Chapter 1

Updates on Metabolism in Lactic Acid Bacteria in Light of “Omic” Technologies

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1.1. Sugar Metabolism

Sugars are the primary carbon and energy source for LAB that are grown for fermented food and feed production as well as in laboratory media. Many different transport systems are involved in LAB carbohydrate uptake, including phosphotransferase systems (PTS), ATP-binding cassettes (ABC), and glycoside–pentoside–hexuronide transporters. In *Lactococcus lactis*, glucose is imported by either a mannose or cellobiose PTS or one or more non-PTS permease(s) (Castro et al. 2009). LAB prefer glucose but can also metabolize several common hexoses; however, the ability to ferment other sugars is strain dependent. Dairy LAB can use milk’s most abundant sugar, lactose, as a carbon source, whereas plant-associated bacteria utilize a large variety of other carbohydrates, including β-glucosides (Aleksandrzak-Piekarczyk 2013). Furthermore, phenotypic and genotypic analyses of twenty *L. lactis* subsp. *lactis* and *cremoris* genotypes showed strain-to-strain variations (Fernandez et al. 2011). These two groups had distinctive carbohydrate fermentation and enzyme activity profiles with *cremoris* genotypes exhibiting broader profiles.

Monosaccharides incorporated by the cell or liberated in the cytoplasm by disaccharide hydrolysis enter glycolysis at the glucose-6P (G6P) level or are processed by the Leloir pathway (Figure 1.1). In *L. lactis*, lactose that is transported by the PTS system is hydrolyzed to galactose-6P, transformed by the tagatose pathway (Tag6P), and then enters glycolysis at the triose phosphate level. In some LAB, only the glucose moiety of lactose is fermented, while the galactose moiety is excreted; this leads to galactose accumulation in the medium, which yields poor-quality dairy products (Neves et al. 2010; Aleksandrzak-Piekarczyk, 2013). Galactose can be imported by the non-PTS permease GalP and metabolized via the Leloir pathway (*galMKTE*). Alternatively, galactose can be imported by PTS<sub>Lac</sub> (*lacFE*) and further metabolized to triose phosphates by the Tag6P pathway (*lacABCD*). Recently, an alternative uptake route was discovered that consists of galactose translocation via the

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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 decarboxylase (ALD); 20, 2,3-butanediol dehydrogenase (BDH); 21, diacetyl reductase (DR); 22, glucose-6-P dehydrogenase (G6PDH); 23, 6-P-gluconate 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 (UDP); 29, phosphoglucomutase (PGM).
galactose PTS, followed by Gal6P dephosphorylation to galactose, which is further metabolized via the Leloir pathway (Neves et al. 2010). This knowledge has been used to genetically engineer strains that enhance galactose consumption rates by up to 50% (Neves et al. 2010).

Sugar fermentation leads to the formation of lactic acid alone or in combination with other organic acids and ethanol. Variations in the metabolic products of LAB have yielded three categories of fermentation: homofermentation, mixed-acid metabolism, and heterofermentation (Figure 1.1). These three types of fermentation and their regulatory mechanisms have been comprehensively reviewed in the first edition of this book (Mayo et al. 2010). Currently, the control and regulation of glycolytic flux in LAB are not fully understood (Martinussen et al. 2013). The control of glycolytic flux in *L. lactis* is not due to the actions of a single enzyme, sugar transport, or ATP-dependent mechanism. However, a combination of these mechanisms cannot be ruled out as a possible explanation (Martinussen et al. 2013).

In recent years, studies have shifted from digestible disaccharides to indigestible higher oligosaccharides as interest in intestinal microbial ecology and the commercial use of prebiotic oligosaccharides has emerged. Mono- and disaccharide metabolism is well understood; however, few data are available on the metabolism of higher oligosaccharides, which are abundant in cereals, milk, fruits, and the upper intestinal tract of animals. The metabolism of four major oligosaccharide groups have been examined in detail: (i) starch, maltodextrins, and isomalto-oligosaccharides (IMO); (ii) fructo-oligosaccharides (FOS); (iii) β-galacto-oligosaccharides (βGOS); and (iv) raffinose-family oligosaccharides and α-galacto-oligosaccharides (ROF and αGOS, respectively) (Gänzle and Follador 2012).

In addition to metabolizing sugar, LAB are able to direct sugar towards exopolysaccharide (EPS) biosynthesis. These long-chain saccharides are loosely attached to the cell surface to form capsule-like structures or are secreted into the environment. EPS production by lactobacilli has been previously discussed in a comprehensive review (Badel et al. 2011). Several studies of biopolymer diversity in LAB from cereal (Bounaix et al. 2009, 2010; Palomba et al. 2012) and in intestinal LAB (Salazar et al. 2009; Gór ska-Frączek et al. 2011, 2013; Sims et al. 2011) have also been published since the first edition of this book.

EPSs come in many different structures, sizes, and sugar compositions and are classified into two groups: homopolysaccharides (HoPS), which consist of one type of monosaccharide (α-D-glucan, β-D-glucan, fructan, or a polygalactan); and heteropolysaccharides (HePS), which consist of different types of monosaccharides (D-glucose, D-galactose, L-rhamnose, and their derivatives). HoPS are synthesized extracellularly by highly specific glycosyltransferase enzymes as well as glucan- or fructan-sucrases. HoPS synthesis specifically requires sucrose as a substrate and the energy generated by its hydrolysis. The crystal structures, reaction and product specificities of glucansucrases as well as structural analyses of α-glucan polymers have been recently reviewed (Leemhuis et al. 2013).

HePS are synthesized from glucose, galactose, or other monosaccharides by the combined actions of several types of glycosyltransferases. HePS biosynthesis involves four major consecutive steps: (i) sugar transport into the cytoplasm, (ii) sugar-1P synthesis, (iii) polymerization of repeating unit precursors, and (iv) EPS export outside the cell. The synthesis of two EPSs in *Lactobacillus johnsonii* FI9785 is dependent on the 14-kb eps gene cluster; however, the precise regulation of EPS biosynthesis has yet to be identified (Dertli et al. 2013). It is thought that EPS production can be regulated at each of these four steps. Genome sequencing of EPS-related genes and their organization (Koryszewska-Baginska et al. 2014) may provide additional insight into whether this is the case.

### 1.1.1. Practical Aspects of Sugar Catabolism

The ability of LAB to ferment sugars has been widely utilized in various foods’ production. However, LAB also have the potential for increasing the production value of biofuels and biochemical products due to their robustness and tolerance for ethanol, low pH, and high temperatures (Martinussen et al. 2013). Focus has increased on optimizing lactate production from natural substrates such as starchy
or lignocellulosic materials from agricultural, agro-industrial, and forestry sources due to their abundance, low price, high polysaccharide content, and renewability (Okano et al. 2009; Abdel-Rahman et al. 2011; Castillo Martinez et al. 2013). In addition, EPS from LAB can play an important role in the food industry as an emulsifier, thickener, viscosifier, and stabilizer. EPS has been used in the rheology and texture of fermented milks (Ramchandran and Shah 2009) and other fermented products, such as sourdough (Katina et al. 2009; Galle et al. 2010) and cereal-based beverages (Zannini et al. 2013). It may also improve the quality, safety, and acceptability of gluten-free bread (Moroni et al. 2009) and replace hydrocolloids in sorghum sourdough (Galle et al. 2011). EPSs from LAB are also of great interest to agro-food industries since their vast structural diversity may lead to innovative applications. However, the majority of LAB only produce low levels of polysaccharides; therefore, optimized methodologies for increased EPS production and recovery are still required (Notararigo et al. 2013).

EPS from LAB also has beneficial physiological properties for humans (Patel et al. 2011). Cell-bound EPSs from Lactobacillus acidophilus 606 (Kim et al. 2010) and Lactobacillus plantarum 70810 (Wang et al. 2014) have been shown to have antitumor properties. EPSs have also been shown to exhibit immunomodulatory activity for macrophages (Liu et al. 2011; Ciszek-Lenda et al. 2011) as well as intestinal epithelial cells (Patten et al. 2014). In addition, EPSs produced by Lactobacillus reuteri can inhibit enterotoxigenic Escherichia coli-induced hemagglutination of porcine erythrocytes, which further indicates that EPS has therapeutic potential (Wang et al. 2010). EPS from Lact. plantarum 70810 has a metal binding capacity and could be used as a potential biosorbent for lead removal from the environment (Feng et al. 2012). LAB can also produce a variety of functional oligosaccharides that can be used as prebiotics (Pepe et al. 2013), nutraceuticals, sweeteners, humectants, drugs against colon cancer, and immune stimulators (Patel et al. 2011). Some probiotic LAB can also utilize prebiotic compounds, including non-digestible FOSs, inulin-type fructans, or β-glucans (Russo et al. 2012), which stimulate the growth of beneficial commensals in the gastrointestinal tract.

1.2. Citrate Metabolism and Formation of Aroma Compounds

In addition to sugars, several LAB species can metabolize citrate. Citrate fermentation in LAB leads to the production of volatile compounds. In fermented dairy products, these compounds are C₄ compounds, such as diacetyl, acetoin, and butanediol, which are responsible for the typical aroma of many fermented dairy products. Therefore, citrate metabolizing LAB, such as L. lactis subsp. lactis biovar. diacetylactis (L. diacetylactis) and some Leuconostoc and Weissella species, are currently used as starter and adjunct cultures for the production of these C₄ compounds. However, in other fermented products, such as wine, beer, and sausages, the volatile compounds produced from the fermentation of citrate by LAB are considered off-flavors, and their presence should be avoided. Citrate utilization by LAB has been previously described in detail (Quintans et al. 2008) and summarized in the first edition of this book (Mayo et al. 2010). Therefore, in this chapter, we are presenting only citrate metabolism in LAB in the context of recent achievements.

1.2.1. Citrate Transport

Citrate transport is a limiting step for citrate utilization and is performed by a variety of membrane-associated permeases. In contrast, volatile compounds formed in the cytoplasm are secreted without requiring specific transporters. Most LAB species internalize citrate using a 2-hydroxy-carboxylate (2-HCT) transporter, which can transport dicarboxylic and tricarboxylic acids. The 2-HCT family of transporters includes CitP from Lactococcus, Leuconostoc, and Weissella (Pudlik and Lolkema 2010). CitP is an antiport transporter that exchanges H-citrate⁻ and lactate¹⁻ to generate a membrane potential (Figure 1.2). In L. diacetylactis, CitP is encoded by the citQRP operon located on the “citrate
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plasmid” (Drider et al. 2004; Kelly et al. 2010). In *L. diacetylactis*, transcription of the promoters that control *cit* operons are specifically activated by low pH environments as an adaptive response to acid stress. This has been confirmed by transcriptomics analysis of *L. diacetylactis* in milk (Raynaud et al. 2005) and cheese (Cretenet et al. 2010). In *Weissella paramesenteroides* and *Leuconostoc mesenteroides* subsp. *cremoris*, the *citP* genes (*citMCDEFGRP*) are located in a plasmidic or chromosomal *citI* cluster, respectively (Martín et al. 1999, 2000; Bekal et al. 1998). Enlarged citrate plasmids (15 to 23 kbp) have also been observed in wild (non-starter) strains of *L. diacetylactis* (Drici et al. 2010; Kelly et al. 2010).

In *Enterococcus faecalis* and *Lactobacillus casei*, citrate is transported by CitM and CitH transporters, respectively, both belonging to the family of citrate-metal symporters (CitMHS) (for a review see Lensbouer and Doyle 2010). CitMHS transporters transport citrate in cation (Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, or Fe\textsuperscript{3+}) complexes. Recently, CitH in *Lact. casei* has been shown to be a proton symporter that transports a Ca\textsuperscript{2+}-citrate complex and uses Ca\textsuperscript{2+} as a substrate (Mortera et al. 2013). Other types of citrate

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**Figure 1.2.** Citrate metabolism in *Lactococcus*, *Leuconostoc*, and *Weissella* 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.
transporters have also been identified in *Lact. plantarum*, *Oenococcus oeni* (for a review see Mayo et al. 2010), and the atypical citrate-fermenting wild *L. diacetylactis* strain (Passerini et al. 2013b).

### 1.2.2. Conversion of Citrate into Pyruvate and Production of Aroma Compounds

Once inside the cell, citrate is converted into acetate and oxaloacetate in a reaction catalyzed by the citrate lyase (CL) enzyme complex (Figure 1.2). Next, oxaloacetate is decarboxylated by oxaloacetate decarboxylase (OAD), which generates pyruvate and CO₂ (Figure 1.2). Analysis of various LAB genomes have identified genes encoding the α-, β-, and δ-subunits of OAD (Makarova et al. 2006). However, the physiological role of OAD remains poorly understood in LAB species; only enzymes from *E. faecalis* (Repizo et al. 2013) and *Lact. casei* (Mortera et al. 2013) have been recently investigated. In other LAB species, including *L. diacetylactis*, *W. paramesenteroides*, *Leuc. mesenteroides*, *Lact. plantarum*, and *O. oeni*, oxaloacetate is decarboxylated by the soluble and cytoplasmic malic enzyme (ME) (Sender et al. 2004). Surprisingly, the *cit* locus of *E. faecalis* has been recently shown to contain genes that encode both OAD and ME (Espariz et al. 2011).

Pyruvate metabolism in LAB can yield different end products, including lactate, formate, acetate, and ethanol as well as the important aroma compounds diacetyl, acetoin, and butanediol (Neves et al. 2005).

### 1.2.3. Conversion of Citrate into Succinate

Some LAB species cannot truly convert citrate into pyruvate. Instead, the CitT transporter generates succinate via malate and fumarate. Furthermore, the complete tricarboxylic acids (TCA) pathway has recently been identified in the *Lact. casei* genome using *in silico* analysis (Díaz-Muñiz et al. 2006). In this LAB species, the dominant end-products of citrate metabolism were acetic acid and L-lactic acid at both excess and limiting amounts of carbohydrates. Trace amounts of D-lactic acid, acetoin, formic acid, ethanol, and diacetyl confirm OAD activity; however, succinic acid, malic acid, and butanediol were not observed (Díaz-Muñiz et al. 2006; Mortera et al. 2013).

### 1.2.4. Bioenergetics of Citrate Metabolism

The co-metabolism of glucose and citrate produces different physiological effects in homofermentative and heterofermentative LAB. In homofermentative LAB, citrate utilization has a protective effect against acid stress. In heterofermentative LAB, “citrolactic” fermentation generates one extra mol of ATP per mol of citrate. In milk, *L. diacetylactis* metabolizes lactose and produces lactic acid, which is exchanged by the antiporter CitP for citrate during excretion. *L. diacetylactis* is homofermentative and converts glucose into lactate, generating two moles of ATP per mol of glucose. The NAD⁺ consumed in the first steps of this pathway is regenerated during the transformation of pyruvate into lactate, thereby 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 α-acetolactate synthesis and the subsequent production of aroma compounds. Similar to *L. diacetylactis*, the higher biomass of *Lact. casei* during Ca²⁺-citrate and carbohydrate co-metabolism has been attributed to counteracting the growth inhibition of carbohydrate metabolism that is induced by acidification in its final stages (Mortera et al. 2013).

### 1.3. The Proteolytic System of Lactic Acid Bacteria

*L. lactis* strains isolated from dairy products are characterized by a high number of amino acid auxotrophies and the ability to utilize milk proteins as an amino acid source. These properties are common to dairy strains even from distant geographic locations in Asia, Europe, North America, and
New Zealand (Rademaker et al. 2007; Kelly et al. 2010). The use of environmental proteins as an amino acid source in LAB explains the importance of their proteolytic system. LAB depend on this system to obtain the essential amino acids that are then used as precursors for not only peptides and proteins but also for many other biomolecules. Amino acids are the precursors of aromatic compounds, which are important to the final flavor of food products. Proteolytic activity also generates other molecules, such as bioactive peptides, that have functions related to the probiotic properties of LAB.

The proteolytic system in LAB can be divided into several steps: protein degradation, peptide transport, peptide degradation, and amino acid catabolism (Figure 1.3).

1.3.1. Protein Degradation

Studies of protein degradation in LAB initially focused on casein degradation using *L. lactis* as a model organism. Casein hydrolysis in LAB is initiated by a cell-envelope proteinase (CEP), which degrades proteins into oligopeptides, and gene deletion studies have shown that LAB are unable to grow in milk in the absence of a functional CEP. However, since CEP is extracellular, peptides produced by CEP can also be consumed by protease-negative variants, allowing them to survive in culture (Bachmann et al. 2012).

Lactocepins are a diverse group of CEPs that belong to the subtilisin protein family of serine proteases. CEPs are anchored to the cell wall via sortase A (SrtA) (Dandoy et al. 2011). LAB lactocepins are encoded by *prtP, prtB, prtS*, and/or *prtH*, which differ in their number of functional domains. CEP distribution varies widely among strains. Overall, the most abundant paralog among LAB is *prtH3*, which is carried by over 80% of strains tested, followed by paralogs of *prtH* and *prtH4* (Broadbent et al. 2011). Most LAB possess only one CEP. However, four CEP genes (*prtH,*
prtH2, prtH3, and prtH4) have been identified in Lactobacillus helveticus CNRZ32 (Jensen et al. 2009). The presence of several protease genes in Lact. helveticus could explain its high proteolytic efficiency. Only prtH2 is common to all characterized Lact. helveticus strains (Genay et al. 2009). However, analysis of the protease in BGRA43 showed that the only active gene was prtH. CEP activation requires the maturation protease PrtM. Two PrtMs (PrtM1 and PrtM2) have been identified in Lact. helveticus CNRZ32 (Savijoki et al. 2006). Additional studies (Genay et al. 2009; Broadbent et al. 2011) have reported that PrtM1 is required for PrtH activation, while PrtM2 plays a role in the activation of other CEP paralogs.

As mentioned above, CEP activity was initially evaluated using casein as a substrate. However, LAB strains isolated from non-milk or non-fermentation environments have also exhibited casein hydrolysis. For instance, Lact. helveticus BGRA43, which is isolated from human feces, has strong proteolytic activity and is able to completely hydrolyze αs1-, β-, and κ-caseins (Strahinic et al. 2013). In addition, the lactocepin secreted by Lactobacillus paracasei VSL#3 can selectively degrade cell-associated and tissue-distributed IP-10 and other proinflammatory chemokines in vitro (von Schillde et al. 2012). These findings indicate that lactocepin, which is encoded by prtP, is highly selective despite targeting a broad spectrum of cleavage sites. Therefore, additional protein characteristics, such as surface charge and/or three-dimensional structure, determine whether a protein is cleaved or not. The selective degradation of pro-inflammatory chemokines could be also related to differences in the regulation of prtP expression.

In Streptococcus thermophilus, the cell wall associated proteinase PrtS is highly conserved (95% identity) with the PrtS of Streptococcus suis. Although recent studies have suggested that PrtS contributes to the virulence of Strep. suis (Bonifait el al. 2010), the primary role of PrtS in Strep. thermophilus strains is cleaving casein to oligopeptides. This function is clearly related to the adaptation of Strep. thermophilus to dairy environments; analyses of prtS distribution in Strep. thermophilus found that it occurs infrequently in historical specimens but frequently in more recent industrial ones. Furthermore, this “ecological” island confers an important metabolic trait for milk adaptation and appears to be laterally transferred in Strep. thermophilus. Together, these data suggest that Strep. thermophilus evolved via gene acquisition and selection as the result of the environmental pressures of food production (Delorme et al. 2010).

The second stage of protein degradation is the transport of di-, tri-, and oligo-peptides into the cell. Three oligo-, di-, and tri-peptide transport systems (Opp, Dpp and DtpT, respectively) have been described in LAB. Lact. acidophilus, Lactobacillus brevis, Lact. casei, Lactobacillus rhamnosus, and L. lactis, possess all three of these peptide transport systems. Some Lact. helveticus strains, such as DPC4571, also have three peptide transport systems, while others, such as H10, only have two (Opp and DtpT). These results indicate that the proteolytic systems differ between different strains of even the same species. Finally, Lact. reuteri only has one functional peptide transport system (DtpT) (Liu et al. 2010).

### 1.3.2. Peptidases

Once casein-derived peptides are taken up by LAB cells, they are degraded via the concerted action of peptidases with differing and partially overlapping specificities. Peptidases are a very important part of the proteolytic system in LAB and are involved in peptide hydrolysis and the release of essential amino acids. Peptidases can be divided into two main groups: endopeptidases and exopeptidases. Endopeptidases hydrolyze the internal peptide bonds of oligopeptides to generate peptides that can be further degraded by exopeptidases. Exopeptidases act on the terminal ends of oligopeptides to generate smaller peptides or amino acids.

The main LAB endopeptidases that have been characterized are PepO, PepF, PepG, and PepE, all of which act on NH2-Xn↓Xn-COOH substrates. PepO is encoded by three paralogous genes (pepO,
pepO2, and pepO3). In Lact. helveticus strains, these genes are identical; however, strain heterogeneity may be observed due to the loss of gene function or sequence polymorphisms, which may affect the specificity or relative activity of the individual peptidase enzymes (Broadbent et al. 2011).

Three paralogous genes also encode PepF (pepF, pepF1, and pepF2). In L. lactis, pepF1 is located on the chromosome, while pepF2 is located on a plasmid. The location of pepF2 explains the variability in its absence or presence among different Lactococcus strains (Liu et al. 2010).

The PepE and PepG proteins are absent in lactococci and streptococci. In Lactobacillus, two paralogous genes have been described for PepE (pepE and pepE2). In Lact. helveticus, these genes are almost universally conserved among strains from different origins, including cheese, whey, whiskey malt, and commercial cultures. PepE also plays an important role in the debitterness function of Lact. helveticus (Broadbent et al. 2011).

Exopeptidases are traditionally classified by their specificity, and four exopeptidase groups have been identified in LAB: aminopeptidases, dipeptidases, tripeptidases, and proline-specific peptidases. Aminopeptidases hydrolyze a single amino acid from the N-terminal oligopeptide NH2–X|Xn–COOH. They can be divided into either general or specific aminopeptidases. General aminopeptidases (PepN and PepC) are present in all genomes, usually as one gene per genome. Some LAB genomes have two homologs that potentially have the same function; for example, there are two PepC homologs in Lact. johnsonii (Liu et al. 2010). Specific aminopeptidases are categorized according to the type of residue they hydrolyze. PepS, which has only been described in Strep. thermophilus, is specific for aromatic residues. PepA is specific for Glu and Asp residues; it is found in streptococci and some Lactobacillus and Lactococcus strains but is absent in Pediococcus and Oenococcus. PepM is specific for methionine residues and is present in Lactobacillus, Lactococcus, Streptococcus, Pediococcus, and Oenococcus strains (Liu et al. 2010).

Dipeptidases are exopeptidases specific for NH2–X|X–COOH dipeptides. The PepD dipeptidase family has a broad specificity for various dipeptides. The pepD genes are distributed heterogeneously in LAB genomes and can vary in number from 0 to 6 paralogs. PepV is also encoded by multiple paralogous genes. It is present in all LAB and also has a broad specificity.

Tripeptidases release the amino acid from the N-terminal position of NH2–X|X–X–COOH tripeptides. They have a broad specificity but preferentially target hydrophobic peptides and do not hydrolyze proline residues. The only tripeptidase currently identified in LAB is PepT. The pepT gene is present in all LAB genomes and can occur as two paralogous genes in some LAB (Lact. acidophilus, Lact. johnsonii, and Lactobacillus gasseri).

Proline-specific peptidases can hydrolyze proline residues from the N-terminal position of peptides. Proline iminopeptidase (PepI) has aminopeptidase activity towards N-terminal proline peptides and prefers tri-peptides (NH2–Pro|Xn–COOH). The prolinase PepR has a broad specificity for dipeptides (NH2–Pro|X–COOH). These proline-specific peptidases are absent from all L. lactis strains. The activity of cell extract from Lact. helveticus and Lact. rhamnosus for several proline dipeptides was significantly reduced in PepR-deletion mutant. Those observations suggest that PepI and PepR may contribute to the specific proteolytic capacity for breaking down peptides containing proline in Lactobacillus strains (Liu et al. 2010).

PepP is a proline peptidase that can cleave any N-terminal amino acid linked to proline in an oligopeptide (NH2–X|Pro–Xn–COOH). PepQ is also a proline peptidase but is specific for proline dipeptides (NH2–X|Pro–COOH). One pepP gene is found in each LAB genome, except in Lactobacillus sakei and Pediococcus pentosaceus. The absence of pepP in both these genomes is likely due to gene loss. PepQ genes are equally distributed in all LAB genomes as one copy per genome. However, the Lactobacillus delbrueckii subsp. bulgaricus (Lact. bulgaricus) strains have two pepQ paralogs; one paralog is clustered with other orthologs, while the second is located in a separate cluster. This may be the result of an ancient duplication or horizontal gene transfer (HGT) event. PepX is a proline-specific endopeptidase (NH2–X–Pro|Xn–COOH) that is present in all LAB
genomes as one gene per genome. However, some LAB genomes have two peptidase homologs, and these are normally the genomes of strains from dairy environments. PepX2 is a PepX homolog of *L. lactis* subsp. *lactis* IL1403. This putative pepX2 gene was originally annotated as a hypothetical protein named ymgC. The only members of the PepX2 (YmgC) group are from *L. lactis* subsp. *lactis* IL1403 and *Pediococcus* (Liu et al. 2010).

### 1.3.3. Technological Applications of the Proteolytic System

The proteolytic system provides LAB with the amino acids they require to grow. For instance, *Lact. bulgaricus* and *Lact. helveticus* have a very extensive set of proteolytic enzymes, which is consistent with previous findings that *Lact. bulgaricus*, rather than *Strep. thermophilus*, serves as the proteolytic organism in yogurt. *Lact. helveticus* is a proteolytic adjunct culture of cheese that is used to degrade bitter peptides. Interestingly, *Lact. bulgaricus* encodes the Dpp system, which prefers the uptake of hydrophobic di/tripeptides. This complements the presence of *Strep. thermophilus*, which encodes the general di/tripeptide transporter DtpT and suggests that more peptides can be utilized by both bacteria when they are grown together. LAB from plants, such as *Lact. plantarum*, *O. oeni*, and *Leuc. mesenteroides*, encode fewer proteolytic enzymes, which is consistent with their fiber-rich ecological niche that contains less proteins. Milk proteins have also been used as raw material for obtaining bioactive peptides. Various peptides with immunostimulating, antimicrobial, opioid, anticancer, mineral binding, and antihypertensive properties have been isolated as proteolytic products in LAB (Griffiths and Tellez, 2013).

### 1.3.4. Amino Acid Catabolism

The catabolism of amino acids has quality (flavor compound formation) and safety (biogenic amine synthesis) implications for fermented foods. Amino acid catabolism is also thought to have an important role in the abilities of LAB to obtain energy in nutrient-limited conditions. Furthermore, amino acid catabolism has also been identified as a mechanism of pH control.

Amino acid degradation is important for the synthesis of volatile compounds and the transamination of some amino acids: methionine, branched-chain, and aromatic amino acids. Transamination is the main degradation pathway that leads to the formation of α-keto acids, which are then degraded into various aromatic compounds. The conversion of amino acids to keto- and hydroxyl acids is initiated by lactobacilli, and *Lactococcus* strains further convert these products to carboxylic acid. This cooperation between LAB and non-starter LAB can enhance cheese flavor.

Leucine, valine, and isoleucine catabolism can be divided into two parts. The first comprises the main degradation pathway; an aminotransferase reaction converts the amino acid to α-keto acid or, in the case of leucine, α-keto isocaproate (KICA). The resulting compounds are then converted to aldehyde, alcohol, or carboxylic acid via three different pathways: α-keto acid decarboxylation, oxidative decarboxylation, or an alternative dehydrogenation route that generates α-hydroxy-isocaproate (HICA) (Smits et al. 2005). Aromatic amino acids are also degraded by the amino transferase AraT. Transamination generates indole pyruvate, phenyl pyruvate, and p-hydroxy-phenyl pyruvate from tryptophan, phenylalanine, and tyrosine, respectively. Alpha-ketoacids generated by aromatic amino acid transamination are further degraded to various compounds by enzymatic (dehydrogenation, decarboxylation, or oxidative decarboxylation) or chemical reactions.

Sulfur compounds are generated by sulfur amino acid catabolism and are potent odorants that contribute flavor to many fermented foods. Methionine catabolism produces various volatile sulfur compounds (VSCs) such as H₂S, methanethiol, dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) (Fernández et al. 2000). The enzymes in LAB strains from raw goats’ milk cheeses crucial for VSC formation from L-methionine have very diverse enzyme capabilities.
Lactococci had higher C-S lyase and aminotransferase activities specific for sulfur-containing compounds than Lactobacillus and Leuconostoc (Hanniffy et al. 2009).

Amino acid catabolism also generates biogenic amines (BA). BA are basic nitrogenous organic compounds that are primarily formed by amino acid decarboxylation. BA are present in a wide range of foods, including dairy products, and can accumulate to elevated concentrations. Consuming foods with high amounts of these amines has toxicological effects. Tyrosine, histidine, lysine, ornithine, and arginine can all be decarboxylated into tyramine, histamine, cadaverine, putrescine, and agmatine, respectively. Agmatine can be further deaminated to produce putrescine. Amino acid decarboxylation pathways involve the transport of the amino acid into the cell, decarboxylation, and transport of the resulting BA out of the cell. The amino acid/BA interchange is performed by a transporter protein, and the tyramine, histamine, and putrescine biosynthesis pathways have been characterized in LAB. The genes encoding the decarboxylase and transporter proteins are located on the chromosomes for most LAB strains. Although BA production in LAB has been proposed as strain specific, genomic analysis of *E. faecalis*, *Enterococcus durans*, and *Enterococcus faecium* have shown that tyramine production is species specific (Ladero et al. 2012). Tyramine biosynthesis requires tyrosine, the decarboxylase (TDC), and a transporter protein (TyrP). The genes that encode TDC and TyrP form a chromosomal cluster, and a third open reading frame that encodes a protein homologous to tyrosyl tRNA synthase is always present in this cluster, upstream of the *tdc* gene (Linares et al. 2012). In cheese, Lactobacillus and Enterococcus genera are the main producers of tyramine.

In dairy products, the agmatine pathway (AgDI) has been proposed as the main route for putrescine synthesis since the ornithine decarboxylase pathway is not present in dairy LAB. These bacteria make putrescine from agmatine in three steps that are catalyzed by agmatine deiminase (AgDI), putrescine transcarbamylase (PTC), and carbamate kinase (CK). An antiporter then exchanges putrescine for agmatine. In addition to putrescine, ATP and NH$_3$ are also products of this set of reactions. The agmatine pathway has been identified in *E. faecium*, *E. faecalis*, *Lact. brevis*, and Lactobacillus curvatus. In the last two species, both the tyramine and putrescine pathways are linked on the chromosome. Some Lactococcus strains are also able to produce putrescine from agmatine (Ladero et al. 2012). In Lactococcus, some strains carry the gene cluster and some have an insertion element that renders the cluster non-functional. These combinations suggest that these strains putatively evolved from an ancestral strain that carried the gene cluster; the gene cluster could then accumulate mutations or even be lost if agmatine was not present in the strain’s environment.

The agmatine pathway is similar to the arginine deiminase pathway (ADI), which is comprised of three reactions catalyzed by arginine deiminase, ornithine carbamoyl-transferase, and CK. The ADI pathway is widely distributed among LAB and has been described in strains from the Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Streptococcus, and Weissella genera.

Histamine is another BA that occurs frequently in fermented products. It is produced from histidine in a reaction catalyzed by histidine decarboxylase (HdcA). Histamine synthesis also requires an antiporter protein that interchanges histidine and histamine. The histamine gene cluster has a gene encoding for the HdcB protein, which is cotranscribed with *hdcA* and is necessary for HdcA maturation (Tripi et al. 2011). A gene encoding a protein homologous to histidyl tRNA synthetase is also found in this cluster, although its position varies from one LAB species to another (Calles-Enriquez et al. 2010). The HDC cluster has been characterized in different species of Lactobacillus, *O. oeni*, *Strep. thermophilus*, Tetragenococcus muriaticus, and Tetragenococcus halophilus and is located on the chromosome, except for Lactobacillus hilgardii (Lucas et al. 2005).

BA synthesis always requires the presence of amino acids; however, other parameters such as pH, carbon source, or temperature differ from one BA to another (Linares et al. 2012).
1.4. LAB Metabolism in Light of Genomics, Comparative Genomics, and Metagenomics

The use of next generation sequencing technology revolutionized microbial science by rapidly increasing the number of publically available genomes. The first LAB genome was published in 2001 (Bolotin et al. 2001) and, since then, nearly 100 complete genomes from typical LAB species (excluding enterococci) have been deposited at NCBI, and more than 600 whole genome sequencing projects with a permanent draft or incomplete status have been reported, according to GOLD (as of 20 May 2015). Among draft genome sequences, a dozen Lactobacillus strains have been generated as part of the Human Microbiome Project (The Human Microbiome Jumpstart Reference Strains Consortium et al. 2010). Furthermore, the technological advances and cost reductions of sequencing have allowed the resequencing of the L. lactis MG1363 genome and its comparison to the genome of its derivative L. lactis NZ9000 (Linares et al. 2010). This comparison identified six differences that were not attributable to known errors in the published sequence. Two specific mutations were found in the ptcC promoter, which plays a key role in regulating cellobiose and glucose uptake. A comparative analysis of the genomes of six Lactobacillus and several other LAB has also identified nine niche-specific genes (O’Sullivan et al. 2009). A comparison of 20 complete Lactobacillus genomes showed that their bacterial pan-genome consists of approximately 14,000 genes, including a core genome of 383 orthologous genes (Kant et al. 2011).

The analysis of mixed cultures, which is very important in industrial fermentations, has also become possible using metagenomic approaches. Novel molecular technologies such as metagenomics can be used to analyze the metabolic potentials of LAB. In one study, LAB isolates from African pearl millet slurries and the metagenomes of amylaceous fermented foods were screened for genes involved in probiotic functions and starch metabolism (Turpin et al. 2011). Metagenomic approaches were used to monitor changes in bacterial populations, metabolic potential, and the overall genetic features of the microbial community during a 29-day fermentation process of the traditional Korean food kimchi (Jung et al. 2011). The resulting metagenomic sequences were categorized by their functions. Sequences in the carbohydrate category indicated that the kimchi microbiome was enriched with genes associated with mono-, di-, and oligosaccharide fermentation. Similarly, sequences in the fermentation category were enriched for various genes involved in lactate fermentation and acetoin and butanediol metabolism. The number of metabolic genes involved in carbohydrate metabolism and fermentation generally increased as kimchi fermentation progressed.

In addition to fermented food products, the human gastrointestinal tract and its complex microbial community have also garnered considerable interest. More than three million bacterial genes in the human gastrointestinal tract have been characterized (Qin et al. 2010; Methé et al. 2012).

1.5. Novel Aspects of Metabolism Regulation in the Post-genomic Age

Transcriptional regulation is essential for microorganism adaptations to changes in external or intracellular conditions. In bacteria, transcription can be activated or inhibited by various transcription factors (TFs) that recognize specific cis-regulatory DNA elements and TF-binding sites (TFBSs) in gene promoter regions. The set of genes or operons under direct control of the same TF is known as a regulon, and all the regulons in an organism form a transcriptional regulatory network.

In recent years, the number of dairy and non-dairy LAB genomic sequences has dramatically increased. A study of 30 LAB representatives found that 3.5% (Strep. thermophilus, Lact. delbrueckii, Lact. helveticus) to 7.5% (Lact. plantarum) of the proteome helped regulate transcription (Figure 1.4; Ravcheev et al. 2013). In addition, the total amount of TFs varied significantly between species, ranging from 63–64 (Lact. helveticus and Strep. thermophilus, respectively) to 240 (Lact. plantarum). Among 30 Lactobacillales genomes, putative TFs were identified in 49 protein
families (average of 36 TFs per genome), and approximately 90% belonged to 24 major families (at least two TFs per genome) (Ravcheev et al. 2013). The most represented TF family was the Xre family (298 TFs in total), and a dozen or even several dozen Xre members were identified in each genome (average of 19 per genome). Other TF families were considerably less represented; those that had at least four representatives per genome included TetR, GntR, MarR, OmpR, LacI, LysR, MerR, and AraC (Figure 1.4) (Ravcheev et al. 2013).

Other systematic genome-wide analyses of transcriptional regulatory networks have been performed for single LAB species, including *Lact. plantarum* (Wels et al. 2011) and *L. lactis* (de Jong et al. 2012, 2013). These sequence- and motif-based reconstructions of gene regulatory networks have led to the development of several analysis tools, such as PEPPER, RegTransBase, PRODORIC, RegPrecise, FITBAR, RegAnalyst, and MGcV (Overmars et al. 2013). Tools for discovering new motifs have also been developed, including MEME (Bailey et al. 2009), Tmod (Sun et al. 2009), GIMSAN (Ng and Keich 2008), and EXTREME (Quang and Xie 2014).

![Figure 1.4. (Continued)](image-url)
Figure 1.4. Distribution of predicted transcription factors (TFs) in selected Lactobacillaceae (a) and Streptococcaceae (b) genomes. The least‐represented TF families (AbrB, AsnC, BirA, CcpN, CodY, ComK, CtsR, DtxR, Fis, Fur, GlnL, GutM, HrcA, HTH_11, IclR, LexA, NiaR, NrdR, NtrR, PF04394, PurR, Rex, ROK, SdaR, SfsA, SorC, YobV) are not presented on the ideograms. Row data derive from (Ravcheev et al. 2013). Graphical presentation of these data has been developed with Circos (v0.64) (Krzywinski et al. 2009). Strains of presented Lactobacillales species: L. lactis cremoris (Lactococcus lactis subsp. cremoris SK11), L. lactis lactis (Lactococcus lactis subsp. lactis IL1403), S. thermophilus (Streptococcus thermophilus CNRZ1066), S. agalactiae (Streptococcus agalactiae 2630V/R), S. uberis (Streptococcus uberis 0140J), S. equi (Streptococcus equi MGCS10565), S. dysgalactiae (Streptococcus dysgalactiae GGS_124), S. pyogenes (Streptococcus pyogenes M1 GAS), S. galolyticus (Streptococcus galolyticus UCN34), S. mutans (Streptococcus mutans UA159), S. suis (Streptococcus suis 05ZYH33), S. mitis (Streptococcus mitis B6), S. pneumoniae (Streptococcus pneumoniae TIGR4), S. gordonii (Streptococcus gordonii CH1), S. sanguinis (Streptococcus sanguinis SK36), L. sakei (Lactobacillus sakei 23K), L. casei (Lactobacillus casei ATCC 334), L. rhamnosus (Lactobacillus rhamnosus GG), L. delbrueckii (Lactobacillus delbrueckii ATCC BAA-365), L. acidophilus (Lactobacillus acidophilus NCFM), L. helveticus (Lactobacillus helveticus DPC 4571), L. johnsonii (Lactobacillus johnsonii NCC 533), P. pentosaceus (Pediococcus pentosaceus ATCC 25745), L. brevis (Lactobacillus brevis ATCC 367), L. plantarum (Lactobacillus plantarum WCFS1), L. fermentum (Lactobacillus fermentum IFO 3956), L. reuteri (Lactobacillus reuteri JCM 1112), O. oeni (Oenococcus oeni PSU-1), L. mesenteroides (Leuconostoc mesenteroides ATCC 8293), L. salivarius (Lactobacillus salivarius UCC118).
Despite extensive analyses of the available genomes, comprehensive, experimental studies of LAB regulatory networks remain lacking. Most gene regulation studies in LAB focus on individual regulons in primarily model species, such as *L. lactis* subsp. *cremoris* MG1363 or *L. lactis* subsp. *lactis* IL1403. These studies have identified both global (CcpA – catabolite control protein A) and specific (ClaR, FruR, MalR, and XylR from the RpiR, DeoR, LacI, and AraC families, respectively) TFs for different α- or β-glucosides, fructose, galactose, lactose, maltose, sorbose, or xylose assimilation genes (reviewed by Aleksandrzak-Piekarczyk 2013; de Jong et al. 2012). CcpA is a component of carbon catabolite repression (CCR), which also includes HPr, HPr kinase, and the glycolytic enzymes fructose 1,6-bisphosphate and glucose-6-phosphate (Deutscher 2008; Görke and Stülke 2008). Large-scale approaches, such as transcriptomics and proteomics, have shown that CcpA is also involved in the regulation of tens of genes associated with membrane transport, nucleotide and nitrogen metabolism, protein biosynthesis, and folding (Mazzeo et al. 2012; Zomer et al. 2006). In addition, CcpA-dependent CCR has been shown to impede the expression of two operons involved in citrate metabolism in *E. faecalis* (Suárez et al. 2011).

Many genes undergo diverse regulation by CcpA or other TFs. In milk, transcriptomics has identified several regulatory networks in *L. lactis* (de Jong et al. 2013). A comparative systems biology approach revealed a crucial role for phosphate in regulating central metabolism and glucose uptake in *L. lactis* and *Streptococcus pyogenes* (Levering et al. 2012). Comparing kinetics models in both strains revealed variations that can be explained by differences in phosphate levels in their natural environments.

Recent studies have sought to determine the scope of proteolytic systems and amino acid biosynthesis regulation in *L. lactis* cells. Previous studies identified a significant role for CodY, a global transcriptional regulator, in negatively regulating components of the proteolytic system, which are stimulated by the intracellular pool of branched chain amino acids (BCAAs) (den Hengst et al. 2005). Subsequent studies have reported on the CodY-dependent regulation of several proteolytic system components in other LAB, including *Strep. thermophilus* (Liu et al. 2009), *Streptococcus pneumoniae* (Hendriksen et al. 2008), *Streptococcus mutans* (Lemos et al. 2008), and *O. oeni* (Ritt et al. 2009) but not in lactobacilli. The latest studies have indicated that a proteolysis regulator separate from CodY exists in lactobacillaceae, enterococcaceae, leuconostocaceae, carnobacteriaceae, listeriaceae, exiguobacteria, and bacillaceae. This has been thoroughly examined in *Lact. helveticus*, and a novel BCARR protein has been identified that represses proteolysis gene expression in response to BCAAs (Wakai and Yamamoto 2013). No BCARR orthologs are present in streptococci, including lactococci, which have a CodY homolog.

Amino acid assimilation is crucial for LAB that are generally auxotrophic for amino acids. In addition to RNA structural switches, regulating sulfur amino acid metabolism is also dependent on LysR-family transcriptional regulators, including CmbR in *L. lactis* and CysR, HomR, and MetR in *Strep. mutans* (Liu et al. 2012 and references within). Global ArgR and AhrC regulons have been established by transcriptome analyses, which indicate that both regulators are dedicated to controlling arginine metabolism in *L. lactis* (Larsen et al. 2008). Interestingly, pneumococcal ArgR and AhrC are not involved in regulating arginine biosynthesis and breakdown, as is the case in other bacteria, and instead control the expression of genes associated with arginine and peptide uptake (Kloosterman and Kuipers 2011). AhrC has also recently been reported to have a role in *Strep. pneumoniae* (Kloosterman and Kuipers 2011) and *E. faecalis* (Frank et al. 2013) virulence. Very few global studies of modifications in LAB cell metabolism and the various regulatory mechanisms involved with amino acid shortages have been performed. One study analyzed the global transcriptome and proteomic response of *L. lactis* during progressive isoleucine starvation. CodY appeared to be specifically dedicated to regulating the supply of isoleucine, while the other regulators identified were associated with growth rate and stringent response (Dressaire et al. 2011).
1.6. Functional Genomics and Metabolism

1.6.1. Transcriptomics, Proteomics, and Metabolomics

One of the greatest achievements of recent molecular biology is the development of high-throughput functional genomics approaches, such as transcriptomics, proteomics, or metabolomics. Transcriptomics have been used to analyze the LAB response to different growth conditions, culturing regimens, and various stresses (de Vos 2011). A variety of studies, including attempts at functional genomics, have contributed to the discovery and further characterization of respiratory metabolism in many LAB species (for recent reviews see Lechardeur et al. 2011a; Pedersen et al. 2012). Respiration was found to be activated by cofactors such as exogenous heme or heme and menaquinone. Some LAB species were also found to use nitrate as a terminal electron acceptor (Brooijmans et al. 2009). Furthermore, activating electron transport chains leads to higher biomass production and increased robustness, which is beneficial for industrial applications (Pedersen et al. 2012).

Transcriptional analysis identified genes involved in oxygen respiration. For example, the ygfCBA operon from \textit{L. lactis}, which encodes a putative transport system and regulator, is strongly induced by heme and is involved in heme tolerance and homeostasis (Pedersen et al. 2008). YgfC (or HrtR4) is a heme-responsive repressor that regulates hrtRBA (formerly ygfCBA) operon expression in response to free heme molecules (Lechardeur et al. 2011b). Recently, the crystal structures of HrtR have confirmed that it senses and binds a heme molecule to regulate expression of the heme-efflux system that is responsible for heme homeostasis in \textit{L. lactis} (Sawai et al. 2012). A community transcriptomic approach revealed huge differences in gene expression profiles between \textit{Lact. plantarum} from the human intestine and that from laboratory media (Marco et al. 2010). Moreover, similar expression profiles were observed in mice and humans, which suggests that there are some common intestinal adaptation mechanisms. The metabolic capacity of \textit{Lact. plantarum} is specifically adapted in the intestine for carbohydrate acquisition and the expression of exopolysaccharide and proteinaceous cell surface compounds. The transcriptome response in complex food ecosystems has also been analyzed in bacteria in yogurt fermentation. In milk, \textit{Strep. thermophilus} LMD-9 in the presence of \textit{Lact. bulgaricus} overexpresses genes involved in amino acid transport and metabolism as well as DNA replication (Goh et al. 2011). The metatranscriptomic approach in yogurt showed that specific compounds and metabolic pathways are involved in interactions between \textit{Strep. thermophilus} and \textit{Lact. bulgaricus} strains (Sieuwerts et al. 2010). Similar approaches have been applied to complex communities in fermented foods, such as kimchi (Nam et al. 2009) or sourdough (Weckx et al. 2011).

Proteomic tools can also be used to improve the understanding of metabolism in food microorganisms. Recently, the proteomic approach was used to quantitatively analyze the bacterial enzymes released in Swiss-type cheese during ripening (Jardin et al. 2012). This approach has also been used to investigate stress responses in LAB to acidic growth conditions (Nezhad 2010) or low temperatures (Garnier et al. 2010). Recent modeling approaches of protein and mRNA stability based on transcriptome and proteome \textit{L. lactis} data have also increased the quantitative abilities of functional genomics (Dressaire et al. 2009; Picard et al. 2009).

Global metabolomics studies of LAB have not been frequently reported (de Vos 2011). One study of folate-overproducing \textit{Lact. plantarum} cells (Wegkamp et al. 2010) found that reductions in growth rate occurred when the folate gene cluster was overexpressed. In another study, the response of \textit{L. lactis} to acid stress was analyzed at the metabolite level and complemented by performing a genome-wide transcriptome analysis (Carvalho et al. 2013). The H+/lactate stoichiometry of lactic acid export was determined, and the study findings were used to propose a metabolic model that accounted for the molecular mechanisms of the \textit{L. lactis} response to acid stress.
1.6.2. Global Phenotypic Characterization of Microbial Cells

Phenotype MicroArray™ (PM) technology (Biolog Inc.) is a system of cellular assays designed for the simultaneous and high-throughput screening of a large variety of phenotypes. This technology can be used to complement traditional genomic, transcriptomic, and proteomic approaches. PM can test for nearly 2000 microbial phenotypes (C, N, P, and S metabolism; pH growth range; pH control; sensitivity to NaCl and other ions; and sensitivity to chemical agents) to gain a comprehensive overview of pathway functions in a single experiment (Bochner 2009). In recent years, several studies have presented the results of PM analysis in LAB. PM is most often used to analyze gene function by comparing LAB cell lines that differ by a single gene mutation. For example, the role of the lactate dehydrogenase enzyme (LDH) in \textit{L. lactis}, \textit{E. faecalis}, and \textit{Strep. pyogenes} was identified by screening a large variety of carbon sources using PM arrays. The \textit{ldh} deletion mutants were not able to utilize all carbon sources as efficiently as their corresponding wild-types, which explained why their growth was impaired in a rich but not chemically-defined medium (Fiedler et al. 2010). In another study, the \textit{liaS} gene in \textit{Strep. mutants} was found to encode a cell-envelope stress-sensing histidine kinase, which is thought to be important for the expression of virulence factors (Zhang and Biswas 2009). The \textit{liaS} mutant was more tolerant to various inhibitors that targeted protein synthesis, DNA synthesis, and cell-wall biosynthesis.

PM can also be used to analyze naturally occurring microbial strains, the biological properties of these strains, the effects of chemicals on their cells, and as a tool in bioprocess optimization. For instance, PM was used to understand the phenotypic manifestations of bacterial environmental adaptation (Di Cagno et al. 2010). In this study, the metabolisms of 72 \textit{Lact. plantarum} isolates from raw vegetables and fruits were compared on a variety of carbon sources. Eight clusters of \textit{Lact. plantarum} isolates were categorized based on their metabolism on 27 carbon sources, which interestingly, was not associated with their original habitats (Di Cagno et al. 2010). In another study, combined genomic, transcriptomic, and PM analyses were used to understand the survival capacity of microbes in sourdough (Passerini et al. 2013a). Bacteria that could effectively utilize five-carbon (C5) and six-carbon (C6) sugars from lignocellulose would significantly improve the economic conversion of lignocellulose to biofuels and bioproducts. PM analyses indicated that the \textit{Lactobacillus buchneri} strain can metabolize a broad spectrum of carbon sources, including various C5 and C6 monosaccharides, disaccharides, and oligosaccharides. Furthermore, \textit{Lact. buchneri} had better metabolic rates under anaerobic conditions, high tolerance to ethanol and other stresses, and therefore was an attractive candidate for biomass conversion to various bioproducts (Liu et al. 2007).

1.7. Systems Biology of LAB

Different modeling approaches have been successfully applied to LAB monocultures (Santos et al. 2013). Kinetics models are used to integrate experimental data from biochemical studies of transport, enzyme kinetics, as well as flux and metabolites measurements (Teusink et al. 2011). In \textit{L. lactis}, kinetics modeling has been primarily used to model glycolysis (Hoefnagel et al. 2002a, 2002b; Voit et al. 2006; Andersen et al. 2009; Oh 2011; Levering et al. 2012). Genome sequencing, genome analysis, and omic techniques (proteomics, transcriptomics, metabolomics, etc.) have been used to construct genome-scale metabolic models (Teusink et al. 2006; Pastink et al. 2009; Santos et al. 2009; Flahaut et al. 2013), which can help elucidate many cellular biochemical pathways. Coupling genomic data with metabolic fingerprinting using high-throughput techniques can further facilitate the extraction of relevant biological information. Such techniques have been used to characterize citrate utilization and the subsequent generation of flavor compounds (Bachmann et al. 2009; de Bok et al. 2011). The latest developments in and future of modeling lie in mixed culture and complex ecosystem applications (for a review, see Santos et al. 2013). These analyses have been
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applied to food consortia, such as *Strep. thermophilus* and *Lact. bulgaricus* in yogurt (Sieuwerts 2009) as well as the gut microbiota of the human gastrointestinal tract (de Graaf et al. 2010). Unravelling the physiology of multi-species microbial communities is a great challenge and will require the combination of phylogenetic, metagenomic, and meta-functional genomics data (Martins dos Santos et al. 2010).

Furthermore, systems biology is another powerful tool that can be used to decipher metabolism in LAB. Systems biology studies microorganisms as integrated and interacting networks of genes, proteins, and biochemical reactions (Teusink et al. 2011; de Vos 2011).

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