutrients (Velasco et al. 2006), type of carbon and nitrogen sources (Korakli et al. 2003; Sánchez et al. 2006; Velasco et al. 2006), temperature, and pH (Zisu and Shah 2003; Sánchez et al. 2006).

Finally, it is worth mentioning the protective role of sugars in the preservation and storage of LAB species. Sugars such as trehalose, sorbitol, mannitol, sucrose, lactose, and mannose increased the viability of the cells during drying and freezing processes (reviewed by Santivarangkna et al. 2008). In addition, the mechanism involved in the protective effect of glucose on Lactobacillus rhamnosus GG during gastric transit has been shown to rely on an increased activity of the F$_0$F$_1$-ATPase enabling proton exclusion (Corcoran et al. 2005).

1.3. Proteolysis and Amino Acid Catabolism

It has been well established that LAB species are auxotrophs for a variable number of amino acids, depending both on the species and on the strain. Therefore, for proper growth on most systems LAB depend on a fully active proteolytic system to meet their amino acid requirements. The proteolytic components of dairy LAB are among the best characterized to date, not only as a consequence of their impact on the physiology of LAB, but also because of their involvement in the development of texture and flavor in dairy products (for recent reviews see, Smit et al. 2005; Savijoki et al. 2006). The recent analysis of LAB genomes has further contributed to a complete characterization of the proteolytic systems in the sequenced species (Mayo et al. 2008). The genome of Lact. plantarum is the only LAB which encodes enzymes for the biosynthesis of all amino acids, except for leucine, isoleucine, and valine (Kleerebezem et al. 2003). In contrast, Lact. acidophilus is likely to be auxotrophic for 14 amino acids (Altermann et al. 2005), and Lactobacillus johnsonii appears incapable of de novo synthesis of most, if not all, amino acids (Pridmore et al. 2004).

The structural components of the proteolytic systems can be divided into three groups on the basis of their function: (1) proteinases breaking proteins into peptides; (2) transport systems that
translocate the breakdown products across the cytoplasm membrane; and (3) peptidases that degrade peptides into free amino acids (Fig. 1.2). These amino acids will be further degraded by strain-dependent metabolic pathways to generate the actual volatile compounds responsible for the aroma profile of fermented products (Fig. 1.3).

1.3.1. Proteinases

A single cell wall-bound extracellular proteinase (CEP) is generally considered to be responsible for the initial breakdown of caseins. Gene deletion studies have shown that LAB strains cannot grow in milk in absence of a functional CEP. This enzyme is a serine protease belonging to the subtilisin family. Five different types of this enzyme have been characterized from LAB: PrtP from L. lactis and Lact. paracasei, PrtH from Lactobacillus helveticus, PrtR from Lact. rhamnosus, PrtS from Strep. thermophilus, and PrtB from Lact. delbrueckii subsp. bulgaricus (Lact. bulgaricus). All enzymes are synthesized as pre-pro-proteins, of approximately 2000 residues, consisting of several functional domains (Siezen 1999).

In general, LAB species possess only one CEP each; however, the presence of two enzymes has been described in some lactobacilli (Pederson et al. 1999). A membrane-bound lipoprotein, PrtM, has been found to be essential for the autocatalytic activation of the proteinase in L. lactis and Lact. paracasei. Moreover, prtP and prtM genes are divergently transcribed (Haandrikman et al. 1991). In contrast, the maturation gene has not been identified in the CEP-flanking regions of Lact. helveticus, Lact. bulgaricus, and Strep. thermophilus (Fernández-Esplá
Figure 1.3. Catabolic pathways of the principal amino acids involved in the production of aroma compounds. Continuous lines show enzymatic reactions catalyzed by the enzymes indicated. Dotted and broken lines show spontaneous chemical reactions and poorly defined pathways, respectively. The most important compounds formed are shown in bold.
et al. 2000; Germond et al. 2003). However, recent microarray analysis has shown an increase expression of a homologous of \textit{prtM} in \textit{Lact. helveticus} CNRZ32 when growing in milk (Smeianov et al. 2007), suggesting that at least in this organism a PrtM-like protein may play a role in CEP maturation. Expression of \textit{prtP} in \textit{L. lactis} is repressed in the presence of rich nitrogen sources such as casein hydrolysates, casamino acids, and specific dipeptides. The expression is under the control of the general regulator CodY, which senses the internal branched-chain amino acid (BCAA) pool (Guédon et al. 2001). Regulation of the proteolytic system in lactobacilli is much less studied, and, as in \textit{L. lactis}, rich nitrogen sources represses proteinase expression (Hebert et al. 2000; Pastar et al. 2003). Some species of LAB do not exhibit CEP-like activity, such as \textit{Lact. plantarum} (Kleerebezem et al. 2003) and other lactobacilli species from sourdough (Vermeulen et al. 2005). This suggests that caseinolytic proteinases may function as an adaptative factor, which has been acquired by LAB of dairy and intestinal origin.

Intracellular proteinases involved in regulatory networks and stress responses of LAB, required for normal growth under different stress conditions, such as FtsH, HtrA, and Clp-proteases (Poquet et al. 2000; Foucaud-Scheunemann and Poquet 2003; Savijoki et al. 2003) might also play a role during maturation of fermented products.

1.3.2. \textbf{Peptide and Amino Acid Uptake Systems}

The second step in protein degradation includes the transport of di-, tri-, and oligo-peptides into the cell by the action of different peptide uptake systems (Fig. 1.2). Three functional peptide transport systems have been described in \textit{L. lactis}: DtpT, Opp, and Opt (previously referred to as Dpp). DtpT is a proton motive force-dependent transporter whose specificity is limited to di- and tripeptides (Kunji et al. 1993). This peptide uptake system is also present in \textit{Lact. helveticus}, \textit{Lactobacillus sanfranciscensis}, and \textit{Strep. thermophilus}. Opp and Opt are transporters belonging to the ABC transporter superfamily, which transport di-, tripeptides, and oligopeptides (Detmers et al. 1998). They have been identified in different species of LAB, such as \textit{L. lactis}, \textit{Lact. bulgaricus}, \textit{Lact. helveticus}, \textit{Lact. plantarum}, \textit{Lact. sanfranciscensis}, and \textit{Leuconostoc mesenteroides}. Opp was characterized first and is composed of five proteins: an oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC), and two nucleotide-binding proteins (OppD and OppF; Tynkkynen et al. 1993). Opt differs from Opp by its genetic organization and peptide uptake specificity. The Opt system contains two different peptide-binding proteins. Initially, the Opt system was only related with the transport of di- and trihydrophobic peptides. However, analysis of Opp-deficient \textit{L. lactis} strains revealed the implication of Opt in the transport of oligopeptides (Foucaud et al. 1995). Differences in specificity between the Opp and Opt are likely to be responsible for the previously observed variability in peptide uptake within different strains of \textit{Lactococcus} (Charbonnel et al. 2003). Specificity has been shown to be imposed by the (oligo)peptide-binding proteins (Doeven et al. 2005). A second set of Opp-transporter encoding genes, named \textit{opp2}, was found in \textit{L. lactis} MG1363, although this latter Opp system is either not expressed or not functional (Doeven et al. 2005). All these systems (Opp, Opt, and DtpT) have been found to be functional in only a minority of \textit{L. lactis} strains (such as MG1363, SK11, and Wg2; Doeven et al. 2005). The Opp from IL1403 and SKM6 lacks the OppA component. Thus, most likely, uptake of oligopeptides in these strains seems to be driven by Opt.

In \textit{Strep. thermophilus}, the oligopeptide transport system presents some differences from that of \textit{Lactococcus}. It has been called Ami, since it shows the highest identity with the Ami system of \textit{Streptococcus pneumoniae} (Garault et al. 2002). However, the specificity of the \textit{Strep. thermophilus} oligopeptide uptake is broader than those of streptococci and similar to that specified by OppA from \textit{L. lactis} (Garault et al. 2002). The Ami transporter is composed of three oligopeptide-binding proteins (AmiA1, AmiA2, and AmiA3), two-membrane
proteins (AmiC, AmiD), and two ATPases, which provide energy to the system (AmiE, AmiF).

A variable number of genes encoding putative amino acid permeases and transporters of several classes have been identified in the genome of LAB species (Makarova et al. 2006). However, specificity of most transporters is not yet known.

1.3.3. Peptidases

Inside the cells, peptides are degraded by the concerted action of an array of peptidases with distinct but overlapping specificities (Kunji et al. 1996a). Peptidases can be divided into two types: endopeptidases, which hydrolyze internal peptide bonds, and exopeptidases, which remove amino acids from an extreme of the peptide chain. In LAB a majority of the exopeptidases are aminopeptidases and their specificity depends on the peptide length and the nature of the N-terminal amino acid residue (Kunji et al. 1996b; Fig. 1.2).

Endopeptidases. PepO was the first endopeptidase characterized from a LAB species. It is a 70-kDa monomeric metalloprotease capable of hydrolyzing peptides from 5 up to 35 residues, but unable to hydrolyze intact caseins. The inspection of sequenced genomes has shown that all LAB encode putative PepO homologs. L. lactis MG1363 encodes a second endopeptidase with properties similar to PepO, named PepO2. Additionally, the analysis of the Lact. helveticus CNRZ32 genome revealed a third PepO endopeptidase, PepO3, which is a functional paralog of PepO2, and both endopeptidases (PepO2 and PepO3) could play a key role in the Lact. helveticus CNRZ32 ability to reduce bitterness in cheese (Savijoki et al. 2006). Duplication of peptidases would enhance the ability of Lactobacillus species to exploit protein-rich environments (Makarova et al. 2006). A second endopeptidase with a different specificity from that of PepO has been designated as PepF1. It is a 70-kDa monomeric metallopeptidase, which hydrolyzes peptides containing between 7 and 17 amino acids with a rather broad specificity. In L. lactis subsp. cremoris NCDO763 two copies of this gene have been found, one, pepF1, is plasmid encoded, whereas a second copy, pepF2 (80% identity), is located in the chromosome. PepF has also been identified in other LAB genomes, such as Lact. helveticus, Lact. brevis, Strep. thermophilus, O. oeni, Leuc. mesenteroides, Lactobacillus gasseri, and Lact. delbrueckii. Other endopeptidase genes have further been identified in Lact. delbrueckii (PepG; van de Guchte et al. 2006) and Lact. helveticus (PepE and PepE2; Callanan et al. 2008).

Aminopeptidases. The aminopeptidases identified in LAB can be divided into three groups: aminopeptidases with broad specificity, specific aminopeptidases for acidic or basic amino acids, and those specific for hydrophobic or aromatic residues. Aminopeptidases with broad specificity, PepC and PepN, have been identified in Lactococcus, Lactobacillus, and Strep. thermophilus. PepC belongs to the C1 family of cysteine peptidases and has a significant activity on basic, acidic, hydrophobic/uncharged, and aromatic residues. PepN presents a higher activity on basic residues followed by hydrophobic/uncharged residues and, in general, low or undetectable activity on Asp, Glu, and Gly (Savijoki et al. 2006). Aminopeptidases acting on acidic residues, PepA, also referred to as glutamyl aminopeptidases, have been identified in L. lactis, Strep. thermophilus, and Lact. gasseri genomes. This enzyme belongs to M42 family of metallopeptidases. Since bitter peptides are mainly formed by hydrophobic and aromatic residues, an aminopeptidase specific for aromatic amino acids could have an important role in the development of flavor in dairy products. An enzyme with this activity, PepS, has been identified in Pediococcus pentosaceus, Strep. thermophilus, Leuc. mesenteroides, Lact. casei, and Lact. sakei. PepL, an aminopeptidase that has only been identified in Lact. delbrueckii, displays high specificity for Leu and Ala residues (Savijoki et al. 2006).

Dipeptidases. Several dipeptidases have been described in LAB with a broad specificity (PepD and PepV) and with specificity to peptides containing proline (PepQ and PepR; see below). PepD has
only been characterized from *Lact. helveticus*. It has an activity similar to that reported for PepV but it is unable to hydrolyze Val-X and Ile-X dipeptides (Savijoki et al. 2006). Analysis of available LAB genomes has lead to the identification of encoding proteins similar to PepV and PepD dipeptidases in other *Lactobacillus* species as well as in *L. lactis* and *Strep. thermophilus*.

**Tripeptidases.** These enzymes hydrolyze tripeptides with a wide range of amino acid composition although they do not hydrolyze tripeptides containing Pro in the middle position. The tripeptidase PepT has been identified in *L. lactis*, *Strep. thermophilus*, and *Lact. helveticus*. Other tripeptidases have been purified from other LAB (*Lact. sakei*, *Lact. delbrueckii*, *Ped. pentosaceus*) and, like PepT, they are all metallopeptidases having a high activity on tripeptides composed of hydrophobic/uncharged and aromatic residues.

**Proline-specific peptidases.** This group of peptidases includes aminopeptidases and dipeptidases (PepQ, PepR, PepI, PepP, and PepX), which are specific for Pro residues. PepQ is a prolidase enzyme specific for X-Pro dipeptides although in some cases is able to hydrolyze other dipeptides. This enzyme has been characterized from different *Lactobacillus* and *Lactococcus* species. PepR is a dipeptidase identified only in some *Lactobacillus* species with prolidase activity, specific for Pro-X dipeptides, which has a limited activity in larger peptides. PepI releases Pro from the N-terminus of tripeptides, but not from tetra- and penta-peptides. PepP is an aminopeptidase releasing the N-terminal amino acid from peptides with the X-Pro-Pro-(Y)_n composition. This enzyme has been characterized from *L. lactis* and has high activity on peptides ranging from three to nine residues, with the highest rate of hydrolysis on pentapeptides. PepX releases X-Pro dipeptides from substrates containing three to seven amino acid residues. This enzyme is widely distributed among dairy LAB, and it surely is the most thoroughly studied Pro-specific peptidase. Prolidase enzymes such as PepQ can subsequently hydrolyze the dipeptides released by PepX (Savijoki et al. 2006).

### 1.3.4. Regulation of the Proteolytic System

The proteolytic system of *L. lactis* has been shown to respond to changes in nitrogen availability. It is now clear that the transcriptional regulator CodY negatively regulates the expression of several components of the proteolytic system in this species by binding to their cognate promoter regions (den Hengst et al. 2005). Repression is modulated by the intracellular pool of BCAAs (Guédon et al. 2001). However, not all proteolytic enzymes respond to this regulation. Expression of *pepP* has been demonstrated to respond to catabolite repression and is controlled by CcpA (Guédon et al. 2001).

The regulatory mechanisms are much less studied in *Lactobacillus*. Peptide concentration in the growth medium has been shown to control PrtH and PrtR biosynthesis in *Lact. helveticus* and in *Lact. rhamnosus*, respectively. A recent systematic study on *Lact. acidophilus* using expression profiling with whole genome microarrays has revealed that a two-component regulatory system acts as a pleiotropic regulator in controlling the expression of at least 80 genes, including major components of the proteolytic system (Azcárate-Peril et al. 2005). To date, only one regulatory protein directly controlling the expression of proteolytic genes has been identified in *Lactobacillus*. In *Lact. delbrueckii* subsp. *lactis* and *Lact. bulgaricus*, Pepr1, a CcpA-like regulator, has been demonstrated to bind to the promoter regions of *pepQ* and *pepX*, acting as a transcriptional activator. In this case, expression of PepQ depends on carbohydrate concentration and composition rather than on peptide concentration; this contrasts with the biosynthesis of other components of the proteolytic system in *Lact. bulgaricus* (Morel et al. 2001).

### 1.3.5. Physiological Role and Technological Aspects of Proteolysis

Analyses conducted on mutant strains of *L. lactis*, *Lact. helveticus*, *Lact. rhamnosus*, and *Strep. thermophilus* defective in components of the proteolytic system have shown that PrtP and Opp are crucial for growth in a medium with casein as the sole source
of amino acids (Christensen et al. 1999). Numerous LAB strains with mutations in single peptidase genes have also been examined for their ability to grow in milk; although minor changes in growth were observed, it can be concluded that none of the individual peptidases is essential for growth in milk because its activity is compensated by other peptidases (Kunji et al. 1996b). Proteolysis is considered one of the most important biochemical processes involved in the ripening of many fermented dairy products. It releases amino acids from caseins, which, in turn, are major precursors of specific flavor compounds: alcohols, aldehydes, acids, esters, and sulfur compounds. As mentioned before, the peptidolytic activity (PepN, PepX, PepO2, and PepO3) is also important in reducing the accumulation of hydrophobic peptides to reduce bitterness in cheese (Sridhar et al. 2005).

Besides the importance of proteolytic enzymes in the final organoleptical properties of fermented products, certain LAB strains are further known to be able to liberate bioactive peptides encrypted in proteins, which are thought to have a role in promoting health (Korhonen and Pihlanto-Leppälä 2002). To date, strains of LAB endowed with such properties include strains such as *Lact. helveticus* CP790, *Lact. rhamnosus* GG, *Lact. bulgaricus* SS1, and *L. lactis* subsp. *cremoris* FT4 (Gobetti et al. 2002; Muguerza et al. 2006; see Chapter 13 for more details). Several reports have indicated that bioactive peptides may also be produced *in vivo* by intestinal LAB after the intake of milk proteins (Meisel 2004). Production of such peptides at an industrial scale for use as dietary supplements is currently receiving increased interest (Meisel 2004).

1.3.6. Amino Acid Catabolism and Flavor Formation

The catabolism of amino acids has implications with regard to quality (formation of flavor) and safety (synthesis of biogenic amines) of fermented foods. Amino acid catabolism is further believed to have an important role in LAB physiology for obtaining energy in nutrient-limited conditions, participating in pH homeostasis. However, most pathways remain only partially characterized in LAB. BCAAs (valine, isoleucine, and leucine), aromatic amino acids (tyrosine, tryptophane, and phenylalanine), sulfur-containing amino acids (methionine and cysteine), and threonine are the main amino acid sources for flavor (sweaty, sour, and sweet aroma; Fig. 1.3). Other amino acids can participate as well; for instance, the catabolism of aspartic acid has been recently shown to be involved in production of both acetoin and diacetyl (Le Bars and Yvon 2008).

BCAAs. BCAAs and sulfur-containing amino acids can be degraded by two distinct pathways: transamination and degradation (Fig. 1.3). Transamination can be catalyzed by two amino-transferases, BcaT and AraT (Yvon and Rijnen 2001), which convert amino acids into their corresponding α-ketoacid. BcaT orthologs are present in all lactococcal and streptococcal strains while they are absent in a number of lactobacilli. The gene encoding AraT has been experimentally characterized in *L. lactis* and putative araT genes have been found in all LAB genomes except those of *Lact. sakei* and *Lact. brevis*. The araT gene is monocistronically transcribed and it is under the control of CodY. The transaminase reaction is commonly linked to the deamination of glutamate to α-ketoglutarate, catalyzed by glutamate dehydrogenase (GDH). GDH activity varies widely among LAB, and most strains of *L. lactis* apparently lack this activity (Lapujade et al. 1998) while it is present in the majority of *Strep. thermophilus* strains (Helink et al. 2004). In lactobacilli, GDH activity is also strain dependent (Kieronczyk et al. 2003). Inspection of available LAB genomes has revealed that GDH-encoding genes are present in some strains (e.g., *Lact. plantarum*, *Lact. sakei*, *Strep. thermophilus*) but absent in others (e.g., *L. lactis*, *Lact. casei*, *Lact. delbrueckii*).

The α-ketoacids can be further converted to aldehydes, carboxylic acids, and hydroxyacids in three different routes: oxidative decarboxylation to carboxylic acids, decarboxylation to aldehydes, and reduction to hydroxyacids (Fig. 1.3). α-Ketoacids can be decarboxylated to aldehydes by α-ketoacid decarboxylases. In LAB this activity has been
reported in *L. lactis* var *maltigenes*, *Lact. casei*, and *Lact. delbrueckii* subsp. *lactis* (Helinck et al. 2004) and in some nondairy *L. lactis* strains (Smit et al. 2004). Recently, this enzyme has been characterized from two different *L. lactis* strains and shown to require thiamine pyrophosphate as a cofactor. The encoding gene (*kdcA*, also called *kivD*) shows homology with indole pyruvate decarboxylases (de la Plaza et al. 2004; Smit et al. 2005). Aldehydes produced in these reactions can then be reduced to alcohols by alcohol dehydrogenases or oxidized to carboxylic acids by aldehyde dehydrogenases (Yvon and Rijnen 2001).

Oxidative decarboxylation of BCAAs to carboxylic acids is uncommon in LAB. This reaction leads to the formation of carboxylic acids without transitory formation of aldehydes. In the first step, the α-ketoacids are reductively decarboxylated to their corresponding acyl-CoA by α-keto acid dehydrogenase (KaDH), an enzyme complex composed by four subunits. Acyl-CoA is further converted to the corresponding carboxylic acid by a phosphotransacylase and acyl kinase (ACK; Fig. 1.3). Orthologous genes of components from the oxidative decarboxylation pathway (*ptb-buk-bkdDABC*) have only been found in the *Lact. casei* genome. Nevertheless, homologs of the *ptb* gene, *buk* gene, and *bkdDABC* genes were found encoded separately in different positions of the chromosome in various other LAB, for instance in *Strep. thermophilus*. This finding agrees well with the experiments of Helinck et al. (2004), who showed these enzyme activities in *Strep. thermophilus* strains. Caution is required, however, since the best homologs of KaDH in many LAB are annotated as either pyruvate or acetoin dehydrogenase complex, and it is not clear whether these complexes have overlapping substrate specificity (Liu et al. 2008).

Keto acids can also be reduced to hydroxy acids by hydroxy acid dehydrogenase (HADH). Two stereospecific enzymes, D-HADH and L-HADH, are distinguished, which belong to the larger D-LDH and L-LDH protein families, respectively. L-HADH from *Weissella confusa* (formerly *Lactobacillus confusus*) has been characterized, and a D-HADH encoding gene has been cloned from *Lact. casei*. Although there is no literature evidence that hydroxy acids can directly lead to flavor formation, the fact that precursors of hydroxy acids and some flavor compounds are shared imply that the activity of HADH could have a negative effect on flavor formation by shunting flavor precursors into off-flavors.

ADH and aldehyde dehydrogenase (ALDH) catalyze the conversion of aldehydes to alcohols and carboxylic acids, respectively. Most LAB genomes encode multiple ADH members, but only a single ADH/ALDH ortholog; these activities have never been studied in LAB species.

Methionine, cysteine, and threonine. Sulfur compounds produced from the catabolism of sulfur-containing amino acids are potent odorants in many fermented foods. For this reason Met and Cys catabolism has been deeply studied in the last years. The metabolism of sulfur-containing amino acids is complex, considering the existence of multiple alternative pathways/enzymes and a species and strain distribution of these activities. Met can be metabolized by (1) conversion to cystathionine through S-adenosyl methionine, thus linking Met and Cys pools; (2) deamination to α-oxo-γ-methylthiobutyrate by an aminotransferase reaction; and (3) simultaneous deamination and dethiomethylation to methanethiol by Met lyases (Fig. 1.3). The conversion of Met into methanethiol via lyase can be catalyzed by three PLP-dependent enzymes: cystathionine β-lyase (CBL), cystathionine γ-lyase (CGL), and Met γ-lyase (MGL). MGL is widely distributed among bacteria, but it has not been identified in LAB. CBL and CGL can use various sulfur-containing substrates including Met to produce methanethiol via α- and γ-elimination. CBL- and CGL-encoding genes have been identified in many LAB genomes, but these lyases possibly play a minor role in Met degradation, at least in *L. lactis* (Fernández et al. 2000). Met can also be degraded via transamination (Fig. 1.3). The resulting product, 2-oxo-4-(methylthio) butyric acid (KMBA), can be subsequently transformed into methanethiol by an unknown pathway (Yvon and Rijnen 2001). In lac-
tobacilli and Strep. thermophilus, transamination by AraT and BcaT is the main pathway for Met degradation (Amárita et al. 2001). The resulting KMBA can be chemically converted to methylthioaldehyde, methanethiol, and dimethyl sulfide. Methanethiol can be auto-oxidized to dimethyl sulfide and dimethyl trisulfide (Fig. 1.3). However, KMBA can also be degraded to methanethiol and 2-hydroxyl-4-methylthiobutyric acid by an enzymatic pathway in some lactococcal strains. Decarboxylation of KMBA to methional has been reported in one strain of L. lactis although by unknown pathway (Amárita et al. 2002).

Very few data are available on Cys catabolism in LAB. Amino acid utilization studies have shown that most LAB can utilize Cys, although this ability is strain-dependent (Williams et al. 2001). In addition, it has been reported that CGL can degrade Cys and cystine (the latter being the best substrate) to ammonia, hydrogen sulfide, and pyruvate (Bruinenberg et al. 1997). This activity, also referred to as Cys desulfhydrase, has been reported in Streptococcus anginosus and other streptococci (Yoshida et al. 2003). Cys could also be utilized for Met biosynthesis via cystathionine, which might be subsequently degraded.

Threonine can be degraded to acetaldehyde (Fig. 1.3), a major flavor component of yoghurt. A Thr aldolase has been described as the key enzyme of the Thr catabolic pathway in LAB (Christensen et al. 1999). This enzyme catalyzes the splitting of Thr into acetaldehyde and glycine. The Thr aldolase activity can be attributed in bacteria to two different enzymes: serine hydroxymethyltransferase (SHMT) and to a low-specificity Thr aldolase (LTA). SHMT-encoding genes are present in all LAB genomes, while genes encoding LTA have yet to be identified. Biochemical and genetic studies have shown that Thr aldolase in LAB is possibly due solely to SHMT enzyme (Chaves et al. 2002). Thr can also be deaminated to 2-oxobutanoate, the precursor compound for the biosynthesis of BCAAs. Deaminase-encoding genes have been found in L. lactis, streptococci, Lact. casei, Lact. sakei, Leuc. mesenteroides, and Ped. pentosaceus.

1.3.7. Biogenic Amines Pathways from Amino Acids

Catabolism of some amino acids can produce biogenic amines (BAs), which can cause food poisoning. BAs are mainly formed by decarboxylation of the amino acids tyrosine, histidine, lysine, and ornithine (Fig. 1.3), from which tyramine, histamine, cadaverine, and putrescine, respectively, are produced. Tyrosine, histidine, and ornithine decarboxylase activities have been reported in LAB species and strains of Lactobacillus, Enterococcus, Oenococcus, Tetragenococcus, and Carnobacterium. Genes encoding decarboxylase enzymes are found in clusters, which include genes encoding amino acid-BA antiporters, and in some cases (histamine and tyramine) aminoacyl tRNA synthetases. The similar organization of different clusters, their distribution, and their high similarity of sequence suggest a horizontal transference from a common source (review by Fernández and Zúñiga 2006). Lysine decarboxylase has never been identified in LAB.

Arg decarboxylase (ADC) converts Arg into agmatine. This activity has been reported only in Lactobacillus hilgardii X1B (Arena and Manca de Nadra 2001). Furthermore, several LAB can use agmatine via the agmatine deiminase pathway to produce putrescine. This pathway is constituted by three enzymes: agmatine deiminase, putrescine carbamoyltransferase, and carbamate kinase. The agmatine deiminase pathway was first described in Enterococcus faecalis (Simon and Stalon 1982) and genome analyses and recent results obtained by Lucas et al. (2007) revealed the presence of agmatine deiminase genes in different Lact. brevis, Lact. sakei, and Ped. pentosaceus strains.

1.3.8. Arginine Metabolism

Arginine pathways have been deeply studied in LAB species, although it is not involved in either flavor or BA formation. The most common pathway for the catabolism of arginine in LAB is via the arginine deiminase (ADI) pathway. The ADI
pathway comprises three reactions catalyzed by ADI, ornithine carbamoyl-transferase and carbamate kinase (Fernández and Zúñiga 2006). Degradation of Arg through the ADI pathway results in production of ATP and ammonia. Therefore, the ADI pathway is thought to provide energy and protection against an acidic external pH. Nevertheless, its relevance as an energy source or as a protective system against acidic environments varies among LAB. In general, arginine induces the expression of ADI enzymes and some carbohydrates repress their synthesis, being controlled by catabolite repression.

1.4. Citrate Metabolism and Formation of Aroma Compounds

In addition to sugars, several LAB species have the capability of metabolizing citrate, a process requiring citrate transport, conversion of citrate into oxaloacetate, and then into pyruvate and CO₂ (Fig. 1.4). Citrate fermentation by LAB leads to the production of 4-carbon compounds, such as diacetyl, acetoin and butanediol, which have aromatic properties and impart the typical aroma to many dairy products. Strains of L. lactis subsp. lactis biovar. diacetylactis (L. diacetylactis) and species of Leuconostoc and

![Figure 1.4. Citrate metabolism in Lactococcus and Leuconostoc species. Key for the enzymes: CL, citrate lyase; OAD, oxaloacetate decarboxylase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; ALS, α-acetolactate synthase; ADC, α-acetolactate decarboxylase; DAR, diacetyl acetoin reductase; BDH, 2,3-butanediol dehydrogenase; Tppi, thiamine pyrophosphate.](image-url)
Weissella are used by the fermentation industry as diacetyl producers. Other LAB species such as *Lact. plantarum* and *O. oeni* use the citrate present in raw materials to produce a secondary fermentation in wine, beer, and sausages, which confers off-flavors to the fermented products. Thus, either beneficial or detrimental, the use of citrate by LAB has a great industrial interest.

1.4.1. Citrate Transport

Transport of citrate is performed by specific membrane-associated permeases, and constitutes the limiting step for citrate utilization. Biochemical characterization of the permeases has shown that citrate can be incorporated by diverse mechanisms. Most LAB species internalize citrate by a 2-hydroxy-carboxylate type of transporters, which transports dicarboxylic and tricarboxylic acids (Sobczak and Lolkema 2005). Among the citrate transporters of this family, the well-characterized CitS and the CitP from *Lactococcus*, *Leuconostoc*, and *Weissella* are included. CitS is a symporter, which uses a Na<sup>2+</sup>-gradient to transport citrate, whereas CitP is responsible for the antiport of H-citrate<sup>2−</sup> and lactate<sup>1−</sup> generating a membrane potential. In LAB the genes encoding CitP are identified in plasmids and show a 99% nucleotide sequence identity, suggesting recent acquisition by horizontal transfer (Drider et al. 2004). Genes from different species are located in unrelated plasmids and present a different genetic organization (Sesma et al. 1990; Martín et al. 2000). In contrast, in *Ent. faecalis* and *Streptococcus mutans* citrate is transported by CitM and CitH transporters, respectively, belonging to the recently described family of citrate-metal symporters (CitMHS) (Korithoski et al. 2005; Blancato et al. 2006). Uptake of citrate by these transporters occurs in the form of complexes with cations (either Ca<sup>2+</sup>, Mn<sup>2+</sup>, or Fe<sup>3+</sup>). Additionally, the genes encoding CitM and CitH are located in the chromosome, associated to citrate fermentation clusters. Citrate transporters of other types may be found in *Lact. plantarum* (Kleerebezem et al. 2003) and *O. oeni* (Augagneur et al. 2007).

1.4.2. Conversion of Citrate into Pyruvate

Once inside, citrate is converted into acetate and oxaloacetate in a reaction catalyzed by the citrate lyase (CL) enzyme. CL is an enzymatic complex, which catalyzes the conversion of citrate in a multi-reaction process. CL has also been purified from *Leuc. mesenteroides* 195 strain (Bekal et al. 1998), and the CL-coding genes have been cloned from this strain (Bekal et al. 1998), from a plasmid belonging to a *Weissella paramesenteroides* strain (Martín et al. 1999, 2000), and from the chromosome of *L. diacetylactis* CRL264 (Martín et al. 2004).

The second step of citrate metabolism, catalyzed by oxaloacetate decarboxylase (OAD), is the decarboxylation of oxaloacetate, generating pyruvate and CO<sub>2</sub>. Analysis of the genomes of various LAB indicate that a homolog of the *Klebsiella pneumoniae* OAD, the best characterized bacterial enzyme of this type, is only present in *Strep. mutans*, *Ent. faecalis*, *Lact. sakei*, and *Lact. casei*. In most citrate fermenters, OAD seems to be a 40-kDa polypeptide sharing high amino acid identity with proteins of the malic enzyme family. OAD is encoded by either citM in *Weissella* (Martín et al. 2000), or mae in all other LAB (Bekal et al. 1998; Bolotin et al. 2001). In agreement, co-transcription of these genes with that encoding CL has been detected in *Leuconostoc*, *Weissella*, and *L. diacetylactis* (Bekal et al. 1998; Martín et al. 2000, 2004).

1.4.3. Conversion of Pyruvate into Aroma Compounds

Metabolism of pyruvate can yield in LAB different end-products such as lactate, formate, acetate, ethanol, and the aroma compounds of four carbons (C<sub>4</sub> compounds) diacetyl, acetoin, and butanediol (Neves et al. 2005; Fig. 1.4). The metabolic biosynthetic pathway from citrate to diacetyl was revealed in *L. diacetylactis* by use of nuclear magnetic resonance techniques. Ramos et al. (1994) demonstrated that the main route of diacetyl synthesis is via the intermediary α-acetolactate (Fig. 1.4). α-Acetolactate synthase (ALS) is the key enzyme in the synthesis of C<sub>4</sub> compounds by catalyzing the
condensation of two pyruvate molecules to generate α-acetolactate. Once synthesized, α-acetolactate is unstable and is readily decarboxylated to acetoin by α-acetolactate decarboxylase (ALD), or by nonenzymatic decarboxylation (in the presence of oxygen) to diacetyl. Acetoin can also be synthesized from diacetyl by diacetyl reductase (DAR). This enzyme also possesses acetoin reductase activity, yielding 2,3 butanediol from acetoin, while the reverse reaction is catalyzed by 2,3 butanediol dehydrogenase (BDH). The balance between the end-products of citrate fermentation will depend on the redox state of the cells (Bassit et al. 1993).

The genes encoding both ALS, als and ALD, aldB have been cloned and characterized from L. diacetylactis (Marugg et al. 1994; Goupil-Feuillerat et al. 1997). AldB plays a dual role, catalyzing the second step of the acetoin biosynthetic pathway, and regulating the pool of α-acetolactate in the cell during BCAA metabolism. Transcriptional analysis of the aldB expression revealed that this gene forms an operon with leu, ilv, and aldR genes. In fact, the aldB is located downstream of genes encoding enzymes involved in the biosynthesis of BCAAs (leucine, valine, and isoleucine) and precedes the aldR gene, which encodes a putative regulator whose function has not been completely elucidated (Goupil-Feuillerat et al. 1997). This second role is very important as BCAAs are essential for protein synthesis and, consequently, the expression of this enzyme is strictly regulated at both the transcriptional and post-transcriptional levels (Goupil-Feuillerat et al. 1997, 2000).

DAR catalyzes two stages of citrate metabolism: the conversion of diacetyl into acetoin and the reduction of acetoin to 2,3-butanediol. Conversion of 2,3-butanediol into acetoin is catalyzed by BDH. In L. diacetylactis, two proteins seem to possess DAR and BDH activities. The DAR enzyme of Leuconostoc pseudomesenteroides is encoded by the plasmidic butA gene (Rattray et al. 2000). Bioinformatic analysis of the butA gene from this species allowed identifying the butA gene in the L. diacetylactis IL1403 genome (García-Quintáns et al. 2008). Both enzymes share 81% of amino acid identity. Preceding the L. diacetylactis IL1403 butA is the butB gene, whose product was annotated as BDH in the lactococcal genome sequence. BDH belongs to the Zn-containing alcohol dehydrogenases. The genes butB and butA are also linked to the L. lactis subsp. cremoris (MG1363 and SK11) chromosome. However, in other LAB species, such as Leuc. pseudomesenteroides, butA is carried on plasmids (Rattray et al. 2003), which may facilitate its horizontal transfer. The two DARs purified from lactococci have less affinity for 2,3-butanediol than for diacetyl and acetoin. They both have optimal alkaline and acidic pH, respectively, for its BDH and DAR activities. Therefore, it is conceivable that at acidic pH an increase of 2,3-butanediol occurs; a fact that has experimentally been observed in L. diacetylactis (Ramos et al. 1994).

1.4.4. Regulation of Citrate Utilization

The biochemical machinery for sensing, transport, and utilization of citrate is grouped into operons, showing highly conserved components in all LAB species. A citMCDEFXG (cit) operon has been also identified in the L. diacetylactis chromosome. However, the citrate permease citP is not part of the operon; instead, it is encoded on the citrate transport operon, citQRP, located in the citrate plasmid (López de Felipe et al. 1995). Similarly, a cit operon (citMCDEFGRP) encoding CitM, CL, and CitP is found in W. paramesenteroides. Upstream of this operon is the divergently transcribed citI gene (Martín et al. 1999, 2000). Two unique and different models of regulation for citrate metabolism have been described in LAB. In L. diacetylactis, specific transcriptional activation of the promoters controlling cit operons takes place at low pH; this activation is thought to provide an adaptive response to acidic stress. In W. paramesenteroides, the CitI transcriptional regulator functions as a citrate-activated switch allowing the cell to optimize generation of metabolic energy.

1.4.5. Bioenergetics of Citrate Metabolism

During growth in milk, L. diacetylactis metabolizes lactose-producing lactic acid, which is exchanged
for citrate during excretion by the antiporter CitP. As a homofermentative organism, \textit{L. diacetylactis} converts glucose into lactate, producing 2 moles of ATP per mol of glucose metabolized. The NAD$^+$ consumed in the first steps of the pathway is regenerated during the transformation of pyruvate into lactate, thus maintaining the redox potential. In the presence of glucose and citrate, each mol of citrate produces one mol of pyruvate without generating NADH. This excess of pyruvate is diverted to the synthesis of \(\alpha\)-acetolactate and, subsequently, to the production of aroma compounds. The utilization of citrate results in a growth advantage for \textit{L. diacetylactis} when the medium has a low pH (García-Quintáns et al. 1998; Magni et al. 1999; Martín et al. 2004). Recent results have shown that contrary to what has been argued, the beneficial effect of citrate on growth under acid stress is not primarily due to a concomitant alkalinization of the medium (detoxification effect), but seems to stem from less expenditure of the ATP obtained from glucose to achieve pH homeostasis (Sánchez et al. 2008).

Glucose metabolism in heterofermentative \textit{Leuconostoc, Oenococcus,} and \textit{Lactobacillus} species takes place through the pentose phosphate pathway, a heterolactic fermentation characterized by lactate, ethanol, and acetate as end-products (Zaunmüller et al. 2006). In this case the main part of the acetylphosphate formed during the glucose degradation is reduced to ethanol by the phosphoketolase pathway, regenerating the NAD$^+$ consumed. However, the presence of citrate together with glucose provokes a shift to the heterofermentative route. When citrate is present, the NAD$^+$ is regenerated during the pyruvate reduction to lactate. The acetylphosphate is therefore converted to acetate by means of the acetate kinase, generating an extra mol of ATP (Sobczak and Lolkema 2005; Fig. 1.1C).

\section*{1.5. Functional Genomics and Metabolism}

The recently completed genome sequence of LAB species (Mayo et al. 2008) creates an excellent opportunity to examine how metabolic pathways function in a global perspective. As microbial genomes reflect the metabolism, physiology, biosynthetic capabilities, and adaptability of the organisms to varying conditions and environments, the availability of genome sequences has also expanded our knowledge of LAB’s metabolic potential and bioprocessing capabilities (Teusink and Smid 2006). As an example in the following paragraph, the respiration capacity of \textit{L. lactis} and its industrial-derived applications are briefly presented (Duwat et al. 2001; Gaudu et al. 2002).

Sugar fermentation was long considered to represent the sole means of energy metabolism available in LAB, producing organic acids (mainly lactic acid) as a final product. Early evidence by Sijpsteijn in 1970 for the respiratory capability of LAB was largely overlooked (as reported in Duwat et al. 2001), and only after analysis of the complete genome sequence of \textit{L. lactis} IL1403 (Bolotin et al. 2001) a respiratory capacity for this species in the presence of exogenous heme was first envisioned (Duwat et al. 2001). Subsequent research confirmed that \textit{L. lactis} certainly has the ability to respire in the presence of oxygen (Duwat et al. 2001; Gaudu et al. 2002), provided the growth medium contains heme because this bacterium does not have a functional biosynthetic pathway for this compound (Bolotin et al. 2001). Under respiratory conditions, acetate, acetoin, and diacetyl are produced from pyruvate at the expense of lactic acid (Duwat et al. 2001). An important consequence of respiration is a more efficient conversion of the carbon source into biomass, resulting in higher cell yields and increased survival after growth. The initial results obtained with the well-characterized laboratory strain \textit{L. lactis} IL1403 were optimized for different industrial \textit{L. lactis} strains and also for \textit{Leuconostoc} species (Pedersen et al. 2005). These results are of industrial significance and allowed the development of a patented process for the production of LAB starter cultures (Duwat et al. 1998). \textit{L. lactis} strains were also assayed in aeration in the absence of added heme to distinguish simply aeration from true respiration (Pedersen et al. 2005). Numerous genes were differentially expressed under these two conditions. Approximately half of these genes have unknown
function, indicating that more research is still needed to fully understand the physiology of respiration in this species. However, increased biomass after aerated incubation in the presence of heme was not obtained for *Strep. thermophilus*, *Lact. bulgaricus*, and *Lact. helveticus*. Analysis of the complete genome sequences of all these species (Bolotin et al. 2004; van de Guchte et al. 2006; Callanan et al. 2008) does not reveal the presence of genes for cytochrome oxidase or for the biosynthesis of quinones, features which are believed to be essential for respiration (Gaudu et al. 2002).

Starter cultures obtained by the respiration technology have been assayed in pilot scale tests for Cheddar cheese production and compared with starters produced under standard conditions (Pedersen et al. 2005). Manufacture parameters were all within the normal range. Indeed, sensory differences were not perceived by trained panels after maturation. Industrial scale trials of Cheddar, Feta, and Cottage cheese have already been done, and again no significant differences were seen in the manufacturing parameters, cheese microbiology, chemistry, texture, or flavor development (Pedersen et al. 2005).

1.6. Metabolic Engineering of LAB Metabolism

Cryptic plasmids from many LAB species provided the platform for the development of sophisticated genetic tools (e.g., vectors for cloning [including food-grade vectors], expression, and integration; Mills et al. 2006). Of note is the nisin-controlled gene expression system, which has been in use for more than 10 years (Mierau and Kleerebezem 2005). These and other genetic tools allowed a first engineering and rerouting of LAB metabolism (de Vos and Hugenholtz 2004; Hugenholtz 2008). Among the many examples that can be mentioned with regard to illustrating the high potential of engineering the metabolism of LAB species, the conversion of *L. lactis* into a homoalanine producer as the only fermentation end-product was reported as early as in 1999 (Hols et al. 1999). The conversion of this species into a high diacetyl producer by combining NADH-oxidase overproduction and α-ALD inactivation has also been achieved (Hugenholtz et al. 2000). The other important aroma compound in dairy products is acetaldehyde. It is produced by both *Lact. delbrueckii* and *Strep. thermophilus* in yoghurt fermentation; however, it is hardly produced as a fermentation end-product in *L. lactis*. In lactococcal cells, acetaldehyde can be converted from acetyl coenzyme A by the action of aldehyde dehydrogenase (ADH; Fig. 1.1B). Recently, the efficient rerouting of pyruvate toward acetaldehyde in *L. lactis* has been achieved by functional expression of a pyruvate decarboxylase gene from *Zymomonas mobilis* and the endogenous NOX gene (Bongers et al. 2005).

The controlled overexpression of key genes in the folate biosynthesis cluster has been used to overproduce this vitamin in *L. lactis* cells (Sybesma et al. 2003). Cloning and expression of the whole folate operon in *Lact. gasseri* converted this folate-consumer species into a folate producer (Wegkamp et al. 2004). Furthermore, *L. lactis* has also been used for the production of the nutraceutical compound mannitol (Wisselink et al. 2004). More recently, by reverting the sorbitol catabolic pathway in a *Lact. plantarum*, mutant deficient in both L- and D-lactate dehydrogenase activities has been forced to become a high-level sorbitol producer from fructose-6-phosphate (Ladero et al. 2007).

Overexpression of genes encoding components of the proteolytic system has also been accomplished in several LAB species under cheese conditions (Christensen et al. 1995; Tuler et al. 2002). Cheeses produced with the recombinant overexpressing-strains have frequently received higher scores than those made with the original strains. However, it has been repeatedly reported that amino acid degradation is the actual process controlling flavor formation in cheese from proteins (for recent reviews see Smit et al. 2005; Fernández and Zúñiga 2006). The availability of whole genomes of many LAB species would allow the building of complete metabolic structural models. Data derived from genome sequence information could also be used as tools to direct metabolic engineering strategies to
improve LAB as cell factories (Oliveira et al. 2005; Smid et al. 2005).

1.7. Conclusions
Knowledge of the metabolism of industrially important LAB species has been gathered during the last 25 years. In particular, the metabolism of carbohydrates, citrate, and amino acids, and the key components produced during their degradation are all known. Rudimentary genetic tools have already been used to engineer and reroute some metabolic pathways involved in the synthesis of the desirable compounds that define and are sought for in different fermented products. At present the new techniques and disciplines emerging in the post-genomic era, such as genomics, proteomics, metabolomics, and system biology, open new avenues for interpretation of biological data, making possible the development of new predictive models. In combination with classical and molecular techniques, these new methods will be invaluable in the rational optimization of LAB metabolism in order to obtain more tasteful, aromatic, and safer traditional and new fermented products. This knowledge will ultimately allow a more rational use of LAB species not only in traditional fermentations but also in novel bioprocessing, probiotic, and biotechnological applications.

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