Overview on sugar metabolism and its control in Lactococcus lactis – The input from in vivo NMR

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Abstract

The wide application of lactic acid bacteria in the production of fermented foods depends to a great extent on the unique features of sugar metabolism in these organisms. The relative metabolic simplicity and the availability of genetic tools made Lactococcus lactis the organism of choice to gain insight into metabolic and regulatory networks. In vivo nuclear magnetic resonance has proven a very useful technique to monitor non-invasively the dynamics of intracellular metabolite and co-factor pools following a glucose pulse. Examples of the application of this methodology to identify metabolic bottlenecks and regulatory sites are presented. The use of this information to direct metabolic engineering strategies is illustrated.

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Keywords: LAB; Lactococcus lactis; In vivo NMR; Glycolysis regulation; Metabolic engineering

Contents

1. Introduction ................................................................. 532
2. Strengths and weaknesses of NMR for studying metabolism ................................................ 533
3. Overview of sugar uptake and initial metabolism in L. lactis ............................................. 536
4. Metabolic bottlenecks in L. lactis sugar metabolism .......................................................... 538
   4.1. Pyruvate kinase, a bottleneck that determines the accumulation of starvation metabolites ........ 538
   4.2. α-Phosphoglucomutase, a major bottleneck in galactose metabolism ................................. 541
5. Redirection of carbon flux to the production of desired end-products .................................. 542
   5.1. Disruption of ldh discloses the unexpected capacity of L. lactis to synthesize mannitol ........ 542
6. Control and regulation of the glycolytic flux ............................................................... 544
   6.1. Shift to mixed-acid fermentation and the impact of oxygen ........................................ 545
   6.2. Control of the glycolytic flux ....................................................................................... 547
7. Concluding remarks .......................................................... 549
Acknowledgements ......................................................................................... 549
References ................................................................................................. 549

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1. Introduction

Lactic acid bacteria (LAB) are industrially important microorganisms that are used worldwide in the manufacture of fermented foods and beverages. These microbes produce mainly lactic acid from sugar, providing an effective method of preserving fermented products. The homofermentative and heterofermentative pathways utilized by LAB for the conversion of carbohydrates to lactic acid are well known and have been described in textbooks [1] and reviews [2,3]. The fact that LAB usually lack functional electron chains, grow under low oxygen tension, and rely mostly on fermentative processes to provide energy, considerably limits their metabolic versatility. Nevertheless, minor products of sugar metabolism have been shown to be highly relevant in dairy fermentations. In addition to preservation, LAB also contribute to other product characteristics, such as flavor, texture and, frequently, nutritional value (for reviews see [4–8]). The well established status of LAB as food organisms together with a relatively simple physiology make them suitable targets for metabolic engineering strategies aimed at the improvement of food quality and human health [9]. Of all LAB, Lactococcus lactis is by far the most extensively studied organism. The relative simplicity of L. lactis metabolism that converts sugars via the glycolytic (homofermentative) pathway to pyruvate, generating energy mainly through substrate level phosphorylation (Fig. 1), makes it an attractive target for the development of effective cell factories. Moreover, the availability of a large number of genetic tools [10] and the complete genome sequence [11] consolidated its status as a model for LAB and offers the opportunity of adopting global approaches that, it is to be hoped, will provide a comprehensive picture of how cellular components interact to produce a functional organism [12].

A major breakthrough for using L. lactis as a cell factory was the development of several genetic tools that made the genetic manipulation of this organism straightforward. In particular, the nisin-controlled overexpression system [13], a toolbox allowing modulation of gene expression at a selected level [14], and the pORI/pVE6007 two-plasmid system to obtain clean food-grade deletions of genes of interest [15] are invaluable tools available for directed genetic manipulation of this bacterium. In the last decade several reports on metabolic re-routing of central carbon metabolism in L. lactis were presented and the engineering strategies have been extensively reviewed [16–18]. It is interesting to note that successful cases in metabolic engineering were not accomplished through disruption or overexpression of single genes. Indeed, several examples illustrate this concept [19,20]. Instead, coordinated expression and/or disruption of several genes was required to attain the desired objective. Successful examples were the redirection of pyruvate metabolism to products other than lactate, such as alanine and diacetyl [21,22]. Furthermore, increased production of compounds like exopolysaccharides [23,24] and vitamins [25] has been described. However, engineering such complex biosynthetic pathways still poses a major challenge, and predicting how cell physiology will respond to a genetic modification is not easy. The difficulty originates mainly from the existence of multiple interlocked pathways connected via common metabolites and cofactors through various levels of genetic and metabolic regulation [26,27]. Overall, the consensus is that manipulation of strains leading to the desired metabolic features cannot be achieved without a global understanding of the metabolic network as well as of the interdependent relationships among the different steps.

Regulation of glycolysis in L. lactis has been the subject of intensive research. Key glycolytic enzymes, pyruvate kinase (PK) [28–31], phosphofructokinase [32], fructose 1,6-bisphosphate aldolase [33], glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [34] and lactate dehydrogenase (LDH) [35,36] were characterized, and concentrations of glycolytic intermediates, fructose 1,6-bisphosphate (FBP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PGA), 2-phosphoglycerate, phosphoenolpyruvate (PEP), in cell extracts had been obtained already in the eighties (for a review on early studies see [37]). Under certain conditions a metabolic shift from homolactic (lactate production) to mixed acid fermentation (ethanol, acetate and formate production) can occur in L. lactis (reviewed in [3,38]). The mechanisms underlying this shift have been the object of considerable controversy and a full explanation has yet to be put forward; this topic will be discussed in more detail later in this paper. More recently, the control of the glycolytic flux has been addressed in a number of elegant experiments (for review see [39]), but an answer to the question of what controls glycolysis in L. lactis remains elusive. Despite the wealth of metabolic information collected during years of intensive research and numerous genetic tools available for L. lactis, we are still far from achieving a comprehensive understanding of sugar metabolism and regulatory pathways in this model organism. This goal can only be achieved by resorting to quantitative metabolic models, the development of which requires reliable data on intracellular concentrations of intermediates and metabolic fluxes [40].

Powerful analytical methodologies can now be used to characterize the ensemble of low-molecular mass metabolites in the cell [41]. Nuclear magnetic resonance spectroscopy (NMR) is a powerful technique for studying metabolism, making it feasible to probe complex reaction pathways and control points simultaneously and non-invasively in the live cell, obtain invaluable information on the magnitude of intracellular metabo-
light pools, and calculate absolute fluxes from dynamic labeling experiments and end-products derived from the metabolism of \([\text{[1-}^{13}\text{C}]\text{glucose}\) is shown. It should be stressed that, under anaerobic glycolysis, lactate accounts for about 95% of the label from glucose. The reactions indicated are catalyzed by the following enzymes: 1. phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS, \text{ptnABCD}) or/and non-PTS permeases (genes unknown)/glucokinase (gsk) system; 2. phosphoglucose isomerase (\text{pgi}) and 6-phosphofructo-1-kinase (\text{pfk}); 3. fructose 1,6-bisphosphate aldolase (\text{fba}); 4. triosephosphate isomerase (tpi); 5. glyceraldehyde 3-phosphate dehydrogenase (gapB) and phosphoglycerate kinase (pgk); 6. phosphoglyceromutase (pmg) and enolase (enoA); 7. pyruvate kinase (pyk); 8. lactate dehydrogenase (ldh); 9. pyruvate-formate lyase (pfl); 10. pyruvate dehydrogenase complex (\text{pdhABCD}); 11. acetaldehyde dehydrogenase (\text{adlAB}) and alcohol dehydrogenase (\text{adl}) and acetate kinase (\text{ackA}); 12. \(\alpha\)-acetolactate synthase (als); 13. \(\alpha\)-acetolactate decarboxylase (aldB); 14. 2,3-butanediol dehydrogenase (butB).

This article will review the latest advances in the knowledge of sugar metabolism and its control in \textit{L. lactis}, highlighting the application of in vivo NMR for identifying metabolic bottlenecks and direct metabolic engineering strategies.

2. Strengths and weaknesses of NMR for studying metabolism

Nuclear magnetic resonance spectroscopy is based on the response of nuclides that possess an intrinsic
magnetic moment (\( ^1\text{H}, ^{13}\text{C}, ^{15}\text{N}, ^{19}\text{F}, ^{31}\text{P}, ^{23}\text{Na}, \text{etc.} \)) to an external magnetic field. NMR is a powerful analytical technique that allows qualitative and quantitative characterization of chemical mixtures, the measurement of reaction rates in steady state, the determination of isotopic distribution within molecules and the structure of biomolecules. The idea of using NMR to study living systems dates back to the origins of the technique, but the usefulness of early experiments was limited by the poor quality of the instrumentation available at the time. The development of high-field superconducting magnets together with the emergence of the Fourier transform NMR method, revolutionized the scope of NMR and allowed researchers to benefit from the capabilities of this technique for carrying out measurements directly on live systems [57–59]. Pioneering work by Moons and Richard [60] showed that phosphorylated metabolites could be detected non-invasively in \(^{31}\text{P} \) NMR spectra of intact red blood cells and that intracellular pH could be directly determined from these spectra. Glucose catabolism in living cells of the yeast Candida albicans was investigated by \(^{13}\text{C} \) NMR [61].

To date, the in vivo NMR field has expanded enormously, and nowadays a large number of metabolic parameters can be determined by a variety of NMR techniques. An introduction to NMR and its applications to the study of microbes can be found in recent reviews [62–64].

For studying biological systems, \(^{31}\text{P} \) has been the nucleus that enjoyed the greatest popularity. The \(^{31}\text{P} \) isotope represents 100% of all phosphorus nuclei and has a high relative sensitivity; it allows monitoring of intracellular pools of intracellular inorganic phosphate (P) and several phosphorylated metabolites, such as NTP, thus, providing information about the energetic status of the cell in a non-invasive way [65]. The NMR sensitivity of \(^{13}\text{C} \) is much lower than that of \(^{31}\text{P} \), but \(^{13}\text{C} \) NMR offers some general advantages for the investigation of metabolic pathways and non-invasive measurements of intracellular concentrations [66,67]. Primarily, almost all cellular intermediates contain carbon, allowing simultaneous monitoring of phosphorylated and non-phosphorylated metabolites (e.g. FBP and glucose). Since the natural abundance of \(^{13}\text{C} \) is only 1.1% it is possible to use \(^{13}\text{C} \)-enriched molecules to trace biosynthetic pathways and to measure carbon fluxes (Fig. 1). Furthermore, \(^{13}\text{C} \) NMR has a large chemical shift range and is, therefore, the technique of choice whenever labeled substrates are affordable.

A major drawback of NMR is its intrinsic low sensitivity, which limits in vivo observations to metabolites present at relatively high concentrations (mM range). Therefore, the majority of NMR experiments are conducted with thick suspensions of non-growing cells, to increase the total intracellular space available for detection and, hence, problems in gas or nutrients supply may arise [68]. It is generally impossible to maintain a metabolic steady state over periods longer than a few minutes, and this applies especially to microorganisms, because of their rapid metabolism. It is difficult to extend NMR studies to growing cells under well-defined conditions; however, Hartbrich et al. [69] developed a hydrocyclone bioreactor, coupled directly to a wide-bore NMR spectrometer, with such a high oxygen transfer rate that good oxygenation of yeast cells is ensured even at cell densities as high as 75 g dry mass \(^{-1} \)

[70].

The data obtained from early studies of sugar metabolism in LAB using in vivo NMR were constrained by the experimental set-up, since most studies were performed with dense cell suspensions without control of environmental parameters. Thomson and Torchia [71] re-examined the effects of FBP and P, [72,73] in the modulation of the regulatory enzyme PK by using \(^{31}\text{P} \) NMR. Identical methodology was used to determine changes in the glycolytic intermediates during the switch from homolactic to mixed acid fermentation imposed by using suitable substrates [74]. In both cases, the information obtained contributed to a better understanding of the systems, but determination of individual sugar phosphates was precluded by extensive line broadening and consequent overlapping of the resonances in the \(^{31}\text{P} \)-spectra. Broad \(^{31}\text{P} \) NMR resonances have also been observed with other Gram-positives [74–77] in contrast to Gram-negatives [51,78,79]. Accumulation of high intracellular concentrations of paramagnetic ions (e.g. manganese) [80,81], or a rapid acidification of the medium due to the high glycolytic rate and concomitant acid production are invoked to explain the severe line broadening of most phosphorous resonances, which prevented the direct quantification of metabolites.

The narrower lines and large chemical shift distribution of \(^{13}\text{C} \) spectra, the feasibility of \(^{13}\text{C} \)-labeling experiments and the dissemination of two-dimensional heteronuclear techniques made \(^{13}\text{C} \) over the most popular nucleus in NMR studies of cell metabolism. With regard to LAB, information derived from \(^{13}\text{C} \) NMR experiments strengthened important findings, as exemplified by the elucidation of the pathway leading to diacetyl production [50,82], and the discovery of erythritol as a major product of glucose metabolism in Oenococcus oeni and characterization of its biosynthetic pathway [48]. More recently, \(^{13}\text{C} \) NMR was also used to determine the contribution of the heterolactic pathway and the mannitol pathway in the metabolism of fructose and glucose [83]. Carbon-13 was also successfully used to characterize co-metabolism of glucose and citrate in L. lactis and in Oenococcus oeni. A judicious choice of specific labeling of the two substrates allowed glucose and citrate carbons to be distinguished throughout the metabolic pathways upstream of pyruvate [50,53]. Nota-
bly, NMR provides a straightforward way for detecting the unexpected. These are good examples of the use of NMR spectroscopy to identify new pathways, unknown branches, or clarify regulatory steps in a proposed pathway. All these studies rely on the analysis of labeling patterns of end-products or intermediate metabolites present in cell supernatants or cell extracts.

To take full advantage of the unique non-invasive and analytical features of NMR to monitor the kinetics of the intracellular pools of metabolites directly in living cells has been a major focus of interest in our research team for nearly 15 years. To maintain the cells under specific conditions during the NMR experiment we use a circulating system for on-line NMR, based on fast circulation of the cell suspension from a mini bio-reactor (working volume 50 ml) to the NMR tube and back to the bio-reactor [84]. The experimental set-up enabled control of pH, temperature, and gas atmosphere. Application of the on-line system and the use of a chemically defined growth medium devoid of paramagnetic ions allows changes in the concentrations of several intracellular metabolites to be monitored following a pulse of substrate (Figs. 2 and 3). The technique proved to be very useful in monitoring simultaneously substrate consumption and end-products formation as well as in following changes in the concentration of metabolic intermediates (glucose 6-phosphate, FBP, 3-PGA, PEP, galactose 1-phosphate, glucose 1-phosphate, UDP-glucose, UDP-galactose, mannitol 1-phosphate, etc.) in a non-invasive manner [20,84,85]. Additionally, 13C-enrichment of pyridine nucleotide pools by growing L. lactis on [5-13C]nicotinic acid enabled simultaneous

![Fig. 2. Glucose metabolism in non-growing cell suspensions of L. lactis MG1363 monitored by in vivo 13C NMR.](image-url)

Symbols: (◇) glucose; (■) lactate; (▲) total FBP; (●) 3-PGA; (◆) PEP; (▲) NAD+; (◇) NADH.
measurement of NAD$^+$ and NADH pools and $^{13}$C-glycolytic intermediates in living cells (Fig. 2(a) and (c)) [55]. In parallel experiments, good quality $^{31}$P NMR spectra of L. lactis cell suspensions allowed us to evaluate in vivo the energetic state of the cell and the intracellular concentration of inorganic phosphate (Fig. 3 (a) and (b)) [31,55,84]. This methodology provided a global picture of how L. lactis cells respond to changes in extracellular conditions and allowed the identification of bottlenecks, information used to direct metabolic engineering strategies.

3. Overview of sugar uptake and initial metabolism in L. lactis

The initial event in the metabolism of carbohydrates is their transport across the cytoplasmic membrane. There are three major uptake systems for sugars in bacteria: (i) the phosphoenolpyruvate: (carbohydrate) phosphotransferase system (PTS), which is involved in both transport and phosphorylation of a sugar at the expense of PEP, resulting in accumulation of the corresponding carbohydrate phosphate (group translocation) [86]; (ii) ion-linked sugar transport: secondary transport systems where the sugar uptake is driven by an ion gradient [87]; and (iii) carbohydrate transport ATPases, primary transport systems that couple ATP hydrolysis with translocation (ABC transport systems) [88]. From a bioenergetic point of view, the PTS system is probably the most efficient, since the sugar is translocated and phosphorylated in a single step at expense of one PEP molecule (equivalent to one ATP molecule, since during glycolysis one ATP molecule is derived from one PEP at the level of PK). Instead, for carbohydrates that are actively accumulated by non-PTS systems, more than one ATP equivalent must be expended for both transport and subsequent phosphorylation. The multicomponent PTS system consists of two general proteins, enzyme I (EI), and the HPr protein, and several sugar-specific enzymes II (EII) that can contain up to four domains present as individual polypeptides or linked together in a multidomain complex. Uptake of sugars via secondary transport systems (permeases) is coupled to ion translocation, and sugar transport is followed by a kinase-mediated phosphorylation of the free sugar [87]. The secondary transport system for lactose was the first ion-linked sugar transport discovered in L. lactis [89].
Since then, several secondary transport systems have been described, all belonging to the galactoside-pentose-hexuronide group of transport systems [90].

The metabolism of lactose, glucose, and galactose is of special relevance to the dairy industry. According to the literature, the major transport system mediating the uptake of glucose in \textit{L. lactis} is the mannose-PTS system (PTS\textsuperscript{\text{man}}) (Fig. 4) [91], which, besides glucose, transports 2-deoxy-D-glucose, mannose, glucosamine, and fructose. Glucose is transported and concomitantly phosphorylated by EI\text{II}A to glucose 6-phosphate, the entry point into the glycolytic pathway. A second PTS system, the glucose-PTS system that exhibits specificity to glucose and α-methyl-glucoside has also been described for some strains [92]. Alternatively, glucose can be transported via a permease and subsequently phosphorylated by an ATP-dependent glucokinase [93].

Lactose can also be translocated either via a lactose-PTS to yield lactose 6-phosphate or by a permease (Fig. 4) [94,95]. The product of the PTS\textsuperscript{\text{lac}}, lactose 6-phosphate is then hydrolyzed by a phospho-β-galactosidase, producing galactose 6-phosphate and glucose. After phosphorylation via glucokinase, glucose can enter glycolysis, while galactose 6-phosphate is further metabolized to the glycolytic intermediates DHAP and glyceraldehyde 3-phosphate by three enzymes of the tagatose 6-phosphate pathway [96,97]. The lactose specific components of the PTS, together with the enzymes of the tagatose 6-phosphate pathway are plasmid encoded in most \textit{L. lactis} strains, although in some cases the \textit{lac} genes were found to be chromosomally located [98,99]. Lactose uptake by a permease is followed by hydrolysis via β-galactosidase, yielding glucose and galactose [33]; galactose is further metabolized to glucose 1-phosphate via the three enzymes of the Leloir pathway, encoded by the \textit{gal} genes [100,101]. Glucose 1-phosphate can, after isomerization to glucose 6-phosphate via α-phosphoglucomutase, enter the glycolytic pathway (Fig. 4). In

Fig. 4. Possible pathways for the transport and initial metabolism of carbohydrates (mono- and disaccharides) in \textit{L. lactis}. The reactions indicated are catalyzed by the following enzymes: 1. galactose-PTS (gene unknown); 2. galactose 6-phosphate isomerase (\textit{lacAB}); 3. tagatose 6-phosphate kinase (\textit{lacC}); 4. tagatose 1,6-bisphosphate aldolase (\textit{lacD}); 5. lactose-PTS (\textit{lacFE}); 6. phospho-β-galactosidase (\textit{lacG}); 7. glucose permease (gene unknown); 8. glucokinase (\textit{glk}); 9. mannose/glucose-PTS (\textit{ptnABCD}); 10. lactose permease (\textit{lacY}); 11. β-galactosidase (\textit{lacZ}); 12. galactose permease (\textit{galP}); 13. galactose mutarotase (\textit{galM}); 14. galactokinase (\textit{galK}); 15. galactose 1-phosphate uridylyltransferase (\textit{galT}); 16. UDP-galactose 4-epimerase (\textit{galE}); 17. α-phosphoglucomutase (gene unknown); 18. maltose ABC-transporter permease (\textit{malG}) and maltose binding protein (\textit{malK}); 19. maltose phosphorylase (\textit{malP}); 20. β-phosphoglucomutase (\textit{pgmB}); 21. trehalose-PTS (gene unknown); 22. trehalose 6-phosphate phosphorylase (\textit{trePP}); 23. sucrose-PTS (\textit{sacB}); 24. sucrose 6-phosphate hydrolase (\textit{sacA}); 25. 6-fructokinase (\textit{sacK}); 26. fructose-PTS (\textit{fruA}); 27. 1-phosphofructo-6-kinase (gene unknown); 28. phosphoglucoisomerase (\textit{pgi}); 29. 6-phosphofructo-1-kinase (\textit{pfk}); 30. fructose 1,6-bisphosphate aldolase (\textit{fbp}); 31. EMP enzymes. Circles and double rounded-rectangles represent non-PTS permeases and PTS systems, respectively.
analogy with the tagatose 6-phosphate pathway when translocated by a PTS\textsuperscript{gal}, and by the Leloir pathway when transported via the highly specific galactose permease system [100,102,103]. Insights into glucose and galactose metabolism will be described below.

Two other important sugars in the food industry are fructose and sucrose. Fructose uptake can be mediated by the PTS\textsuperscript{man}, yielding fructose 6-phosphate, or via a specific fructose-PTS, and the resulting fructose 1-phosphate enters glycolysis as FBP after phosphorylation [104]. The uptake of sucrose in some \textit{L. lactis} strains is mediated by a sucrose-specific PTS, and the resulting sucrose 6-phosphate is hydrolyzed by a sucrose 6-phosphate hydrolase yielding glucose 6-phosphate and fructose [105]. In order to enter glycolysis, fructose has to be phosphorylated by an ATP-dependent fructokinase [106].

Maltose is transported by an ATP-dependent permease, and converted to glucose and \(\beta\)-glucose 1-phosphate via the action of a \(P_i\)-dependent maltose phosphorylase [107–109]. Anomerization of \(\beta\)-glucose 1-phosphate into \(\alpha\)-glucose 1-phosphate and entry into the glycolytic pathway is catalyzed via a specific \(\beta\)-phosphoglucomutase [110]. The latter enzyme is also involved in the metabolism of trehalose, which enters the cell via a specific trehalose-PTS system yielding trehalose 6-phosphate that is further converted to glucose 6-phosphate and \(\beta\)-glucose 1-phosphate via trehalose 6-phosphate phosphorylase (Fig. 4) [111]. Genes coding for most of these proteins have been found in the genome sequence of \textit{L. lactis} IL1403 [11], but genes coding for glucose permease(s) and a galactose-specific PTS remain unknown. Recently, a gene coding for \(\alpha\)-PGM in \textit{L. lactis} MG1363 has been identified and its gene product fully characterized [Neves, A.R, Pool, W., Mingote, A., Kok, J., Kuipers, O.P. and Santos, H., unpublished data].

4. Metabolic bottlenecks in \textit{L. lactis} sugar metabolism

4.1. Pyruvate kinase, a bottleneck that determines the accumulation of starvation metabolites

In \textit{L. lactis} glucose is linearly converted to pyruvate through the glycolytic pathway, with production of ATP by substrate level phosphorylation and reducing equivalents (NADH) at the level of glyceraldehyde 3-phosphate dehydrogenase. Reduction of pyruvate to lactate via LDH maintains the redox balance by regenerating NAD\(^+\). Accumulation of FBP to high levels (around 50 mM) is a major characteristic of glucose metabolism in \textit{L. lactis}, whereas starved cells accumulate 3-PGA and PEP (Fig. 2(b)) [37,112]. The finding that FBP is an allosteric regulator (activator) of PK and LDH suggested an important role of this metabolite in the regulation of \textit{L. lactis} metabolism [37]. A simple regulatory model based solely on the allosteric properties of enzymes was proposed: during homolactic fermentation, activation of PK and LDH by the high levels of FBP directs the flux towards the production of lactate. Accumulation of PEP and 3-PGA in starved cells is a consequence of PK inhibition by high \(P_i\) and low FBP concentrations, conditions associated with glucose depletion [37,71,73]. The shift to mixed-acid fermentation (production of acetate, formate and ethanol) has been interpreted as resulting from a reduced FBP pool leading to LDH inactivation, and inhibition relief of pyruvate-formate lyase by triose phosphates [113,114]. The role of FBP on the regulation of pyruvate distribution between LDH and PFL has been downplayed, since it was shown that its intracellular concentration is always sufficiently high to ensure full activation of the former enzyme [115]. It is possible that the role of FBP on regulation was overestimated as a consequence of it being the metabolite whose concentration is highest and thereby more easily measured.

More recently, FBP was shown to be a major signaling molecule for carbon catabolite protein A (CcpA)-dependent catabolite repression and activation of genes in Gram-positive bacteria. Carbon catabolite repression, which is the result of global transcriptional control and inducer exclusion mechanisms, as well as its specific regulatory mechanisms have been thoroughly discussed in a number of recent reviews [116–120]. In brief, interaction of the catabolite co-repressor P-Ser-HPr (PTS subunit HPr phosphorylated at Ser-46) with CcpA allows for the binding of this complex to catabolite response elements (cre) in promoter regions, thereby activating or repressing gene expression. Phosphorylation of HPr at Ser-46 is mediated by the bifunctional enzyme HPr kinase/phosphorylase (HPrK/P); the kinase activity of HPr is allosterically activated by FBP and inhibited by \(P_i\), which serves as a substrate for the phosphatase reaction [120,121]. P-Ser-HPr can also trigger inducer exclusion mechanisms by inhibition of sugar permeases [122,123]. The function of the PTS system in the mechanisms underlying carbohydrate utilization is evidenced by the involvement of the HPr subunit, which is also required for the transport of PTS sugars when phosphorylated at His-15. Therefore, the PTS must be regarded as a functional system which serves both transport and signal transduction purposes [86,116]. It is interesting to note that FBP and \(P_i\), the classical main players of sugar regulation in \textit{L. lactis}, in part due to their dual but antagonistic modulation of PK activity, were shown to be crucial in a global control mechanism. Hence, FBP may provide a link between glycolytic activity and carbon catabolite repression in these bacteria (Fig. 5). In \textit{L. lactis}, CcpA was found to be a transcriptional activator of the \(las\) operon, thus modulating glycolytic activity by controlling the key enzymes, PFK, PK, and LDH;
however, enhancement of the binding of CcpA to cre sites in response to FBP, though suggested, has not yet been proven [124,125].

Undoubtedly the level of FBP is high in energized cells (presence of glucose) (Table 1), but what drives the accumulation of this metabolite is still the subject of discussion. Garrigues and co-workers [115] suggested that inhibition or activation exerted by the ratio NADH/NAD⁺ on GAPDH or LDH is the main issue regulating glycolysis. Accumulation of FBP and the triose phosphates was a consequence of high NADH/NAD⁺ (≥0.05), which restrains GAPDH but not LDH [115]. In line with this interpretation, a mechanistic model for glycolysis in L. lactis rationalized the dynamics of glucose metabolism as being driven by ATP surplus causing NAD⁺ shortage and ultimately FBP accumulation [84]. On the other hand, based on non-invasive NMR measurements of NAD⁺ and NADH, we have shown that the NAD⁺ concentration was constant and maximal while glucose was available in the medium (Fig. 2(c)) [55]. In the light of these findings, accumulation of FBP immediately after addition of glucose was attributed to a low PK activity due to high Pᵢ levels. As a consequence of this initial bottleneck, FBP builds up at the expense of the Pᵢ pool, resulting in the relief of PK inhibition and the establishment of a "quasi steady-state" characterized by a constant level of FBP (in the 50 mM range) and maximal rate of glucose consumption. This simplistic model suggests a major bottleneck at the level of PK. This hypothesis was investigated by overexpression of the gene encoding PK (15-fold when compared to the wild-type level) [31]. It was expected that the maximal level of FBP in this strain would be lower. The small decrease (3 mM) in FBP concentration is not a strong argument in favor of that hypothesis (Fig. 6 and Table 1). Interestingly, overproduction of PK caused acceleration of the rates of FBP depletion and NAD⁺ recovery once glucose was exhausted, and lack of accumulation of 3-PGA and PEP, the two metabolites associated with cell starvation (Fig. 6). These data indicate the occurrence of a metabolic bottleneck at the level of PK in wild-type strains, at least when glucose becomes limiting. The obstruction at the level of PK is primarily ascribed to
Table 1
Maximal concentrations of intracellular intermediates (mM) in cell suspensions of several *L. lactis* strains after a pulse of glucose (20 mM, unless otherwise stated) as assessed by in vivo $^{13}$C NMR.

<table>
<thead>
<tr>
<th>Strain</th>
<th>FBP</th>
<th>3-PGA</th>
<th>PEP</th>
<th>Mtl 1-P/Mtl</th>
<th>Anaerobic O$_2$</th>
<th>Anaerobic O$_2$</th>
<th>Anaerobic O$_2$</th>
<th>Anaerobic O$_2$</th>
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<tbody>
<tr>
<td>MG5267</td>
<td>49</td>
<td>30</td>
<td>13</td>
<td>ND</td>
<td>49</td>
<td>30</td>
<td>13</td>
<td>ND</td>
</tr>
<tr>
<td>NZ2500</td>
<td>49</td>
<td>29</td>
<td>13</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NZ2007</td>
<td>84</td>
<td>55</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MG1363</td>
<td>49</td>
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<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MG1363 (40 mM)</td>
<td>51</td>
<td>10</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FI7851</td>
<td>40</td>
<td>46</td>
<td>17</td>
<td>67/88</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FI9630</td>
<td>38</td>
<td>48</td>
<td>21</td>
<td>100/120</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FI9630 (40 mM)</td>
<td>37</td>
<td>57</td>
<td>23</td>
<td>170/207</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FI10009</td>
<td>37</td>
<td>46</td>
<td>17</td>
<td>76/288</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NOX+</td>
<td>46</td>
<td>10</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NOX-</td>
<td>46</td>
<td>39</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NZ9000PK+ (40 mM)</td>
<td>46</td>
<td>36</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The values reported are averages of two to four experiments and the accuracy varied between 5% and 10%.

- FI7851, derivative of MG1363 with LDH inactivation [20,139].
- FI9630, derivative of MG1363 harboring a *ldh* gene deletion, constructed by double crossover (food-grade strain) [55].
- FI10009, derivative of FI9630 harboring a *mtlF* (PTSmtl) gene deletion, constructed by double crossover [147].
- NOX+ and NOX-, MG1363 derivatives NADH oxidase overproducing or deficient strains, respectively [85].
- NZ9000PK+, NZ9000 derivative overproducing pyruvate kinase [31].

* Value reported was calculated considering mannitol as intracellular.
- ND, not detected; –, not determined.

Fig. 6. Effect of pyruvate kinase overproduction on the metabolism of glucose in *L. lactis* monitored by in vivo $^{13}$C NMR. Time course for the consumption of [1-$^{13}$C] glucose (40 mM) and evolution of the intracellular metabolite pools in a strain overproducing PK, NZ9000[pNZpyk] (a, c) and its control strain NZ9000[pNZ8020] (b, d) under anaerobic conditions (a, b) or oxygen atmosphere (c, d) in a *L. lactis*. In strain NZ9000[pNZpyk] pyruvate kinase was 15-fold induced. Metabolite concentrations were obtained from in vivo $^{13}$C NMR data. Symbols: (●) glucose; (▲) total FBP; (○) 3-PGA; (△) PEP.
accumulation of P_i, a well-known inhibitor of the enzyme, counteracting the activating properties of FBP [29,126]. In fact, it is known that the concentration of P_i rises abruptly when glucose is exhausted (Fig. 3(b)) [55]. The data suggests that P_i is an important regulator of the flux through PK in vivo.

The lower levels of FBP accumulation in a strain overproducing NADH oxidase (Table 1) with a PK activity identical to the wild-type appear not to fit the interpretation of PK inhibition as the main cause of FBP accumulation. This suggests that the reason for FBP accumulation is more complex [85]. In the same line of thought, in wild-type strains under aerobic conditions, the maximal FBP concentration is slightly lower, whereas 3-PGA and PEP pools are considerably higher when compared to anaerobic conditions (Table 1 and Fig. 6(b) and (d)). Under an oxygen atmosphere, as NADH oxidase provides an additional path for NADH oxidation, the lower FBP accumulation is rationalized as an increase of the flux through GAPDH caused by the lower NADH concentration. Furthermore, the higher level of FBP observed under anaerobic conditions in a LDH-deficient strain (Table 1) [19], with an impairment in the NAD^+-regenerating capacity, is also in conformity with our interpretation. At the onset of glucose exhaustion, accumulation of 3-PGA and PEP is driven by PK inhibition; under aerobic conditions, NADH consumption by NADH oxidase obviates the need to regenerate NAD^+ downstream of pyruvate, and thereby to overcome the bottleneck at the level of PK (Fig. 5 for an overview of regulatory mechanisms).

The available data on dynamics of intracellular metabolites in L. lactis can be rationalized as follows: immediately after glucose addition, the build-up of the FBP pool is explained as a consequence of imbalance of PTS, PK and LDH activities. The constriction at the PK level combined with an initial low activity of LDH, due to both high NAD^+ and P_i concentrations and low FBP levels, most likely cause impairment of GAPDH activity; FBP accumulation could be explained as a consequence of inhibition of both GAPDH and PK. A decrease in the P_i pool in parallel with the FBP increase stimulates maximal PK activity. During steady state glycolysis, activation of PK and LDH, together with high PTS activity, allows for full-speed conversion of glucose to pyruvate, and the NADH formed by GAPDH is promptly oxidized through LDH. At the onset of glucose depletion, the PTS becomes substrate-limited, and therefore, all PEP converted to pyruvate has to proceed via the PK step; the availability of pyruvate (for NADH regeneration at the level of LDH) is further reduced by the inhibition of PK due to high P_i concentration. Hence, NADH increases, causing inhibition of GAPDH and consequent reduction in the flux through this step, as denoted by the slow rate of FBP depletion, under anaerobic conditions (Fig. 2(c)). For an overview of regulatory mechanisms in L. lactis see Fig. 5.

4.2. α-Phosphoglucomutase, a major bottleneck in galactose metabolism

In individuals with a deficiency in one of the galactose metabolic enzymes (galactokinase, galactose 1-phosphate uridyl transferase or UDP-galactose 4-epimerase) the intake of galactose results in a disease condition generally called galactosemia [127]. The accumulation of galactose and galactose derivatives (galactitol, galactose 1-phosphate, UDP-galactose) in blood can pose serious health problems, such as the development of cataracts and the damage of woman’s ovaries, among others [128,129]. Several fermented dairy foods, especially yogurt and yogurt-like products, contain significant amounts of galactose [130]. The understanding of galactose metabolism in LAB is therefore of major importance and should allow the improvement of LAB capacity to utilize galactose and to develop galactose-free products.

In L. lactis, galactose that enters the cell via a specific galactose-PTS is metabolized to triose phosphates through the tagatose 1,6-phosphate pathway [102]. On the other hand, galactose taken up by a permease is metabolized to glucose 1-phosphate via the Leloir pathway (Fig. 4) [100,102,103]. Glucose 1-phosphate is converted through the action of α-phosphoglucomutase to glucose 6-phosphate, which enters the glycolytic pathway. The use of one or the other pathway seems to be strain related [102], but in the laboratory organisms L. lactis subs. cremoris MG1363 and L. lactis subs. lactis IL1403 only the Leloir pathway seems to be present [11,101,131]. Indeed, 13C NMR studies on galactose metabolism in strain MG1363 showed the accumulation to high levels of the Leloir pathway intermediates, galactose 1-phosphate and glucose 1-phosphate (Fig. 7(a)). Unlike glucose metabolism, the build-up of the FBP pool results from the consumption of galactose 1-phosphate and glucose 1-phosphate. Together, these data indicate a metabolic bottleneck at the level of α-phosphoglucomutase. This enzyme plays various roles in carbohydrate metabolism and other biosynthetic pathways, by catalyzing the reversible conversion of α-glucose 1-phosphate to glucose 6-phosphate. In L. lactis, when glucose is the substrate, α-PGM is required for the production of nucleotide sugars, the precursors of exopolysaccharides [132] and cell wall polysaccharides [133]. Furthermore, α-PGM is believed to mediate the re-entry in the glycolytic pathway of sugars that have been stored as energy reserves and is essential for the utilization of galactose via the Leloir pathway [100]. A gene encoding α-PGM had not been identified, but α-PGM activity was readily measured in cell extracts of L. lactis [134]. To investigate whether there is a metabolic bottleneck at
the level of \( \alpha \)-PGM the heterologous \( pgmA \) gene from *Streptococcus thermophilus* was overexpressed under the control of the nisin promoter in *L. lactis* NZ9000 ([135]; Neves, A.R., Pool, W.A., Kok, J., Kuipers, O.P. and Santos, H., unpublished results). Interestingly, in a strain overproducing \( \alpha \)-PGM (40-fold enhancement as compared to control) the galactose uptake was improved and the levels of galactose 1-phosphate and glucose 1-phosphate were considerably reduced (Fig. 7(b)). Our data show that in the wild-type strain the carbon flux from galactose to the glycolytic pathway is significantly limited at the level of \( \alpha \)-PGM. Therefore, this metabolic constriction should be taken into account when designing metabolic engineering strategies aimed at improving galactose consumption.

5. Redirection of carbon flux to the production of desired end-products

Initial metabolic engineering in *L. lactis* has focused on rerouting of pyruvate metabolism to increase the production of flavor compounds (Fig. 1). Sugar metabolism was diverted away from lactate production towards production of \( \alpha \)-acetolactate, the precursor of the aroma compound diacetyl by either disruption of LDH or overproduction of NADH oxidase (for a review see [16]). Another example is the conversion of pyruvate into alanine, an amino acid with a sweet flavor, through expression of *Bacillus sphaericus* alanine dehydrogenase in *L. lactis* [21]. Metabolic engineering has been also used to increase the production of the yogurt flavor compound diacetyl by expressing *Zymomonas mobilis* pet operon (pyruvate decarboxylase and alcohol dehydrogenase) in the high-alcohol producing bacterium *Zymomonas mobilis* [136].

In the past few years, attention has been given to the rerouting of glycolysis towards the production of compounds with potential health benefits (nutraceuticals), such as B vitamins (riboflavin, folic acid), exopolysaccharides, natural sweeteners (mannitol, sorbitol, tagatose, trehalose) and antioxidants (mannitol, glutathione) ([18,137,138], www.nutracells.com). The biosynthetic pathways involved are rather complex and successful approaches in metabolic engineering require an understanding of the metabolic network as well as of the interdependent relationships among the different steps. An example will be presented showing that prediction of how physiology responds to a genetic modification is not always straightforward and how unexpected collateral effects can result from the application of metabolic engineering approaches.

5.1. Disruption of ldh discloses the unexpected capacity of *L. lactis* to synthesize mannitol

Diacetyl is an important flavor compound, conferring the buttery aroma typical of dairy products. This minor end-product of the metabolism of *L. lactis* is produced from oxidative decarboxylation of \( \alpha \)-acetolactate. Given its industrial relevance as an aroma compound, a number of metabolic engineering strategies have been pursued to enhance the product yield of diacetyl in *L. lactis*, a homofermentative organism in which about 95% of carbon in glucose is converted to lactate via the action of lactate dehydrogenase on pyruvate. Therefore, LDH appeared to be the most obvious target in the first approaches to increase the flux from pyruvate to end-products other than lactate [19,21,139]. This strategy, per se, caused a notable shift from homolactic to mixed acid fermentation, but the desired production of diacetyl hardly improved.

Different LDH-deficient strains of *L. lactis* responded differently to overcome the severe impairment in the NAD+ regeneration capacity. *L. lactis* strain NZ2007 with disruption of the *ldh* gene primarily used the conversion of acetate to ethanol as a rescue pathway for ful-
filling the redox balance [140]. Unexpectedly, the disruption of the \textit{ldh} gene in other \textit{L. lactis} strains did not affect their homolactic fermentative behavior, suggesting the induction of an alternative LDH, a hypothesis that was recently confirmed ([141], Gaspar, P., Coelho, P., Neves, A.R., Shearman C.A., Gasson, M.J. and Santos, H., unpublished data). Even more surprising was the response of \textit{L. lactis} FI17851, an \textit{ldh} minus strain constructed in the lab of Mike Gasson [139] and analysed by in vivo NMR in our lab (Fig. 8(a)) [20]. The analysis, in real time, of the metabolite pools derived from the metabolism of glucose by non-growing cells revealed the transient formation of high concentrations of mannitol, which was subsequently metabolized via mannitol 1-phosphate once glucose was depleted. The intracellular concentration of mannitol 1-phosphate was about 20 mM when glucose was still available and reached 80 mM when glucose was exhausted and mannitol was the sole carbon substrate (Table 1). The disruption of the \textit{ldh} gene induced a notable increase in the levels of the two enzymes directly involved in the synthesis of mannitol from fructose 6-phosphate: mannitol 1-phosphate dehydrogenase (34-fold) and mannitol 1-phosphate phosphatase. Therefore, in \textit{L. lactis} FI17851, the transient production of mannitol serves as an alternative pathway for NAD$^+$ regeneration. A similar kind of strategy, i.e., mannitol production, had been previously observed in a strain of \textit{Lactobacillus plantarum} deficient in both \textit{D-} and \textit{L-}lactate dehydrogenases [142], but Neves et al. [20] were the first to report the production of mannitol by strains of the genus \textit{Lactococcus}. This is an interesting finding, since mannitol has been claimed to possess health-promoting properties; thus the enrichment of foods with mannitol by in-situ production during fermentation could be a positive, clean strategy to obtain healthier fermented food products. In the human gut, mannitol can be converted to short-chain fatty acids (such as butyrate), which have been claimed to confer

![Fig. 8. Metabolism of glucose and production of mannitol in \textit{L. lactis} LDH-deficient strains. (a) Sequence of in vivo $^{13}$C-spectra obtained during the consumption of [1-$^{13}$C] glucose (20 mM) by a cell suspension of \textit{L. lactis} LDH-deficient strain (FI7851), under anaerobic conditions at 30 $^\circ$C, as monitored by $^{13}$C NMR. Cells were suspended in 50 mM KP, pH 6.5, at a protein concentration of 13.1 mg ml$^{-1}$. Each spectrum represents 30 s of acquisition. Glucose was added at time zero, each spectrum was acquired during the indicated interval, and processed with 5 Hz line broadening. (b) Consumption of [1-$^{13}$C]glucose (40 mM) and kinetics of product formation and mannitol 1-phosphate pool in \textit{L. lactis} FI9630 a LDH-deficient strain and (c) a derivative disrupted in the mannitol-PTS transport system (FI10089) under anaerobic conditions and pH 6.5. Metabolite concentrations were obtained from in vivo $^{13}$C NMR data. Symbols: (●) glucose; (■) lactate; (○) acetoin; (●) 2,3-butanediol; (●) ethanol; (▲) mannitol; (▲) mannitol 1-phosphate.](image-url)
Table 2
Major end-products (mM) from the metabolism of glucose (20 mM, unless otherwise stated) in several \textit{L. lactis} strains, under anaerobic or aerobic conditions, as measured in total cell extracts by \textsuperscript{13}C and \textsuperscript{1}H NMR.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Ethanol</th>
<th>Acetoacetic acid</th>
<th>Acetoin</th>
<th>2,3-Butanediol</th>
<th>Mannitol*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
<td>\textsuperscript{O\textsubscript{2}}</td>
<td>Anaerobic</td>
<td>\textsuperscript{O\textsubscript{2}}</td>
<td>Anaerobic</td>
<td>\textsuperscript{O\textsubscript{2}}</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>MG5267\textsuperscript{a}</td>
<td>32.8</td>
<td>31.8</td>
<td>1.7</td>
<td>2.6</td>
<td>0.2</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>NZ2500\textsuperscript{b}</td>
<td>30.4</td>
<td>1.0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>1.0</td>
<td>ND</td>
</tr>
<tr>
<td>NZ2007\textsuperscript{c}</td>
<td>8.1</td>
<td>2.2</td>
<td>6.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>MG1363</td>
<td>37.0</td>
<td>31.4</td>
<td>0.5</td>
<td>6.5</td>
<td>0.1</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>MG1363 (40 mM)</td>
<td>73.8</td>
<td>64.1</td>
<td>0.5</td>
<td>10.2</td>
<td>0.1</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>FI7851\textsuperscript{d}</td>
<td>9.9</td>
<td>5.5</td>
<td>2.6</td>
<td>5.9</td>
<td>0.5</td>
<td>1.6</td>
<td>9.5</td>
</tr>
<tr>
<td>FI9630\textsuperscript{e}</td>
<td>5.9</td>
<td>1.8</td>
<td>8.9</td>
<td>1.0</td>
<td>8.0</td>
<td>4.8</td>
<td>ND</td>
</tr>
<tr>
<td>FI9630\textsuperscript{c} (40 mM)</td>
<td>17.7</td>
<td>0.7</td>
<td>3.2</td>
<td>14.1</td>
<td>15.9</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td>FI10089\textsuperscript{g}</td>
<td>10.5</td>
<td>2.3</td>
<td>9.7</td>
<td>4.9</td>
<td>9.1</td>
<td>13.1</td>
<td>10.1</td>
</tr>
<tr>
<td>NOX\textsuperscript{h}</td>
<td>37.4</td>
<td>2.3</td>
<td>12.1</td>
<td>0.3</td>
<td>ND</td>
<td>10.4</td>
<td>ND</td>
</tr>
<tr>
<td>NOX\textsuperscript{c}</td>
<td>37.2</td>
<td>34.1</td>
<td>0.1</td>
<td>3.7</td>
<td>0.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NZ9000PK+/f (40 mM)</td>
<td>73.2</td>
<td>43.9</td>
<td>0.3</td>
<td>14.1</td>
<td>0.1</td>
<td>ND</td>
<td>9.2</td>
</tr>
</tbody>
</table>

The values reported are averages of two to four experiments and the accuracy ±5%.

\textsuperscript{a} MG5267, lactose-proficient derivative of MG1363; NZ2500, derivative of MG5267 overproducing \alpha-acetolactate synthase, NZ2007, derivative of MG5267 with a LDH inactivation (strains described in [19]; Neves, A.R., Ph.D. Thesis, 2001).

\textsuperscript{b} FI7851, derivative of MG1363 with LDH inactivation \[20,139\].

\textsuperscript{c} FI9630, derivative of MG1363 harboring a \textit{ldh} gene deletion, constructed by double crossover (food-grade strain) \[55\].

\textsuperscript{d} FI10089, derivative of FI9630 harboring a \textit{mfl} \textit{PTSM}\textsuperscript{\textit{mtl}} gene deletion, constructed by double crossover \[147\].

\textsuperscript{e} NOX\textsuperscript{\textit{+}} and NOX\textsuperscript{\textit{−}}, MG1363 derivatives NADH oxidase overproducing or deficient strains, respectively \[85\].

\textsuperscript{f} NZ9000PK+/f, NZ9000 derivative overproducing pyruvate kinase \[31\].

\textsuperscript{g} Value reported was calculated considering mannitol as extracellular.

\textsuperscript{h} ND, not detected; –, not determined.

Protection against the development of colon cancer \[143\]. Moreover, mannitol is a scavenger of hydroxyl radicals and a low-calorie sweetener, partially and slowly absorbed in the small intestine \[144,145\].

The report on mannitol production by LDH-deficient strains of \textit{L. lactis} fostered further metabolic engineering strategies directed to improve the yield of mannitol. We observed that the mannitol produced by strain FI17851 was taken up and rapidly metabolized after glucose depletion; therefore, it was apparent that the design of a mannitol-producing strain would have to consider the ability of \textit{L. lactis} to utilize mannitol as energy source for growth \[146\]. Thus, the disruption of the mannitol transport system in \textit{L. lactis} was envisaged in the metabolic strategy pursued in our team. A food-grade LDH-deficient strain was used as genetic basis for knocking out the genes \textit{mtlA} (encoding EII\textsubscript{C}M\textsubscript{tl}) or \textit{mtlF} (encoding EII\textsubscript{A}M\textsubscript{tl}). Non-growing cells of the double mutants (\textit{\textit{ΔldhAmtlA}}) and (\textit{\textit{ΔldhAmtlF}}) yielded mannitol, ethanol, 2,3-butanediol, and lactate as major end products, with approximately one-third of the carbon from glucose being channeled to the production of mannitol (Fig. 8(b) and (c) and Table 2) \[147\]. However, only very low levels of mannitol were produced by this strain during growth, and obviously this handicap should be overcome. A distinct engineering strategy was pursued by the team at WCFS with over-expression of the genes encoding the enzymes catalyzing the two reactions that convert fructose 6-phosphate into mannitol, in an attempt to force the mobilization of fructose 6-phosphate to the desired end-product. The mannitol 1-phosphate dehydrogenase gene from \textit{Lactobacillus plantarum} was overexpressed in different genetic backgrounds, but only 1% of glucose was converted to mannitol during growth \[148\]. However, when this strategy was combined with overexpression of the gene encoding mannitol 1-phosphate phosphatase of \textit{Eimeria tenella}, very high yields (up to 50%) of glucose-mannitol conversion were achieved \[149\]. The reported inability of this strain to utilize mannitol is probably due to a fortuitous deficiency in the mannitol transport system. Therefore, it seems that the major bottleneck for mannitol production resides at the level of mannitol 1-phosphate phosphatase, and the overexpression of the respective gene in an LDH-deficient background was a useful metabolic engineering approach.

6. Control and regulation of the glycolytic flux

As already mentioned, despite the relative metabolic simplicity and the wealth of information collected during several decades of research, we are still far from having a comprehensive understanding of sugar metabolism and regulatory pathways in \textit{L. lactis}. Furthermore, satisfactory answers to questions such as “which mechanisms govern the switch to mixed acid fermentation” or “what controls the glycolytic flux in \textit{L. lactis}” have
yet to be put forward. This task has mainly been hampered by the complexity of metabolic networks in living cells; metabolism is a highly branched system connected not only via carbon metabolites but also via redox (NADH/NAD\(^+\)) and energy (ATP/ADP/P\(_i\)) cofactors, and subjected to regulation at several levels.

6.1. Shift to mixed-acid fermentation and the impact of oxygen

Early studies reported a metabolic shift from homolactic (lactate production) to mixed acid fermentation (ethanol, acetate and formate production) in glucose-limited chemostat cultures [113] and during the metabolism of galactose [102] or maltose [74]. A deviation from homolactic fermentation has also been reported under aerobic conditions [150]. The regulation of this shift from homolactic to mixed acid fermentation was originally explained with models based on the allosteric modulation of two enzymes competing for pyruvate: LDH and PFL (Fig. 5) (for a review on pyruvate metabolism see [3]). During homolactic fermentation, regulation of the carbon flux was associated with high levels of FBP, which activates LDH and PK, directing the flux towards the production of lactate [113,114,151]. Conversely, the formation of end-products other than lactate was rationalized as being due to the reduction of LDH activity caused by lower levels of the effector, FBP, and to the relief of pyruvate formate lyase (PFL) inhibition by the concomitant decrease of DHAP and glyceraldehyde 3-phosphate [102,114]. The oxygen-induced metabolic shift would be explained by activation of the pyruvate dehydrogenase complex under aerobic conditions [150]. This concerted control of pyruvate metabolism resulted, under carbon-limited conditions, in the redirection of carbon to acetate/ethanol production and synthesis of ATP under conditions in which sugar metabolism would be insufficient to supply anabolic pathways with the energy required to fulfill cellular needs.

More recently, the observation that intracellular FBP is, under all conditions examined, sufficiently high to ensure full activation of LDH has questioned its role on the regulation of pyruvate distribution between LDH and PFL [115]. Garrigues and co-workers [115] proposed the role of the NADH/NAD\(^+\) ratio (redox state) in the modulation of both GAPDH and LDH activities, and suggested that homolactic metabolism was a consequence of high NADH/NAD\(^+\) ratios (0.05 or above), which restrain GAPDH but not LDH. This causes the accumulation of metabolites upstream of the former enzyme, such as FBP and the triose phosphates known to inhibit PFL. Mixed acid metabolism, observed for galactose or lactose, sugars taken up from the medium at significantly lower rates than glucose, was a consequence of lowered NADH/NAD\(^+\) ratios (lower than 0.05), relieving GAPDH inhibition and limiting LDH [115]. This metabolic model linking the glycolytic flux with pyruvate metabolism via the redox state of the cell obtained with strain L. lactis subs lactic NCDO 2118 (isolated from plant material) was further tested with L. lactis subs. cremoris strains MG1363 and MG1820 (laboratory strains derived from dairy strains), which differ in their ability to metabolize lactose rapidly. The conclusion was that the model postulating modulation of GAPDH activity by the NADH/NAD\(^+\) ratio as the main factor regulating the metabolic shift could be extended to other strains [152]. However, a similar study conducted with strain L. lactis subs pfl IL1403 showed that the type of fermentation was homolactic, regardless of the sugar used (glucose or galactose) and significant variations in both growth rate and sugar consumption [131]. This was interpreted as being due to low concentrations of both PFL and alcohol dehydrogenase. PFL was shown to be approximately three times higher during growth on galactose compared to glucose [153], leading to the suggestion that the level of PFL, rather than its allosteric regulation, controls the shift to mixed-acid fermentation in strain MG1363 [154,155]. Whether regulation of the metabolic shift is a consequence of translation activation of the pfl gene or allosteric relief of PFL activity both lead to a redirection of the flux away from lactate production. In a recent work it was found that cells grown on maltose have an increased level of alcohol dehydrogenase (and also phosphate acetyltransferase), which explained the deviation to mixed-acid products [156]. The finding that the pool of ATP and ADP completely inhibits this activity emphasized the role of alcohol dehydrogenase in the regulation of the shift to mixed acid fermentation, since high adenine nucleotides are associated with homofermentation [157]. Despite the wealth of information available (allosteric modulation of enzymes, metabolite levels, and more recently transcript and protein levels) the factors behind the metabolic switch to mixed acid fermentation remain elusive. However, at least two different allosteric mechanisms, involving the phosphorylated pools (FBP/P\(_i\)/ADP/ATP) and the redox balance (NADH/NAD\(^+\)) are likely to be involved. The contribution of each mechanism to the overall regulatory process is not fully known, but the possibility that the two mechanisms operate simultaneously should not be disregarded. Very recently, it was shown that LDHs are allosterically co-regulated by two mechanisms, the FBP/P\(_i\) pool or NADH/NAD\(^+\); some L. lactis strains are predominantly regulated by one of the mechanisms, whereas the majority bears the two types of regulation, operating to different extents depending on the strain [158]. Therefore, care should be taken when attempting to extrapolate regulatory models from one strain to others.

In spite of most regulatory models proposed so far being based on the in vitro kinetics of enzymes and determination of intracellular metabolites in cell
extracts, some studies have started to raise evidence supporting transcriptional control of central catabolic pathways [124,131,153,156]. A relevant example is the transcriptional activation of the las operon (pk, pk and ldh) by CcpA [124]. The product of phosphofructokinase, FBP, is a major allosteric effector of CcpA, PK, and LDH. Hence, both the transcriptional and allosteric activation play a part in the most pertinent feature of \textit{L. lactis}, which is the efficient production of lactate (Fig. 5).

\textit{L. lactis} is a Gram-positive facultative anaerobe with a fermentative metabolism, therefore constrained by the requirement to balance reactions producing and consuming NADH. However, some \textit{L. lactis} strains can benefit from the presence of oxygen and their growth is positively affected by low oxygen levels [159,160]. Sensitivity to aeration is extremely variable and strain dependent, and oxygen can partially or completely inhibit growth of some \textit{L. lactis} strains [150,159,161]. The ability to grow and survive in the presence of oxygen has been related to the presence of flavin-type NADH oxidases, NADH peroxidase and superoxide dismutase [150,159–164].

It is relevant to this discussion that oxygen has been shown to influence the carbon metabolism of \textit{L. lactis} [85,114,159,162,165]. Under anaerobic conditions, sugars may be converted to lactate or, alternatively, to mixed acid products formate, ethanol and acetate (Table 2). Oxygenation of cultures results in an altered redox state and greater NADH oxidase activity [85,159,165]. The catabolic carbon fluxes and the redox metabolism become uncoupled due to operation of an extra pathway for NAD$^+$ regeneration involving NADH oxidases; as a consequence, sugar catabolism is shifted towards mixed/flavor fermentation with formation of acetate and acetoin in detriment of lactate production [165,166]. This shift is not only related to the induction of the O$_2$-consuming oxidizing enzymes, but also to the competition for NADH between LDH and/or acetaldehyde and ethanol dehydrogenases and NADH-oxidases [85]. On the whole, the requirement to produce reduced end-products is diminished, and acetate (yielding extra ATP) or the production of redox neutral acetoin are favored (Table 2). The flavor diacetyl, a derivative of $\alpha$-acetolactate, can also be formed in the presence of oxygen [22,85].

Although the aerobic metabolic shift was explained on the basis of substrate level regulation, one can infer that altered gene expression in response to aeration can also contribute to the fate of pyruvate. Indeed, LDH levels under aerobic conditions have been shown to diminish to approximately half of that measured under anaerobiosis [159], and the transcription of the \textit{adhE} gene, encoding alcohol dehydrogenase, is repressed under vigorous aeration [167], while pyruvate dehydrogenase and $\alpha$-acetolactate synthase activities are induced under mild aeration [168]. Since the lactococcal PFL is inactivated by O$_2$ [153], acetate production, in \textit{L. lactis}, occurs mostly via the PDHc complex, though in certain LAB, such as \textit{Lactobacillus plantarum}, a pyruvate oxidase has been shown to play a key role in the aerobic metabolism [169]. In a recent study, this activity was identified in \textit{L. lactis}, but at very low levels, hence the contribution to the conversion of pyruvate is probably not significant [170].

The effect of oxygen on the distribution of end-products has long been disclosed, but its impact on the glycolytic metabolite pools only recently was investigated [85]. In our group, we resorted to in vivo $^{13}$C NMR analysis of non-growing cell suspensions to obtain a reliable picture of the oxygen-induced changes in glycolytic metabolite pools (Table 2) [85]. Both the maximum level of FBP and the rate of its consumption, as well as 3-PGA and PEP pools were higher in the presence of oxygen (Fig. 6(b) and (d)). Under an oxygen atmosphere, when NADH oxidase provides an additional path for NADH oxidation, the lower FBP accumulation is due to an increase of the flux through GAPDH caused by the lower NADH concentration. The same reasoning can be applied to explain the faster FBP consumption: GAPDH could sustain a higher flux, since the enzyme was less inhibited by the lower NADH concentrations. In fact, under aerobic conditions, no NADH accumulation was observed by in vivo NMR at the onset of glucose exhaustion (Fig. 9(a)) [85]. At this metabolic stage, accumulation of 3-PGA and PEP is driven by PK inhibition; under aerobic conditions, NADH consumption by NADH oxidase obviates the need to regenerate NAD$^+$ downstream of pyruvate, and thereby to overcome the PK bottleneck. Consequently, 3-PGA and PEP, derived from the metabolism of residual FBP, can accumulate to high levels (Fig. 10).

Surprisingly, genome analysis of \textit{L. lactis} subsp \textit{lactis} IL1403 indicated the presence of nearly all functions needed for aerobic respiration in this organism. \textit{L. lactis} has \textit{men} and \textit{cytABCD} operons, encoding proteins required for menaquinone synthesis and cytochrome \textit{d} biogenesis. It also has three genes involved in the late steps of heme synthesis, but not the genes required for the early steps [11]. Recent work with strain MG1363 has demonstrated the respiratory capacity of \textit{L. lactis} in the presence of exogenous heme and a metabolic shift to mixed-acid/acetoin fermentation during respiration [171]. Furthermore, it was observed that the addition of heme during aerated growth improves both biomass yield and long-term survival; under these conditions growth is diauxic, fermentation occurs first and is followed by respiration that starts at the onset of glucose depletion. Interestingly, CcpA was found to be involved in the regulation of the metabolic switch from fermentation to respiration, by controlling both expression of \textit{noxE} encoding NADH oxidase and heme uptake [172]. CcpA-mediated repression of \textit{noxE} has additional metabolic implications, since it points to the redox status.
(NADH/NAD\(^+\)) as an important signal in the regulation of carbon metabolism under aerobic conditions. The second mechanism implicated in regulation of the metabolic shift at the level of pyruvate is not ruled out by these findings. In fact, involvement of CcpA supports a strong role of FBP in the overall regulation process. On the whole, the data show that the aerobic metabolism of glucose is subjected both to allosteric (redox levels) and transcriptional regulation and the role of NADH oxidases should be considered in studies of metabolic regulation in this organism.

6.2. Control of the glycolytic flux

The information gathered from regulation studies is an important input for the development of predictive kinetic metabolic models that can be used as tools to direct metabolic engineering strategies. However, these
models often cannot consider all possible interactions of the system and therefore their utility is restricted to specific conditions close to those in which the parameters have been determined. In certain cases, the rational modification of metabolic fluxes can be achieved as long as the flux control is known. Determination of fluxes, directly using in vivo NMR or indirectly by resorting to the metabolic flux analysis toolbox, is nowadays straightforward, but it does not provide any quantitative measures of the control of flux. To quantify metabolic control, three major frameworks have been developed: metabolic control analysis (MCA), biochemical systems theory (BST) and Crabtree and Newsholme’s flux-oriented theory (interested readers can refer to the following books: [173–175]). Despite differences in formalism, the three approaches converge in the same basic concepts, such as the use of sensitivity analysis to identify targets for effective genetic (expression, regulation) or biochemical (kinetic) engineering of metabolic pathways. A dynamic model for central metabolism has been developed by Hoefnagel and co-workers [176] and it usefulness to identify key control points in L. lactis has been reported.

In L. lactis, the question as to which enzymes have control on the glycolytic flux has only recently received considerable attention [39]. In the framework of MCA, the control coefficient of a step in a metabolic pathway is defined as the fractional change in flux through the pathway induced by a fractional change in the enzyme activity concerned. Therefore, only the development of genetic tools that allowed for modulation of gene expression at selected levels [14] enabled the determination of control coefficients in the framework of MCA [39]. In the past, modulation of enzyme activities was achieved by utilization of specific irreversible enzyme inhibitors to alter the activity, instead of varying the concentration of the enzyme, which was restricted by the availability of specific irreversible inhibitors. Pooman et al. [177] observed that the capacity of the glycolytic pathway decreased rapidly in L. lactis cells starved of lactose, and that this reduction was accompanied by a decrease in GAPDH activity. The authors resorted to modulation of GAPDH activity by iodoacetate in cell suspensions of L. lactis strains ML3 and Wg2, and found a control coefficient value of 0.9. This high value implied that the overall glycolytic flux was largely controlled by GAPDH. More recently, GAPDH from L. lactis NCDO2118 was purified and characterized [34], and a substantial effect of the NADH/NAD⁺ ratio on the activity of the purified enzyme was observed. Therefore, after taking into account the elasticity of the GAPDH reaction towards the NADH/NAD⁺ ratio, which was shown to vary in the presence of iodoacetate, a flux control coefficient of 0.3 for GAPDH was determined. Though MCA gives a precise quantification of the contribution of each enzyme to the control of the carbon flux, the values obtained cannot be extrapolated to conditions or organisms other than those examined. Nevertheless, the flux control coefficient values found in both studies for GAPDH indicate that the enzyme had a significant influence on the control of the overall flux in L. lactis. This earlier work, based on inhibitor titrations, supported the more recent view that control of glycolysis was dependent on modulation of GAPDH activity by NADH/NAD⁺ in strains NCDO2118 and MG1363 [115,152]. Conversely, we observed by in vivo 13C NMR using non-growing cell suspensions that in a L. lactis LDH-deficient derivative of MG1363, GAPDH was able to support a high flux even in the presence of NADH concentrations much higher than those of the parent strain, indicating that the glycolytic flux in wild type L. lactis MG1363 is not primarily controlled at the level of GAPDH by NADH (Fig. 9(b), compare with Fig. 8(b)) [55]. It could be speculated that contradictory results were due to the use of non-growing cells in this study. More recently, the construction of a series of strains with modulated GAPDH activities showed that GAPDH has no control over the glycolytic flux at the wild-type level in L. lactis MG1363, either in growing or resting cells [178]. These authors speculate that the results obtained with the slow-growing L. lactis NCDO2118 may be an underestimate of the flux control exerted by GAPDH in fast-growing cells as predicted for L. lactis Wg2 or that iodoacetate might inhibit other enzymes besides GAPDH. Additionally, Jensen and collaborators investigated whether phosphofructokinase, pyruvate kinase or lactate dehydrogenase had control over the flux through glycolysis and their conclusion was that there is virtually no flux control by any of these key enzymes [39,179]. Since the control of the flux through a pathway can also reside in processes outside the pathway itself, the demand for ATP was tested by modulating the activity of ATPase [180]. Increasing the expression of ATPases uncoupled biomass production from glycolysis and resulted in lower ATP/ADP ratios. Interestingly, it was found that the glycolytic flux in strain MG1363 was not increased in cells steadily growing on glucose, but it was 3-fold stimulated in non-growing cells. This indicates that under growing conditions, and for this strain, glycolysis is already running at maximal rate. The glycolytic flux in non-growing cells is lower because there are fewer ATP-consuming processes. Therefore, expression of ATPase resulted in increased flux. At the time this manuscript is written, it can be accepted that phosphofructokinase, PK, GAPDH and LDH have virtually no flux control in strain MG1363. The demand for ATP seems to exert some control when the glycolytic flux is much lower than the maximal capacity [39]. Assessment of flux control by other processes, including the other glycolytic enzymes, product efflux and sugar transport should therefore be pursued. Most likely the flux is distributed...
over many steps, perhaps in combination with ATP consuming processes and sugar transport. Our results with MG1363 derivatives carrying deletions in different glucose transport systems showed that the nature of the transporter had great impact on the glycolytic flux and the dynamics of intracellular pools [Pool, W.A., Neves, A.R., Kok, J., Santos, H and Kuipers, O.P., unpublished results]. Hitherto, a satisfactory answer to the question of what controls the glycolytic flux in _L. lactis_ is still lacking.

7. Concluding remarks

The availability of genome sequences paved the way for the use of recently developed methodologies for global analysis such as transcriptomics and proteomics. DNA microarrays provide a powerful tool for analyzing transcription profiles and are expected to give valuable information on metabolic pathways, identification of coordinated regulation of genes and regulatory circuits that mediate cellular responses [181,182]. This technology is now available for _L. lactis_ [183]. Recently, reference maps for the alkaline and acidic proteome of _L. lactis_ have been developed [156,184] and an advanced on-line database for access of proteomes and two-dimensional gels has just been made available for _L. lactis_ [185]. These technologies provide powerful tools to approach biological complexity. Currently, the available data on the time dependent pool concentrations of relevant intracellular metabolites obtained by NMR are used to generate a comprehensive dynamic model for the interplay between intervening metabolites and enzymes. The final ambitious goal is to integrate the huge amount of information provided by metabolic, transcriptional and proteomic analysis in the framework of a dynamic model of _L. lactis_ central metabolism.

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References


