Lactobacilli and pediococci as versatile cell factories - Evaluation of strain properties and genetic tools

Bosma, Elleke Fenna; Förster, Jochen; Nielsen, Alex Toftgaard

Published in:
Biotechnology Advances

Link to article, DOI:
10.1016/j.biotechadv.2017.04.002

Publication date:
2017

Document Version
Version created as part of publication process; publisher's layout; not normally made publicly available

Biotechnology Advances xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Lactobacilli and pediococci as versatile cell factories – Evaluation of strain properties and genetic tools

Elleke F. Bosma, Jochen Forster, Alex Toftgaard Nielsen*

The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet B220, 2800 Kgs. Lyngby, Denmark

ARTICLE INFO

Keywords:
Strain development
Genetic tools
Temperature
Stress tolerance
Second generation biomass
Third generation biomass

ABSTRACT

This review discusses opportunities and bottlenecks for cell factory development of Lactic Acid Bacteria (LAB), with an emphasis on lactobacilli and pediococci, their metabolism and genetic tools. In order to enable economically feasible bio-based production of chemicals and fuels in a biorefinery, the choice of product, substrate and production organism is important. Currently, the most frequently used production hosts include *Escherichia coli* and *Saccharomyces cerevisiae*, but promising examples are available of alternative hosts such as LAB. Particularly lactobacilli and pediococci can offer benefits such as thermotolerance, an extended substrate range and increased tolerance to stresses such as low pH or high alcohol concentrations. This review will evaluate the properties and metabolism of these organisms, and provide an overview of their current biotechnological applications and metabolic engineering. We substantiate the review by including experimental results from screening various lactobacilli and pediococci for transformability, growth temperature range and ability to grow under biotechnologically relevant stress conditions. Since availability of efficient genetic engineering tools is a crucial prerequisite for industrial strain development, genetic tool development is extensively discussed. A range of genetic tools exist for *Lactococcus lactis*, but for other species of LAB like lactobacilli and pediococci such tools are less well developed. Whereas lactobacilli and pediococci have a long history of use in food and beverage fermentation, their use as platform organisms for production purposes is rather new. By harnessing their properties such as thermotolerance and stress resistance, and by using emerging high-throughput genetic tools, these organisms are very promising as versatile cell factories for biorefinery applications.

1. Introduction

In order to satisfy the increasing world-wide demand for fuels and chemicals in a sustainable way, alternatives to fossil resources are needed. Whereas several renewable resources such as wind, water and solar power can be used for supplying energy and fuels, microbial fermentation of biomass-derived sugars in a biorefinery is the most important alternative for chemical production. Some products such as lactic acid have a long history of commercial production via microbial fermentation, but for other products economically feasible production has not yet been achieved. The product, substrate as well as the production host all play a role in determining this. Currently, the most frequently used production hosts include *E. coli* and *S. cerevisiae*, largely due to the availability of an array of efficient genetic engineering tools. However, these organisms are not always the most suitable hosts for biochemicals production, and promising examples of alternative hosts such as thermophiles or Lactic Acid Bacteria (LAB) are emerging due to a range of desirable features (Bosma et al., 2013; Gaspar et al., 2013; Lin and Xu, 2013; Mazzoli et al., 2014). This review provides an overview of the application of LAB for biotechnology purposes in the context of the biorefinery, with an emphasis on lactobacilli and pediococci. First, the introduction will reflect on the requirements of substrates, organisms and products in a biorefinery. Second, this context will be used to examine the properties and metabolism of LAB. This will be illustrated with some experimental data on stress tolerance, substrate utilization, products, temperature range, and genetic accessibility. Third, development of LAB into platform organisms will be further explored by examining genetic tool development and currently available examples of metabolic engineering towards different end products. This section will provide an overview as well as discussion of opportunities for further development. Lastly, concluding remarks will be given on the suitability and future development possibilities of LAB and their products for biotechnology applications in a biorefinery context.

* Corresponding author.
E-mail address: atn@biosustain.dtu.dk (A.T. Nielsen).

http://dx.doi.org/10.1016/j.biotechadv.2017.04.002
Received 13 February 2017; Received in revised form 29 March 2017; Accepted 3 April 2017

1.1. Biorefinery considerations: product, substrate and production host

Chemicals are typically divided into specialty, fine and bulk chemicals. Biofuels and bulk chemicals such as plastics used for packaging are low-cost and produced in large quantities. Specialty and fine chemicals such as drugs and vitamins, on the other hand, are more expensive and produced in smaller quantities (Budzianowski, 2017). Most specialty, fine or bulk chemicals are today produced by chemical synthesis, but in many cases, microbially produced chemicals can directly replace existing products that are now produced from fossil resources. Furthermore, microorganisms can also be used to synthesize compounds that are nowadays not produced via other ways, thereby adding innovative products to the market (Budzianowski, 2017). Microbially produced chemicals that are direct replacements for existing chemicals need to have at least the same or lower price than the existing ones to be economically feasible. This is generally more challenging for bulk than for specialty products, since bulk products are expected to remain very low-cost as long as oil prices do not dramatically increase. In order to enable the production of sustainable bulk biochemicals, it is therefore necessary to develop alternative and cost-efficient production methods. These considerations should be taken into account with the choice of product, but also the substrate and production host to be used in a biorefinery (Bosma et al., 2013; Budzianowski, 2017; Budzianowski and Postawa, 2016).

First generation biorefinery processes use (purified) sugar from food biomass, such as corn, sugar cane and beet, and wheat, thereby interfering with the food and feed chain. Second generation biorefineries use non-edible biomass, in which the focus has so far been mainly on lignocellulosic material. Lignocellulosic biomass can be forestry or agricultural residues, generally the non-edible parts of plants such as sugarcane bagasse and corn stover. The sugar fraction is composed of cellulose and hemicellulose, which are polymers of mainly glucose, xylose and arabinose and are tightly packed together with the structural component lignin (Seidl and Goulart, 2016). Third generation alternatives such as seaweed, household waste, or very low demanding crops are gaining interest and have additional advantages such as that they do not require arable land or freshwater (Jiang et al., 2016; Kawai and Murata, 2016). The second and third generation substrates are more difficult to utilize for the currently used microorganisms. This may lead to high production costs due to need for expensive pretreatment and because the substrates are not completely utilized. Also, no final solution has been found yet for inhibitory and non-fermentable compounds, such as furfural, lignin or salts, that are often found in significant concentrations in hydrolyzed biomass (Jönsson and Martín, 2016).

The most frequently used production organisms so far have been *E. coli* and *S. cerevisiae*, mainly because they are well-studied and have efficient genetic tools available. This has led to the production of a large variety of both natural and non-natural products with impressive titers. Yet, there are only few examples of bulk biochemicals that are being produced at industrial scale, which is largely because of the high costs of the processes. Metabolic engineering has substantially broadened the substrates that can be used by these organisms, ranging from xylose to cellulose and alginate. However, there are many cases in which other organisms have shown to be very promising alternative production hosts with possible superior performance over model organisms. For example, organisms that are naturally and efficiently capable of using complex substrates, are highly resistant to toxic compounds present in or as substrate, medium, process or product, or are able to grow at high temperatures (Bhalla et al., 2013; Boguta et al., 2014). Fermentation at higher temperatures can improve process efficiency by reducing cooling costs and contamination risks and increasing product and substrate solubility. Section 2.3.1 will provide more detailed examples of such cases and explain the associated advantages and disadvantages. The main downside of such non-model organisms is that they are less well-studied and understood, and genetic tools are either absent, underdeveloped or lack throughput, which limits their use as versatile platform organisms. Whereas it might be an option for selected products to use a specific organism only for that single product, it is generally desirable in a biorefinery to use a single platform organism that can be metabolically engineered to produce any desired product in high yields and productivities without (major) byproducts, as this facilitates process optimization, strain handling, etc. Therefore, there is an increasing interest in non-model organisms and the adjustment of existing genetic tools for these organisms is currently accelerating, opening up the possibility of utilizing organisms that have superior characteristics as cell factories. A large part of this review will be dedicated to explain the development of genetic tools in detail for lactobacilli and pediococci, as both these groups of LAB can be found as typical contaminants in today's biorefineries, and therefore can be considered as promising platform organisms for chemical and fuel production.

1.2. Lactic acid bacteria (LAB)

LAB are probably among the microorganisms with the longest track record of use by humans and they have been important for centuries in the production of fermented foods and beverages (Bourdichon et al., 2012; de Vos, 2011; Gaspar et al., 2013). Owing to their long history of safe use, wide variety of metabolic end products and metabolic versatility, applications of LAB nowadays range from the traditional use in food and beverage fermentation to production of fuels and chemicals, food ingredients and pharmaceuticals, to delivery vehicles for vaccines and drugs, as well as pre- and probiotics (Fig. 1) (Gaspar et al., 2013). Whereas a few species such as *Streptococcus pneumoniae* are known as pathogens, the majority of LAB species are known to be harmless or even beneficial, and many species have been Generally Recognized As Safe (GRAS) for use by the FDA (USFDA, 2017).

LAB form a very heterogeneous group of organisms and especially the family of *Lactobacillaceae* is exceptionally large and diverse, which

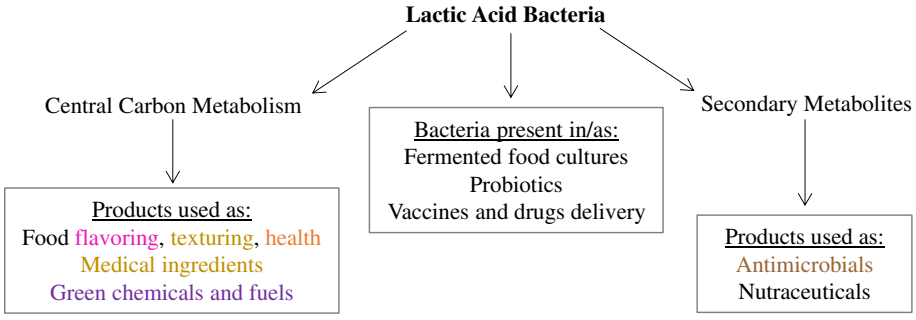

Fig. 1. Overview of applications and products of LAB. The colours of central carbon metabolites correspond with the products in Fig. 3B, where their respective production pathways are shown in detail.

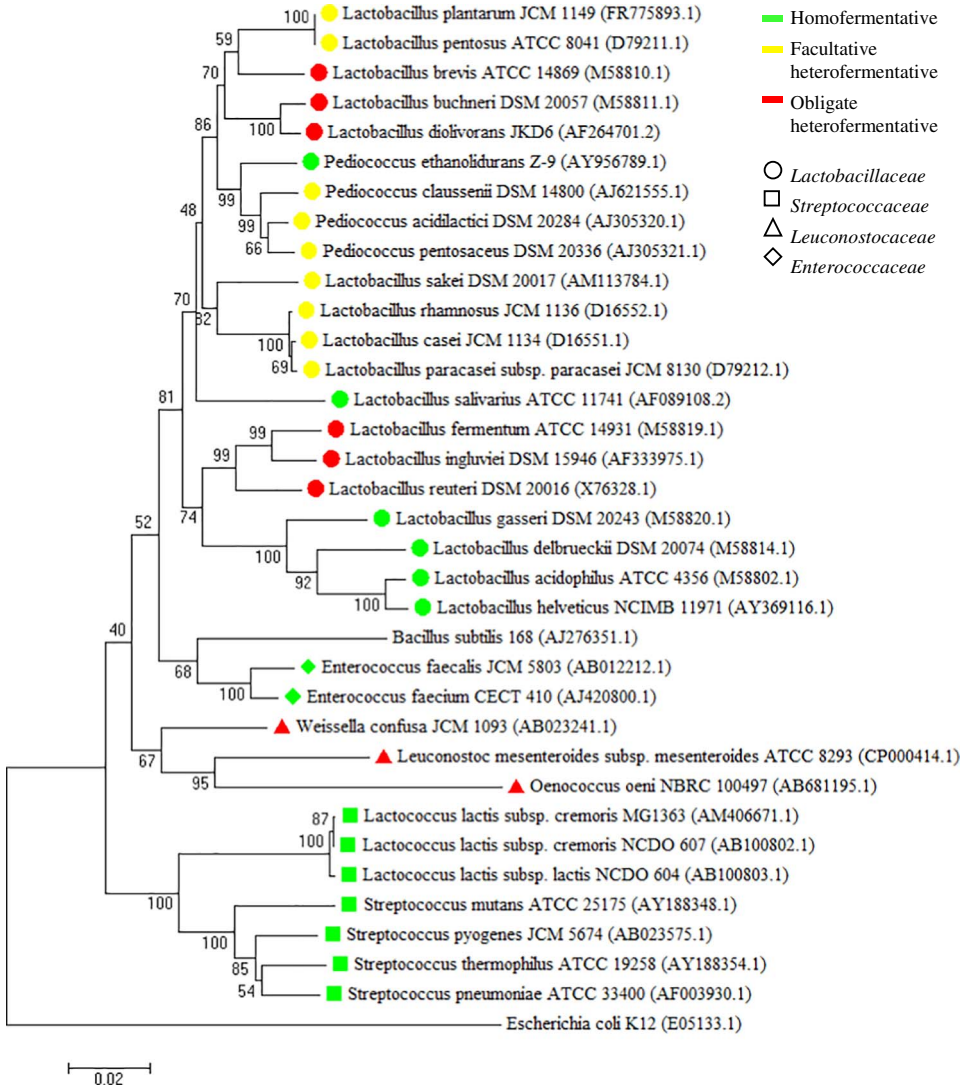

Fig. 2. Neighbor-joining tree of 16S rRNA gene sequences of LAB type strains of species discussed in the text as well as several others representative for other LAB families. The non-type strain *L. lactis* MG1363 is included since this is one of the most-used model LAB. *E. coli* and *B. subtilis* are shown for comparison. The colors correspond to those in Fig. 3A: green are homofermentative species; red are obligate heterofermentative and yellow are facultative heterofermentative species. The different shapes represent families: circles: *Lactobacillaceae*; squares: *Streptococcaceae*; triangles: *Leuconostocaceae*; diamonds: *Enterococcaceae*. Numbers at the nodes represent bootstrap values out of 1000 replicates. GenBank accession numbers are shown in brackets. Mega7 was used for alignment using ClustalW and subsequent construction of the Neighbor-joining tree using the Maximum Composite Likelihood method.

makes taxonomy complicated and phenotypic traits highly variable (Sun et al., 2015; Zheng et al., 2015) (Fig. 2). At the same time, this makes the genus highly interesting to study both from a fundamental and from an applied perspective. Despite the diversity, LAB do share certain phenotypic traits: they are low-GC Gram-positive organisms, facultatively anaerobic or aerotolerant, non-sporulating and non-motile and they ferment a wide range of carbon sources with lactic acid as the main end-product. They are highly directed towards a fermentative lifestyle and depend largely on substrate level phosphorylation for energy production, as they lack a functional respiratory chain (Chassy and Murphy, 1993; de Vos and Hugenholtz, 2004; Endo and Dicks, 2014). They comprise cocci as well as bacilli and have a wide temperature growth range. Although the optimum growth temperature for most LAB is 30 to 37 °C, many species have a very wide growth temperature range with the upper limit often around 45 °C (Franz et al., 2014; Pot et al., 2014). Several species of especially lactobacilli and pediococci, such as *L. delbrueckii* and *P. acidilactici*, are highly thermotolerant and grow well up to around 50 °C (Franz et al., 2014; Monteagudo et al., 1997; Pot et al., 2014). Generally, LAB have a simple metabolism and a small genome of 1.7–2.7 Mb, with a few outliers of 1.23 Mb and 4.91 Mb (Sun et al., 2015). LAB are part of the phylum *Firmicutes*, class bacilli, order *Lactobacillales* and are then divided over six different families: *Lactobacillaceae*, *Streptococcaceae*, *Leuconostocaceae*, *Enterococcaceae*, *Carnobacteriaceae* and *Aerococcaceae* (Fig. 2). *Lactococcus lactis*, belonging to the *Streptococcaceae* family, is one of the most-studied model organisms among LAB. The lactobacilli and pediococci are genera belonging to the *Lactobacillaceae* family, of which especially several lactobacilli have a long track record in fundamental research and applications such as food fermentations and probiotics. While LAB have been used as starter cultures and in food fermentations already for a very long time, their use as platform organisms in a biorefinery is relatively new. Interest in their use as cell factories is increasing due to their beneficial properties such as high tolerance to ethanol, salts, low pH and wide temperature ranges. These and other properties will be discussed in the next sections.

2. Characteristics of lactobacilli and pediococci and evaluation of their potential as platform organisms

The phenotypic variability of LAB is very large and some species or

strains might be more suitable as platform organism than others. Owing to their long history of study, many properties of LAB are known and a selection of possible platform organism candidates can be made, but there is also considerable strain-dependent variation (Huys et al., 2012), making it worthwhile to test for required properties. Descriptions of growth characteristics and transformation have previously been reported for a range of different LAB strains, but these experiments are typically performed in different growth media and under different growth conditions. With this in mind, combined with the large strain diversity known to exist among LAB, we have included a screening of 9 *Lactobacillus* and 18 *Pediococcus* strains for biotechnologically relevant properties. The strains were selected because they met one or more of the following criteria: 1) known to be an industrial ethanol plant contaminant and thus likely to be robust for industrial applications, 2) genetic accessibility has been described for that strain or a close relative, 3) the species or strain can grow on elevated temperatures. Altogether, we aimed at identifying a strain that is robust and suitable for industrial application: i.e. tolerant to several stresses, relatively little nutrient requirements, genetically accessible and able to grow in a wide temperature range. In the sections below, we provide experimental data for the selected strains and use them as examples to review each of these criteria and different organisms in a biorefinery context.

2.1. End product formation and properties of central carbon metabolism in heterofermentative and homofermentative LAB

LAB can be divided into homo- and heterofermentative species (Figs. 2 and 3A). In homofermentative and facultatively heterofermentative LAB, lactic acid is essentially the only product and glucose is catabolized via the Embden–Meyerhof–Parnas (EMP) pathway. Facultative heterofermenters are capable of utilizing pentose sugars via the PKP pathway. Obligate heterofermentative strains, on the other hand, catabolize sugars via the phosphoketolase pathway and hence produce an equimolar mixture of lactate, CO_2 and ethanol or acetate (Endo and Dicks, 2014). Either L-lactate, D-lactate, or both enantiomers are produced depending on whether the strain encodes an L-*ldh*, D-*ldh* or both, as well as on the presence of lactate racemases that interconvert the two enantiomers. All heterofermentative LAB contain a two-domain aldehyde/alcohol dehydrogenase enzyme for the production of ethanol (Zheng et al., 2015). Some homofermentative species also contain this enzyme, in which case they also contain a pyruvate-formate lyase (PFL) for the anaerobic conversion of pyruvate into acetyl-CoA and formate, an enzyme that is not found in heterofermentative LAB (Zheng et al., 2015). Homofermentative LAB include *Enterococcaceae*, *Streptococcaceae* and several *Lactobacillaceae*, while heterofermentation is found in species of *Lactobacillaceae* and in *Leuconostocaceae* (Endo and Dicks, 2014) (Fig. 2). Homofermentative strains are also capable of producing ethanol and acetate during mixed acid fermentation, which is different from heterofermentation since these strains still use the EMP pathway, while only the end products are changed (Fig. 3A). Such a shift to mixed acid fermentation occurs for example during carbon limitation, low growth rates or a change in oxygen concentration (Endo and Dicks, 2014; Goel et al., 2012; Kandler, 1983). In the presence of oxygen, NADH oxidase (NOX) becomes active, and due to its high affinity for NADH it outcompetes LDH for the available NADH, causing a shift from homolactic to mixed acid fermentation under aerobic conditions, with the final products depending on culture pH (Lopez de Felipe et al., 1997). This principle has also been applied in a classic co-factor engineering study, in which *nox* was overexpressed in *L. lactis* to cause a shift towards the mixed acid fermentation products diacetyl and acetoin (Lopez de Felipe et al., 1998).

The use of the PPP for pentose utilization in LAB is rare, but in a strain of the homofermentative species *L. lactis*, the use of PPP for pentose fermentation was shown (Tanaka et al., 2002). Some thermophilic lactate producers such as *Bacillus coagulans* and *Bacillus smithii* have been shown to be homofermentative and use the PPP pathway when grown on pentoses (Bosma et al., 2015; Patel et al., 2006). For lactate production from pentoses, the use of the PKP is disadvantageous since the yields are only half of when the PPP is used, which yields lactate as the sole end product (Fig. 3A). However, for other products, the use of the PKP pathway is not necessarily problematic and can even be beneficial (Henard et al., 2015) and the most important factor is the pentose uptake rate. Regulation of the split ratio between PPP and PKP was shown in an engineered *Lactobacillus brevis* strain to be strongly dependent on the phosphoketolase enzyme (Guo et al., 2014) and hence, this might be a target for future engineering efforts to optimize fluxes though these pathways.

An important difference in central carbon metabolism enzymes between heterofermentative and homofermentative LAB that determines the use of PKP or EMP seems to be the presence or the activity of phosphofructokinae (PFK) and fructose-1,6-biphosphate aldolase (FBA) (Fig. 3A). Although there are contradictory reports, in obligately heterofermentative strains, a *pfk* gene is generally absent and many of these strains also lack *fba* (Sun et al., 2015). In general, surprisingly little is known at the fundamental level about the regulation, gene and enzyme presence and flux distribution of PKP, PPP and EMP pathways in LAB and there are large strain-dependent variations that are not yet fully understood (Burgé et al., 2015; O'Donnell et al., 2013; Papagianni and Legiša, 2014; Saulnier et al., 2011; Zheng et al., 2015; Årsköld et al., 2008). Contrary to what is generally assumed regarding the absence of *pfk* in heterofermentative LAB, for the *L. reuteri* strain ATCC 55730 and for the heterofermentative *Lactobacillus plantarum* and *Lactobacillus salivarius* strains, the presence of all genes necessary for a complete EMP was shown (Årsköld et al., 2008). For *Lactobacillus reuteri* ATCC 55730, the PKP was the main pathway for glucose utilization and the EMP was simultaneously used, serving as a shunt to gain more NADH and ATP (Årsköld et al., 2008). Heterologous expression of *pfk* in *L. reuteri* ATCC 55730 as well as in *L. brevis* strongly enhanced flux through the EMP (Guo et al., 2014; Papagianni and Legiša, 2014). *L. reuteri* type strain DSM 20016 does not seem to encode a *pfk* (Sun et al., 2015), and this strain was recently also shown to barely utilize the EMP (Burgé et al., 2015). However, the relatively low EMP activity in this strain might hint at an alternative PFK activity, as was also assumed in the metabolic model developed for this species (Santos, 2008). The study by Burgé et al. showed large differences in EMP and PKP flux distribution between three different *L. reuteri* strains. The type strain DSM 20016 was shown to barely utilize the EMP but almost solely the PKP (Burgé et al., 2015). In contrast, strain DSM 17938 (a plasmid-free derivative of ATCC 55730, one of the strains shown to encode *pfk*) used exclusively the EMP during exponential growth and switched to the PKP in the stationary phase, and strain ATCC 53608 showed a similar trend but with simultaneous use of the PKP and EMP during exponential growth (Burgé et al., 2015). In summary, care should be taken when comparing different LAB strains, even within the same species, and more research is needed to shed light on the differences and improve the fundamental understanding of central carbon metabolic fluxes. This is also crucial in order to develop metabolic engineering strategies and use LAB as industrially relevant platform organisms.

In addition to the most common end products lactate, acetate and ethanol discussed above, which are produced under most standard fermentation conditions on glucose, LAB are capable of naturally producing many more different end products when grown on other carbon sources than glucose or under aerated conditions (Endo and Dicks, 2014; Kandler, 1983; Papagianni, 2012). The products can include 1,2-propanediol, diacetyl, acetaldehyde, acetoin and alanine, but also exopolysaccharides (EPS) and polyols such as mannitol (Fig. 3B). Details about when and how these products are produced will be discussed in Section 4 that describes metabolic engineering of LAB to further enhance production of these compounds. Also the kinds and amounts of produced organic acids vary highly among species (Özcelik et al., 2016). Complex pathways to for example vitamins are

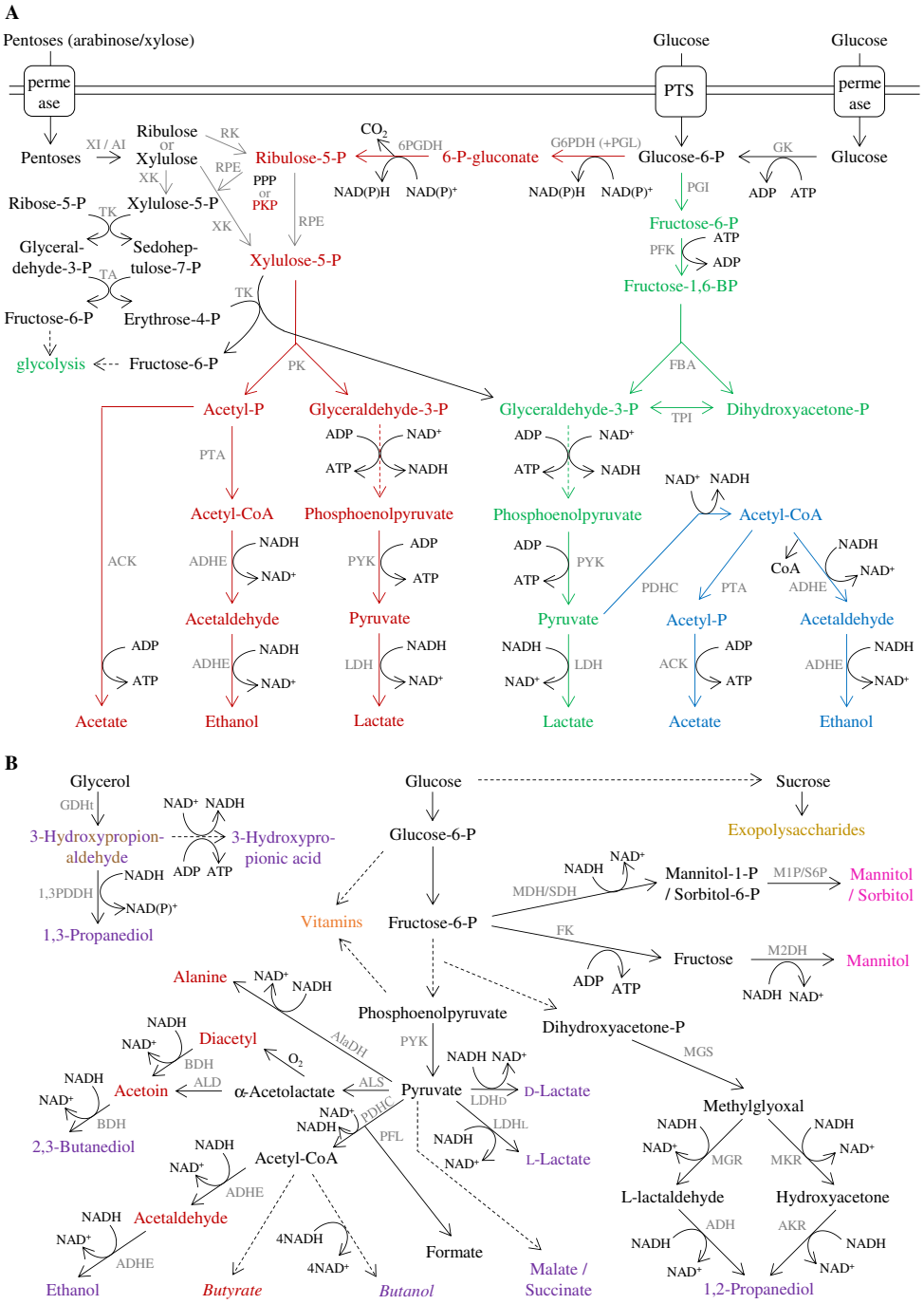

Fig. 3. Overview of naturally present metabolic pathways in lactobacilli and pediococci. A. Metabolic pathways in LAB under standard fermentative conditions. The pathways shown are the major pathways active under 'normal' industrial and laboratory production conditions on glucose or pentoses (xylose or arabinose). Pathways to products only produced under very specific conditions are depicted in part B. Red: Heterolactic fermentation via phosphoketolase pathway (PKP); green: homolactic fermentation via Emden-Meyerhof Parnas pathway (EMP); Blue: mixed acid fermentation in homolactic species via Emden-Meyerhof Parnas pathway; black: pentose phosphate pathway and its connection to PKP and EMP pathways. Grey arrows indicated different options for one molecule. Dashed arrows indicate multiple enzymatic steps. Enzymes are depicted in grey capitals. Enzyme abbreviations: PTS: phosphotransferase system; GK: glucokinase; G6PDH (+ PGL): glucose-6-P dehydrogenase (+ phosphoglucolactonase, this can also be a non-enzymatic step); 6PGDH: 6-P-gluconate dehydrogenase; RPE: ribulose epimerase; RK: ribulokinase; XI/AI: xylose isomerase/arabinose isomerase; XK: xylulokinase; TK: transketolase; TA: transaldolase; PK: phosphoketolase; FBA: fructose bisphosphate aldolase; TPI: triose-phosphate isomerase; PYK: pyruvate kinase; LDH: lactate dehydrogenase; PDHC: pyruvate dehydrogenase complex; PTA: phosphotransacetylase; ACK: acetate kinase; ADHE: bifunctional aldehyde and alcohol dehydrogenase. B. Overview of possible natural end products of metabolism of LAB under different conditions (see text for more details). Dashed lines indicate multiple enzymatic reactions. Colour coding is the same as in Fig. 1: pink: flavoring compounds; yellow: texturing and medical ingredients; orange: health products; purple: chemicals and fuels. For the compounds in italics, some enzymes are present in some LAB (more than in most other species) but metabolic engineering is required to complete the pathway for production. Enzyme abbreviations: GDHt: glycerol dehydratase; 1,3PDDH: 1,3-propanediol dehydrogenase; MDH/SDH: mannitol dehydrogenase/sorbitol dehydrogenase; M1P/S6P: mannitol-6-P dehydrogenase/sorbitol-6-P dehydrogenase; FK: fructokinase; M2DH: mannitol-2-dehydrogenase; MGS: methylglyoxal synthase; MGR: methylglyoxal reductase; ADH: (lact)aldehyde dehydrogenase; PYK: pyruvate kinase; PFL: pyruvate-formate lyase; PDHC: pyruvate dehydrogenase complex; LDH: lactate dehydrogenase; ALS: acetolactate synthase; AlaDH: alanine dehydrogenase; ALD: acetolactate decarboxylase; BDH: butanediol dehydrogenase; ADHE: bifunctional aldehyde and alcohol dehydrogenase.

difficult to engineer and thus it is advantageous if the organism already contains these.

We evaluated the most common central carbon metabolism end products and the enantiomer of lactate produced in the 27 screened strains in MRS medium (Fig. S1). The results are in line with species descriptions in literature, with minor strain-dependent variations in the amounts produced and in the ratio between D- and L-lactate (Fig. 2, Fig. S1). All 18 tested pediococci were found to be homolactic, as well as all *L. plantarum* strains. *L. reuteri*, *Lactobacillus fermentum* and *L. brevis* showed heterofermentation with ethanol as byproduct. All strains produced a mixture of D- and L-lactate except *Lactobacillus delbrueckii*, which produced only the D-enantiomer, and *Pediococcus claussenii*, which produced almost only L-lactate. *Pediococcus ethanolidurans* and *L. brevis* produced minor amounts (0,08 and 0,02 g/L, respectively) of 1,2-propanediol (Fig. S1).

2.2. Substrate utilization

Whereas practically all microorganisms are able to utilize first generation biomass in the form of pure glucose or sucrose, their ability to utilize second and third generation biomass such as lignocellulose or macroalgae are highly species- and strain-dependent. Consolidated bioprocessing (CBP), in which no saccharolytic enzymes are added and the microorganisms directly utilize the polymeric sugars present in lignocellulose, is being investigated with wild-type polysaccharide-degrading organisms such as clostridia, thermoanaerobacteria and geobacilli for ethanol and lactate production (Bosma et al., 2013; Daas et al., 2016; Olson et al., 2015). However, yields of CBP are still relatively low and almost all the organisms only utilize either the hemicellulose or the cellulose fraction and not both. Hence, in industrial CBP processes for bioethanol production using yeast, enzymes still need to be added though at reduced levels. A currently more feasible alternative is the use of simultaneous saccharification and fermentation (SSF), in which saccharolytic enzymes are added to the biomass to degrade the polymers into monomeric sugars that can be fermented by microorganisms in the same reactor. Several SSF processes at elevated temperatures resulting in high lactic acid yields have been described (Ou et al., 2011; van der Pol et al., 2016; Zhao et al., 2013), but co-utilization of pentoses and hexoses is still challenging (Ou et al., 2011; Zhao et al., 2013). Co-utilization of substrates without catabolite repression for the production of lactic acid has been achieved in *E. coli* via metabolic engineering to remove the catabolite repression (Lu et al., 2016) but was shown to naturally occur in *Lactobacillus buchneri* and *L. brevis* (Kim et al., 2009; Liu et al., 2008), in *L. plantarum* after insertion of the xylose-utilization genes (Hama et al., 2015; Zhang et al., 2016), as well as in the thermophile *Geobacillus thermodenitrificans* (Daas et al., 2016). In a recent study, *Lactobacillus casei* was used for the conversion of sugars from hydrolyzed algal cake, the waste product of biodiesel producing-algae (Overbeck et al., 2016).

The substrate utilization range of LAB is broad and highly species- and even strain-specific (Franz et al., 2014; Pot et al., 2014; Zheng et al., 2015). Most LAB are able to utilize both pentoses and hexoses derived from lignocellulose, and some are able to utilize short polymers such as cellobiose, but none are capable of utilizing longer polymers. Polymeric lignocellulosic substrates are also not utilized by current platform organisms such as *E. coli* and *S. cerevisiae*. Whereas *E. coli* naturally utilizes both pentoses and hexoses, *S. cerevisiae* only naturally utilizes hexoses and has been engineered to utilize the pentose fraction (Olofsson et al., 2008). We evaluated the substrate utilization spectra of a subset of the 27 selected strains using API-50 CH tests (Biomerieux). The results were mostly in line with literature but also showed some strain-dependent variation (Table 1). A high strain-specificity and relatively poor reproducibility is known for substrate utilization in LAB (Huys et al., 2012) and therefore screening of strains is worthwhile. Glucose, D-xylose and L-arabinose are the main components of lignocellulose. Glucose and L-arabinose were fermented by all selected strains, and D-xylose was utilized by all strains except *L. reuteri*. Cellobiose was also utilized by all strains.

2.3. Growth conditions

For an industrial production strain, it is crucial that it is robust against contamination with other microbes and phages, but also against several stresses that might occur, especially in large fermenters, such as fluctuations in temperature, pH and oxygen levels. The ability to run the process at high temperature and low pH can substantially decrease the costs (Ou et al., 2009; Zhao et al., 2013). The strain should be able to tolerate high concentrations of the products and substrates, as well as potential inhibitors present in the used substrate, especially if this not a pure sugar but derived from lignocellulosic or other raw biomass (Abdel-Rahman and Sonomoto, 2016; Jönsson and Martín, 2016). Furthermore, the strain should have minimal nutrient requirements. This is especially important when bulk chemicals or fuels are produced that have low added value and should be as cheap as possible to compete with alternatives. Oxygen transfer is often a limiting and expensive parameter for large scale reactors. Also, the theoretical yields are for many products higher under anaerobic conditions, and anaerobic processes are therefore often desirable (Weusthuis et al., 2011). Since strict anaerobes are typically difficult to handle and the medium needs to be made anaerobically, facultative anaerobes or aerotolerant species such as LAB but also thermophilic bacilli have substantial benefits as platform organisms.

For the 27 selected strains, we evaluated robustness by testing growth temperature range, resistance to high salt and ethanol concentrations and low pH, and we tested a sub-selection of strains in less rich media to find strains with less expensive nutrient requirements.

2.3.1. Growth temperature: making use of the temperature tolerance of lactobacilli and pediococci

Using a moderately thermophilic platform organism offers many advantages over mesophilic organisms: substrate and product solubility are higher, metabolic reactions run faster, contamination risk is decreased, cooling costs are lower, and SSF processes run more efficiently at elevated temperature, as has been shown for lactate production with thermophilic *B. coagulans* (Ou et al., 2009). Many commercially available saccharolytic enzymes have an optimum activity temperature of around 50 °C (Ou et al., 2009; van der Pol et al., 2016), which means they perform suboptimal and need to be added in larger amounts when used simultaneously with mesophilic *E. coli* or yeast fermentations, thereby significantly increasing enzyme load and costs (Ou et al., 2009). Furthermore, the production of volatile products at temperatures above their boiling point enables direct product removal, prevents product toxicity and feedback inhibition of production pathways.

Contrary to many mesophiles such as *E. coli* or yeast, several species of lactobacilli are known to tolerate a rather wide temperature range, with most species able to grow at least up to 45 °C (Pot et al., 2014). In the case of pediococci, the species *Pediococcus acidilactici* is known to contain strains that grow well even up to 50 °C, whereas the maximum temperature is highly strain-dependent for *Pediococcus pentosaceus*. Several other species can grow at 40 °C but not at 45 °C (Franz et al., 2014). The advantage of using such thermotolerant LAB over strict or less well-characterized thermophiles such as *Geobacillus* or *Thermoanaerobacter* is that genetic tools are better developed and more readily available or adjustable (Bosma et al., 2013; Mougiakos et al., in press; Taylor et al., 2011).

In general, the results for our 27 selected strains were in line with known temperature limits of the tested species, with the majority of lactobacilli and pediococci able to grow well at 45 °C or higher. Some strain-specificity was also observed, highlighting the need for screening when selecting a production strain. Also, we observed large fluctuations in final OD at the maximum temperature for almost all tested strains

Table 1
Substrate utilization capacitates of selected strains based on API tests.

Carbon source	*L. plantarum* NC8			*L. reuteri* DSM 20016			*L. thermotolerans* DSM 15946			*P. acidilactici* NRRL B-639			*P. ethanolidurans* DSM 22301			*P. pentosaceus* DSM 20206		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
None	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol	−	−	+/− −	−	−	−	−	−	−	−	−	+/− −	−	−	−	−	−	+/− −
Erythrithol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+
L-xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adonithol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-methyl-xyloside	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Galactose	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+/−	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
L-sorbose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	+/− −	+	−	−	−	−	−	−	+/−	+	+	+/−	+/−	+	+/−	+	+
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	+	+	+	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
α-Methyl-D-mannoside	+/−	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
α-Methyl-D-glucoside	−	−	−	−	−	+/− −	−	−	−	−	−	−	−	−	−	−	−	−
N-acetyl-glucosamine	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
Arbutin	+	+	+	−	−	−	−	−	−	+/− −	+	+	+	+	+	+	+	+
Esculin	+	+	+	+/−	+/−	+/−	+/−	+/−	+/−	+	+	+	+	+	+	+	+	+
Salicin	+	+	+	−	−	−	−	−	−	+/− −	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+/−	+	+	−	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+
Melibiose	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−
Saccharose	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−
Trehalose	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Melezitose	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-raffinose	−	+	+	+	+	+	+/−	+	+	−	−	+	−	−	−	−	−	−
Amidon	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glycogen	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Xylitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-gentiobiose	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
D-turanose	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
D-lyxose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-tagatose	−	+/−	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
D-fucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-fucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-arabitol	−	−	+	−	−	−	−	−	−	−	+/− −	+/− −	−	−	−	−	−	−
L-arabitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gluconate	+/−	+	+	+ +/−	+ +/−	+/−	+/−	+/−	+/−	−	+/−	+	−	+/− −	+	−	+/− −	+
2-Keto-gluconate	−	−	+/−	−	−	−	−	−	−	−	−	−	−	−	+/− −	+/−	+/−	+/−
5-Keto-gluconate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

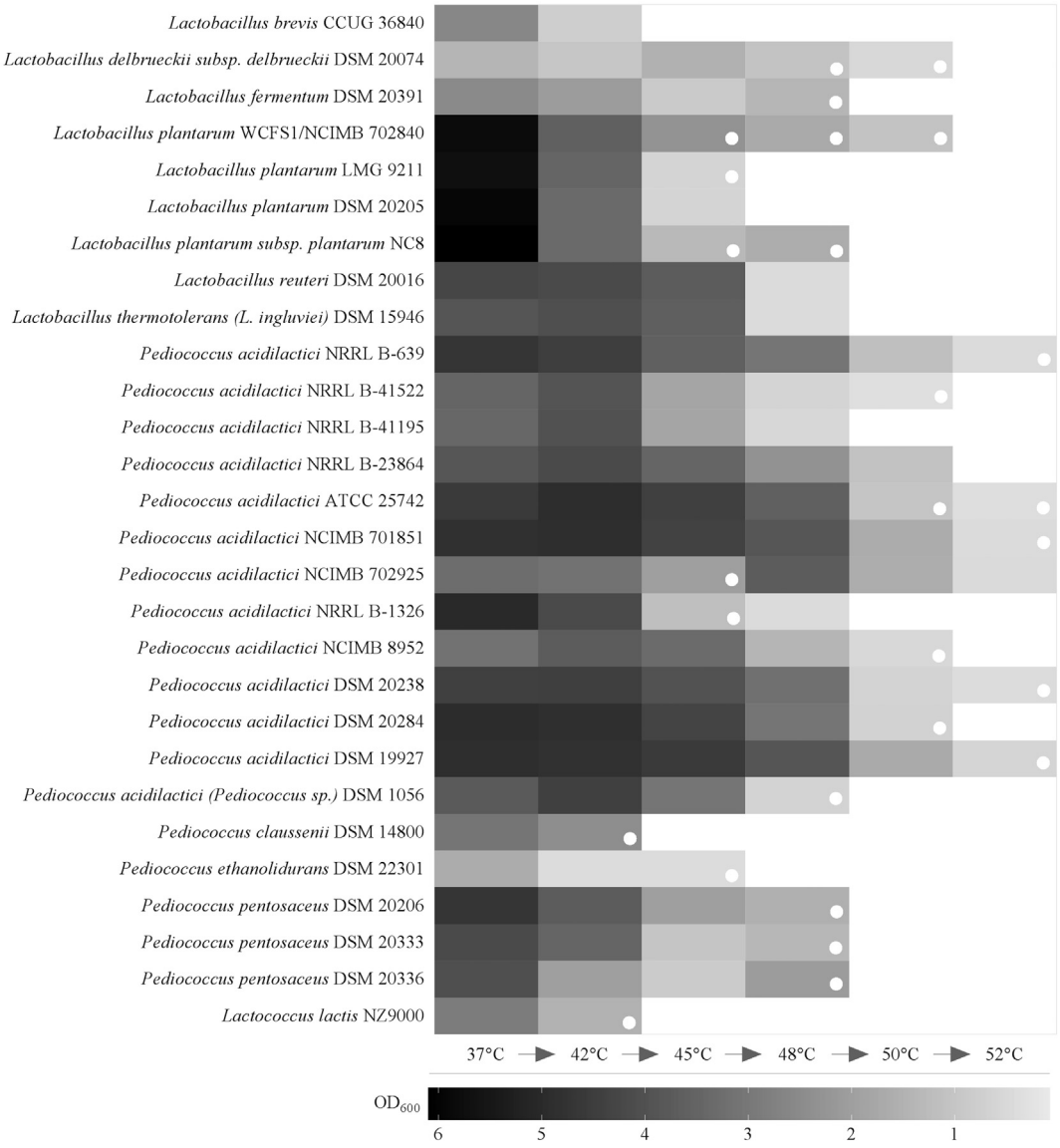

Fig. 4. Growth temperature ranges of screened lactobacilli and pediococci. Data shown are averages of two replicate experiments; for absolute values and standard deviations see Table S2. White dots indicate a standard deviation higher than half of the average, meaning that growth at that temperature was irreproducible. Strains were grown in MRS medium for 12–14 h at each temperature and then 1:50 transferred to the next one, except at 52 °C, in which strains were grown for 24 h. *L. lactis* growth was started at 30 °C.

(Fig. 4, Table S2). It is known that medium composition can have an influence on maximum growth temperature and other growth conditions (Chen et al., 2015; Nakagawa and Kitahara, 1959) and also the state of the cells during transfer as well as adaptation periods are of importance (Chen et al., 2015). Many *P. acidilactici* strains showed reproducible growth at temperatures up to 50 °C – although with relatively low ODs for some of the strains – with more fluctuation starting above that. Strain-dependent variation started to be evident already at 45 °C, where strains NRRL B-41522, NRRL B-41195 and NRRL B-1326 grow noticeably less well than the other strains of this species. At 48 °C, strains NCIMB 8952 and DSM 1056 showed a relatively large decrease in OD compared to 45 °C and performed substantially worse than the remaining strains. Strain NCIMB 702925 showed high variation in density at almost all temperatures. The growth at 37 °C for all *P. acidilactici* strains was strong with an OD_{600} of around 4.0 and the OD at 50 °C was for many strains still around 1.0. Although this is 4 × less than at their optimal temperature, it is still a relatively high density and growth might be further optimized via adaptation or evolution experiments. As also reported in literature (Franz et al., 2014), the *P. pentosaceus* strains showed strain-dependent variability. The strains were able to grow at 45 °C and showed large fluctuation in final densities at 48 °C (Fig. 4, Table S2). A similar irreproducible growth was observed for *L. plantarum* WCFS1 at 48 °C. These results indicate that these strains may be evolved for growth at elevated temperatures. Many LAB are known to produce exopolysaccharides (EPS) (Caggianiello et al., 2016), and care should be taken with interpreting OD-values as these will be influenced by the produced EPS.

2.3.2. Stress tolerance and nutritional requirements

With regard to stress tolerance, lactobacilli and pediococci are among the best recognized organisms, whereas they have less of a reputation with regards to nutritional requirements. Both phenotypes may be linked to their natural habitats: for example, species isolated from animals have lost pathways for synthesis of certain nutrients such as vitamins and amino acids, whereas pathways for uptake and catabolism were gained, since these nutrients are abundantly available in their habitat (Makarova et al., 2006; Sun et al., 2015). Since lactic acid production and concurrent acidification of their environment is their main competitive strategy, LAB (particularly lactobacilli and

pediococci) are among the most acid and low-pH tolerant bacteria. In beer or ethanol plants, their high tolerance to ethanol and hop is important and makes lactobacilli and pediococci the main contaminants (Beckner et al., 2011; Dobson et al., 2002; Geissler et al., 2016; Limayem et al., 2011; Roach et al., 2013). Resistance to high salt concentrations is also very common, with some pediococci being able to grow in > 15% NaCl (Franz et al., 2014). Several recent studies have shown superior tolerance of several lactobacilli and pediococci to inhibitors from pre-treated lignocellulosic biomass when compared to *E. coli* (Abdel-Rahman and Sonomoto, 2016; Boguta et al., 2014; Vinay-Lara et al., 2016; Zhao et al., 2013). Compared to these organisms, model LAB *L. lactis* also performed well in one of these studies, especially under medium conditions that were optimized for this well-studied organism, although it was still outperformed by some of the new isolates (Boguta et al., 2014).

Whereas the natural habitat might play a role in the presence or absence of nutrient pathways and stress resistance, this is not always easy to ascertain since the habitat of isolation is not necessarily where the organism originally evolved from, and a large degree of strain diversity exists (Deguchi and Morishita, 1992; Sun et al., 2015). The latter was shown in an evolution experiment, where different LAB were adapted on minimal media and found to regain biosynthetic capacities for a large number of vitamins and amino acids, indicating that these pathways were not completely lost but merely inactivated by genetic lesions, with different strains showing different adaptive capacities (Deguchi and Morishita, 1992). Large differences between strains of the same species have also been shown for ethanol and hop tolerance (Pittet et al., 2013). A powerful tool for increasing stress resistance or changing other traits of the host organism is genome shuffling. This strategy was successfully applied to several LAB, in which lactic acid production was optimized at pH around 3.8–4.0 (Patnaik et al., 2002; Wang et al., 2007; Ye et al., 2013). The pK_a value of lactic acid is 3.8 and therefore a decrease in pH of the medium below this level is critical for growth inhibition, because the protonated form of lactic acid can diffuse back into the cell (Patnaik et al., 2002).

We screened the 27 selected strains for growth at low pH, high salt and high ethanol concentrations and compared them to several currently used bacterial platform organisms and the model LAB *L. lactis* (Fig. 5). In agreement with literature, most pediococci were found to be able to grow in all three stress conditions. Especially growth at pH 3.8 was efficient in these strains with several strains reaching a higher final density at this pH than in the non-stress (MRS) medium control with pH ~5.6. Some pediococci also still grew up to ~35% of the optical density of the control in 12% ethanol. Tolerance towards low pH and high ethanol was also high in *L. reuteri* DSM 20016. *L. delbrueckii* performed extremely poorly and did not grow in any of the stress conditions. *L. fermentum* and *L. brevis* were not resistant to ethanol and low pH. Several strains performed well at 5% NaCl but showed a sharp decrease in growth at 10% NaCl. We did not find any strain capable of growing in 15% NaCl (data not shown). Depending on the required process and product, these stress profiles are important to keep in mind when choosing a production organism. In general, tolerance to low pH is beneficial also when the product is not an acid, since running fermentations at low pH helps prevent contaminations and can save sterilization costs. Salt resistance is especially important when using seaweed as a substrate, which is currently considered as third generation substrate (Jiang et al., 2016; Kawai and Murata, 2016). The control strains *E. coli*, *P. putida* and *L. lactis* did not grow in 12% ethanol or pH 3.25 and are much more inhibited by low pH (Fig. 5). Their performance on 5% NaCl was comparable to the LAB. *L. lactis* stress mechanisms have been well-studied and its tolerance to several stresses is highly condition-dependent: for example, some resistances appear only in minimal medium conditions or after short adaptation times (Boguta et al., 2014; Hartke et al., 1994; Rallu et al., 1996; Ryssel et al., 2014). Whereas generalized screening conditions will certainly not be optimal for all tested strains, it provides a good indication of the most robust and sensitive strains.

To be economically feasible, fermentations should be performed with as few additional nutrients as possible. Whereas *E. coli* and yeast are known to perform well in minimal media without the addition of expensive yeast extract, LAB are generally known to be fastidious organisms. As mentioned earlier, nutritional requirements are species- and strain-dependent and might be improved by adaptive evolution while genome reshuffling could be an important tool. For a subset of the selected strains we tested growth on limited amounts of undefined medium components (Fig. S2). The amount of growth supported by reduced amounts of rich components varied strongly between species. *L. plantarum* showed a sharp (~3-fold) decrease in OD_{600} between 5.0 and 1.0 g/L yeast extract and peptone but still performed best on the less rich media, which is in accordance with its relatively large genome (~3.3 Mb) containing more biosynthetic pathways than most LAB (Sun et al., 2015). *L. reuteri* DSM 20016 performed comparably, with the OD_{600} on 0.5 g/L yeast extract and peptone still around 1.5, which is ~50% of the control containing 5.0 g/L (Fig. S2). Contrary to *L. plantarum*, for *L. reuteri* there was almost no difference between 5.0 and 1.0 g/L yeast extract and peptone. The different *Pediococcus* species performed comparable to each other, with ODs around half of *L. reuteri* in the respective media. In general, YE appeared to support growth better than peptone. It is known that different types and brands of undefined nitrogen sources such as tryptone, meat extract, peptone or yeast extract have different influences on different strains (Vázquez and Murado, 2008). Our tested compounds may not be optimal for the tested strains, but the results indicate that several strains are well able to grow on less rich medium. This might be further improved via adaptation, and optimization of the nitrogen sources for final selected strains.

The most frequently used undefined growth enhancer Yeast Extract (YE) might also be replaced by cheaper alternatives. Corn Steep Liquor (CSL), which is a by-product of the corn wet-milling industry, is cheaper than YE and has successfully been used in LAB-based lactic acid production, but also in *Aspergillus niger*-based citric acid production (Mazzoli et al., 2014; Salgado et al., 2009). Possibly, an even more promising YE-substitute is vinasses, the main liquid waste of distillation process of lees (the dead yeast remaining after wine-making) and low-quality wines. Vinasses have a high organic content and have been used for efficient lactic acid production by *L. rhamnosus* (Salgado et al., 2009). Also proteinaceous hydrolysates derived from poultry processing leftovers have been shown to enhance LAB growth and production (Lazzi et al., 2013). Using waste resources as nutrient source not only decreases process costs, but also decreases waste streams and contributes to the circular economy (Vázquez and Murado, 2008). The substitution of expensive YE with cheaper, waste-based alternatives is an important and promising field of research (not only for LAB (Kelbert et al., 2015)) and could overcome the disadvantage of LAB being relatively fastidious organisms.

2.4. Genetic accessibility

One of the most crucial criteria for a platform organism is its amenability to genetic modification to enable metabolic engineering towards any desired product of interest in sufficient titers, yields and productivities. *L. lactis* is known to have the highest transformation efficiencies among LAB and genetic tools for this organism are well-developed. Many lactobacilli and pediococci have also been described to be genetically accessible, mainly via electroporation protocols, but efficiencies are generally lower than for *L. lactis* and highly variable among strains and genetic tool development is accelerating only more recently as will be discussed in Section 3. Optimization of electropora-

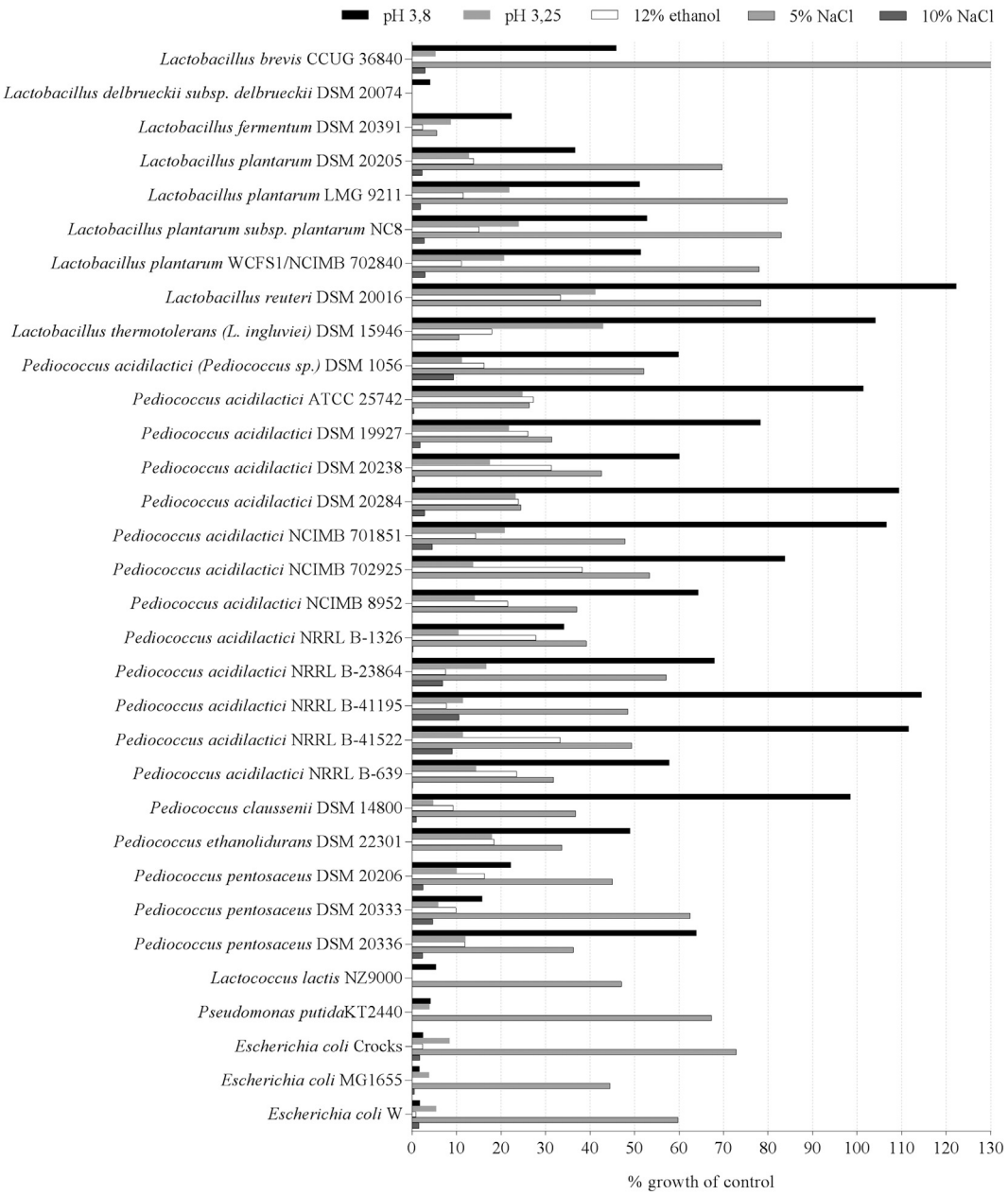

Fig. 5. Tolerance of selected lactobacilli and pediococci to high salt and ethanol concentrations and low pH. Strains were compared to several currently used model organisms shown as the last five strains.

tion protocols has been successfully performed for some strains, for which it resulted in the possibility to use genetic tools that require high efficiencies such as recombineering without the use of selection markers (van Pijkeren and Britton, 2012), as will be discussed in Section 3.

Genetic accessibility as well as optimal transformation conditions are typically highly strain-specific, but it is feasible to obtain a first impression by using a universal screening protocol (Bosma et al., 2015; Landete et al., 2014; Sanoja et al., 1999). We therefore screened several of the selected strains for genetic accessibility (Table 2). Some strains have been previously described as genetically accessible (Table 2), while others have not and to the best of our knowledge, this is the first comparative screening of several *Pediococcus* species for genetic accessibility. We used a protocol described for pediococci (Caldwell et al., 1996) that uses high concentrations of the cell wall weakening agents glycine and DL-threonine as well as a high concentration of sorbitol, or with a protocol used for several lactobacilli (Aukrust and Blom, 1992) (Appendix A). In brief, cells were grown to exponential phase in MRS medium containing different sugars and/or cell wall weakening agents depending on the protocol. After several washing steps in buffer containing high sugar concentrations, cells were transformed with two different high copy plasmids by electroporation and plated on selective medium. With the majority of the strains testing positive for transformation (Table 2), it is clear that genetic accessibility is wide-spread in the tested strains, and especially for the pediococci this has not been shown previously. Efficiencies were generally relatively low, but for some strains already high enough for engineering purposes and it provides a basic proof that DNA uptake is possible in these strains, even with a generalized and probably sub-optimal transformation protocol that can be further optimized. Both glycine and threonine can have beneficial as well as detrimental effects on transformation efficiencies (Rodríguez et al., 2007; Sanoja et al., 1999).

Table 2
Transformation efficiencies for screened lactobacilli and pediococci.

Strain[1]	CFU/μg DNA	
	pIL253 (Ery)	pNZ7021 (Cm)
Lactobacillus plantarum LMG 9211[a]	0[2,3]	500
Lactobacillus plantarum DSM 20205[b]	0[2,4]	1
Lactobacillus plantarum subsp. *plantarum* NC8[c]	140	0
Lactobacillus reuteri DSM 20016[d]	> 1500	> 1500
Lactobacillus thermotolerans (*L. ingluviei*) DSM 15946	1	60
Pediococcus acidilactici NRRL B-639[e]	120	400
Pediococcus acidilactici NRRL B-41522	50	> 1500
Pediococcus acidilactici NRRL B-41195	80	900
Pediococcus acidilactici NRRL B-23864	0	0
Pediococcus acidilactici ATCC 25742[f]	100	30
Pediococcus acidilactici NCIMB 701851	0	0
Pediococcus acidilactici NCIMB 702925	1	20
Pediococcus acidilactici DSM 20238[g]	1	5
Pediococcus acidilactici DSM 19927	15	150
Pediococcus acidilactici (*Pediococcus* sp.) DSM 1056[h]	1100	> 1500
Pediococcus claussenii DSM 14800	500	125
Pediococcus pentosaceus DSM 20206	3	3500
Pediococcus pentosaceus DSM 20333	80	1

Abbreviations: CFU: colony forming units; Ery: erythromycin; Cm: chloramphenicol. Plasmids were introduced by electroporation using three different protocols: one for all pediococci, one for all *L. plantarum* strains and *L. thermotolerans* and one for *L. reuteri* (Appendix A).

[1] Superscript letters indicate the reference in case the strain has previously been shown to be transformable (note that this is mostly with different plasmids and electroporation protocols). All data shown in the table are results obtained in the current study. a: (Sanoja et al., 1999), b: (Alegre et al., 2005), c: (Aukrust and Blom, 1992), d: (Landete et al., 2014), e: (Caldwell et al., 1996), f: (Benachour et al., 1996), g: (Alegre et al., 2005; Rodríguez et al., 2007), h: (Chikindas et al., 1995; Landete et al., 2014).

[2] Slightly resistant, with some colonies obtained on the negative control plate after transformation, while being antibiotic-sensitive before transformation.

[3] This result is in accordance with a previous report describing failure to transform this plasmid into strain LMG 9211 (Sanoja et al., 1999).

[4] This result is in line with a previous report describing the presence of active restriction-modification systems in this strain and the need for in vitro methylation of plasmids prior to transformation (Alegre et al., 2005).

The type and amount of sugars and salt added to the growth medium and wash buffer can also have a large influence, as well as the electroporation settings (Bosma et al., 2015; Rodríguez et al., 2007; Sanoja et al., 1999). Optimization of the growth, washing and electroporation protocols should enable efficiencies that are sufficient for many gene expression and genome engineering purposes.

3. Genetic tool development for metabolic engineering of lactobacilli and pediococci

A wide range of genetic tools has been developed for LAB (Tables 3 and 4, Fig. 6) and metabolic engineering has been successfully applied to several LAB for optimization of production, broadening of the substrate utilization range, or both. At the same time, however, genetic tools are not as highly developed and the availability of engineering tools is far less than for *S. cerevisiae* and *E. coli*, especially for LAB other than *L. lactis*. The importance of *L. lactis* for food production combined with its high genetic amenability and simple metabolism have made it the model organism for genetic work in LAB (de Vos, 2011). Due to the importance of LAB in food applications, genetic tool development for LAB generally focuses on food-grade systems (Landete, 2016). Table 5 provides an overview of the best reported lactobacilli and pediococci as well as *L. lactis* for different products, compared to the best available alternative host under similar process conditions. The best available host is usually *E. coli* or *S. cerevisiae* and the table clearly indicates that the extent of metabolic engineering in lactobacilli and pediococci is far behind that of the other organisms. The same holds true for process

Table 3
Currently available tools for gene expression in lactobacilli and pediococci.

Tool	Purpose	Used in species	Notes	References
NICE (inducible promoter)	Inducible gene expression (food-grade)	*L. lactis*, *L. helveticus*, *L. plantarum*	Tightly controllable in *L. lactis*. Enables high protein production. Less controllable in many lactobacilli - often needs chromosomal integration.	(de Ruyter et al., 1996b; Kleerebezem et al., 1997; Mierau and Kleerebezem, 2005; Pavan et al., 2000)
pSIP (inducible promoter)	Inducible gene expression (food-grade)	*L. sakei*, *L. plantarum*, *L. casei*	Less leaky, and tighter control than with NICE in lactobacilli.	(Jiménez et al., 2015; Sørvig et al., 2005)
Synthetic promoters	Controllable gene expression (constitutive and inducible)	*L. lactis*, *L. plantarum*	In *L. plantarum*, expression was controllable but leaky with all tested systems.	(Heiss et al., 2016; Jensen and Hammer, 1998; Rud et al., 2006; Solem and Jensen, 2002)
Reporter genes	Measuring expression and in vivo and in vitro cell tracking	*L. lactis*, *L. plantarum*, *L. reuteri*	Established reporters: mCherry, GFP, beetle red luciferase.	(Heiss et al., 2016; Jensen and Hammer, 1998; Karimi et al., 2016; Rud et al., 2006; Solem and Jensen, 2002)

Table 4
Currently available tools for creating chromosomal modifications in lactobacilli and pediococci.

Tool	Purpose	Used in species	Notes	References
pORI (HR via non-replicating plasmid)	Clean gene deletion and insertion	*L. lactis*	Requires high transformation and integration efficiencies.	(Law et al., 1995; Leenhouts et al., 1996)
pTRK (= pORI with T_s *repA* on 2nd plasmid)	Clean gene deletion and insertion	*L. acidophilus*, *L. gasseri*	Integration efficiency less dependent on transformation efficiency due to T_s *repA*. Wild-type *repA*, unstable at 43 °C.	(Russell and Klaenhammer, 2001)
pG$^+$host	Clean gene deletion and insertion	*L. lactis* a.o., *L. delbrueckii*	Integration efficiency less dependent on transformation efficiency due to T_s *repA*. Mutant *repA*, unstable at 35 °C.	(Maguin et al., 1996; Serror et al., 2003)
Cre-*lox* system	Gene deletions and insertion with AB_R-gene removal	*L. plantarum*	Allows for removal and re-use of antibiotic resistance marker from genome but leaves small lox sites.	(Lambert et al., 2007)
pTRK + *upp*-based counter-selection	Clean gene deletion and insertion	*L. acidophilus*, *L. casei*, *L. lactis*	Easier plasmid excision and curing due to counter-selection against plasmid.	(Goh et al., 2009; Song et al., 2014a)
ssDNA recombineering	Targeted chromosomal mutations	*L. reuteri*, *L. lactis*, *L. plantarum*, *L. gasseri*[a]	Efficient enough to select for mutations without AB_R selection. Mutation efficiency 0.4–19%.	(van Pijkeren and Britton, 2012)
ssDNA recombineering + CRISPR-Cas9 counter-selection	Targeted chromosomal mutations +	*L. reuteri*	Increased marker-free recombineering mutation efficiency up to 100%.	(Oh and van Pijkeren, 2014)
dsDNA recombineering	Gene deletion and insertion	*L. plantarum*	Efficiencies were too low to enable a markerless/clean system, and insertion of AB_R genes was used for selection.	(Yang et al., 2015)
T_s-plasmid + HR	Clean gene deletion and insertion	*P. acidilactici*	Thermophilic host, T_s uses wide temperature range	(Yi et al., 2016)

[a] The protocol was optimized for *L. reuteri* and *L. lactis*, but for *L. plantarum* and *L. gasseri* only proof of concept was shown.

optimization, although this is not visualized in the table. This however indicates that lactobacilli and pediococci are very promising hosts that are likely to be able to compete with current hosts once they are further engineered and their processes are optimized. To take the development of these organisms into cell factories to the next level, genetic tools are of crucial importance. An extensive overview of available engineered (mainly *L. lactis*) and wild-type LAB for the conversion of several substrates into several chemicals has recently been reviewed elsewhere (Flahaut and de Vos, 2015; Gaspar et al., 2013; Mazzoli et al., 2014). Therefore, the present section focusses strongly on the details and recent developments of the genetic tools necessary to create lactobacilli and pediococci cell factories (Tables 3 and 4, Fig. 6) and Section 4 will give a broad overview of the most recent applications of lactobacilli and pediococci for production purposes to illustrate their possibilities.

3.1. Expression systems

Initial engineering efforts in LAB mainly focused on improving dairy production and flavor properties (de Vos, 1996) and the current emphasis is still mostly on strains that are important for food production and as probiotics. Since many of the genetic elements of LAB relevant for their food applications are related to plasmids and bacteriophages, their genetics were studied at a very early stage, resulting in some of the first characterized replicons for shuttle vectors (de Vos, 2011). Also, *L. lactis* is a very suitable host and model organism for heterologous protein production since it is one of the most genetically accessible Gram positive organisms, and it has a simple secretory machinery. It only produces one secreted protein and one membrane-bound extracellular protease that can both easily be knocked out, enabling easy production and purification of heterologous proteins (Allain et al., 2015; Morello et al., 2008). Hence, expression tools are very well-developed and in general, most tools have been developed for *L. lactis* and later modified for use in mainly *L. plantarum*, but also for *L. reuteri* and L. *brevis*, as well as some pediococci relevant for antimicrobials (Table 3) (Chikindas et al., 1995; Eom et al., 2012) or dairy production (Caldwell et al., 1996).

One of the first tools developed for LAB and still a very commonly used one is the nisin-inducible expression system NICE (de Ruyter et al., 1996a; Kuipers et al., 1998; Mierau and Kleerebezem, 2005) (Fig. 6F, Table 3). In this system, gene expression is induced by the bacteriocin and pheromone nisin, which is a natural compound that can be used in food applications as well. The system was initially developed for *L. lactis*, but soon it was adapted also for use in other LAB, mainly lactobacilli (Kleerebezem et al., 1997; Pavan et al., 2000). A variant of NICE is pSIP, which makes use of the sakacin-inducer peptide from the *L. sakei* bacteriocin sakacin (Axelsson et al., 2003) (Fig. 6F). This system has been used in several lactobacilli, where it was shown to have lower background expression than the NICE-system (Jiménez et al., 2015; Sørvig et al., 2005). Several parameters such as pH and inducer concentrations have recently been further optimized (Nguyen et al., 2015).

In some applications, the use of any external compound is unwanted, expensive, impossible or unnecessary, which triggered the establishment of environmental stimuli-based expression systems. Gene expression induced by environmental stresses ('SICE' for Stress-Induced Controllable Expression) such as low pH, temperature, bile salts or NaCl has been developed in *L. lactis*, mainly for the purpose of drug delivery in the gastrointestinal tract (Allain et al., 2015; Benbouziane et al., 2013; Madsen et al., 1999). As the use of inducer compounds is generally costly and therefore also unwanted in industrial fermentations, such expression systems might also be useful in the development of LAB cell factories for chemical and fuel production, especially if they can be integrated into the genome of the host strain.

The need for inducible promoters other than the established nisin and sakacin systems was the motivation behind a recent study describing novel synthetic regulatable promoters for protein expression

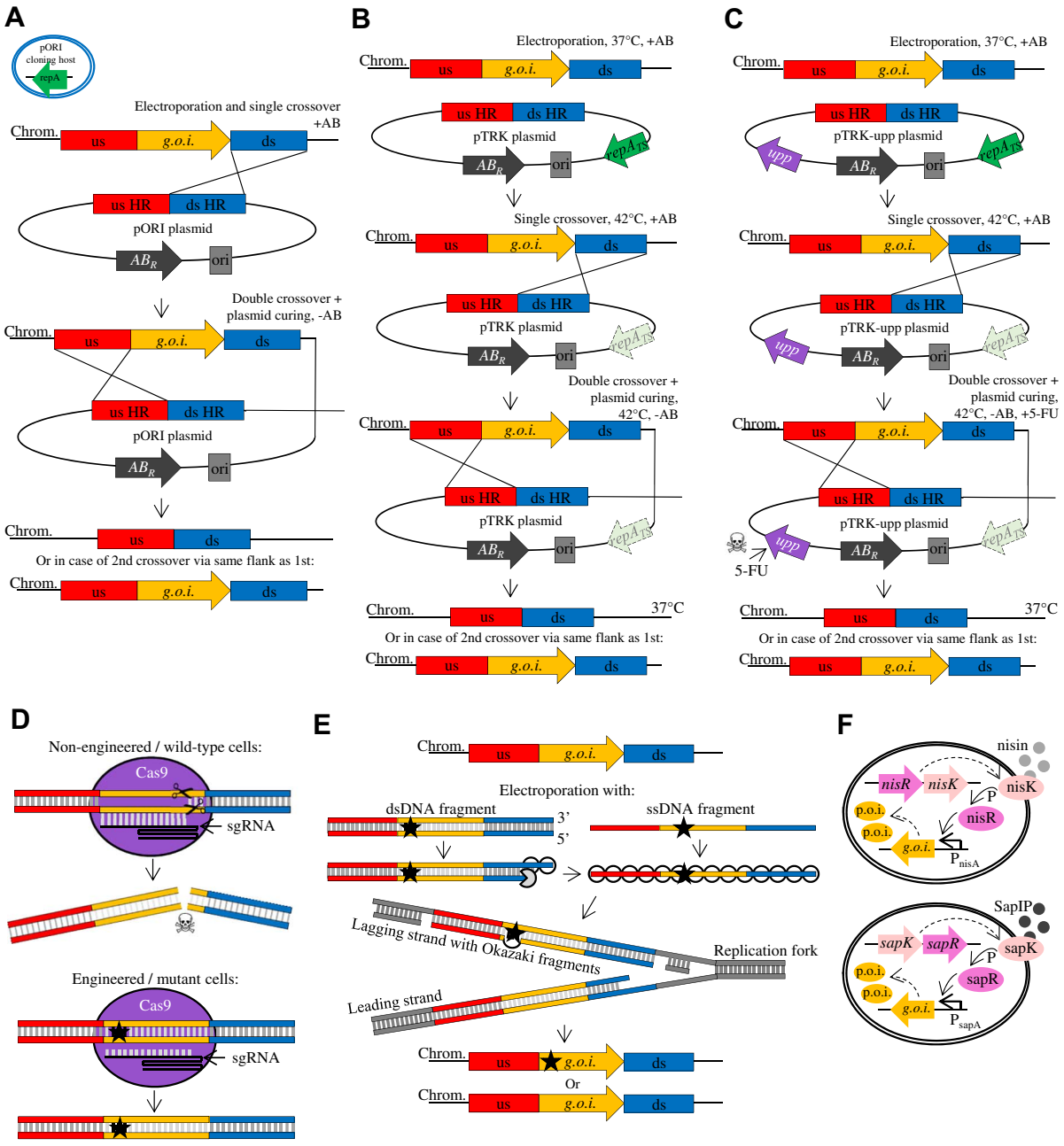

Fig. 6. Schematic overview of available genetic engineering tools for lactobacilli and pediococci. Abbreviations and colour coding: g.o.i.: gene of interest (yellow); us: upstream flanking region of g.o.i. (red); ds: downstream flanking region of g.o.i. (blue); HR: homologous region; Chrom.: chromosome; AB: antibiotic; Ts: thermo-sensitive. Replicons are indicated in green and counter-selection parts in purple. A. pORI system (Leenhouts et al., 1996). B. pTRK system using thermosensitive replicon (Russell and Klaenhammer, 2001). C. pTRK-upp system using 5-FU counter-selection (Goh et al., 2009). D. Cas9 counter-selection. To be combined with HR or recombineering as in A–E so Cas9 acts as counter-selection against wild-type revertant cells (Mougiakos et al., 2016; Oh and van Pijkeren, 2014). The black star indicates a mutation, which can be a point mutation, insertion, or deletion. E. ssDNA and dsDNA recombineering (Boyle et al., 2013; Mougiakos et al., 2016; van Pijkeren and Britton, 2012; Yang et al., 2015). The black star indicates a mutation, which can be a point mutation, insertion, or deletion. White circles indicate ssDNA binding proteins Beta or RecT; grey beak symbol indicates exonuclease Exo or RecE. F. Inducible expression systems based on bacteriocins: NICE system (Kleerebezem et al., 1997; Kuipers et al., 1998) and pSIP system (Axelsson et al., 2003; Sørvig et al., 2005). p.o.i.: protein of interest; SapIP: sakacin induction peptide.

purposes in *L. plantarum* (Heiss et al., 2016). *L. plantarum* is an important host for the food-grade expression of recombinant proteins, both for industrial and medical purposes and several recent studies have focused on optimizing both constitutive and inducible protein expression in this organism (Heiss et al., 2016; Tauer et al., 2014). Using mCherry as a reporter, inducible gene expression was shown with a *Bacillus megaterium*-derived xylose-responsive system, with an *E. coli/L. buchneri*-derived IPTG-inducible *lacI* repressor system, and an artificial T7 RNA polymerase-based system using two plasmids, in which mCherry was expressed from the T7 promoter, and the T7 RNA polymerase was expressed on a second plasmid from the newly developed IPTG-inducible promoter. All systems were inducible but showed varying levels of background expression and further optimization of repressors expression is required to improve the systems (Heiss et al., 2016). To tune constitutive expression, the effects of promoter strength, gene copy number and translation efficiency via modulation of the space between the Shine-Dalgarno (SD) sequence and the start codon were investigated (Tauer et al., 2014). The impact of the space between the consensus sequences has previously also been well-investigated and used for expression modulation in *L. lactis*, where it

Table 5
Examples of metabolically engineered LAB for green chemical and fuel production compared to other platform hosts or natural producers.

Product[a]	Species (np = natural high producer)	Relevant modifications[b]	Substrate[c]	Titer g/L[d]	Yield g/g - % of th. max.[d]	Productivity g/L/h[d]	References
L-lactate	*L. paracasei* (np)	Δ*ldhD*	110 g/L glucose	96.57	0.95–*95*%	6.89 (av.) 12.46 (max)	(Kuo et al., 2015)
	L. paracasei (np)	Δ*ldhD*	220 g/L glucose	195.11	0.99–*99*%	2.2 (av.) 6.3 (max)	(Kuo et al., 2015)
	B. coagulans (np)	–	240 g/L glucose	210.0	0.99–*99*%	3.5 (av.)	(Zhou et al., 2013)
	E. coli	Δ*adhE* Δ*frdABCD* Δ*pta* Δ*pflB* Δ*aldA* Δ*cscR ldhA::ldhL**, ev.	100 g/L sucrose	97	0.90–90%	1.83 (av.) 3.17 (max)	(Wang et al., 2013)
D-lactate	*L. helveticus* (np)	Δ*ldhL*	80 g/L lactose (skim milk)	62	0.92–92%	*3.1* (*av.*) 3.34 (max)	(Kylä-Nikkilä et al., 2000)
	B. coagulans	Δ*ldhL* Δ*alsS* ev.	104 g/L glucose	99.8	0.96–96%	*1.5* (*av.*)	(Wang et al., 2011)
	E. coli	Δ*adhE* Δ*frdABCD* Δ*pta* Δ*pflB* Δ*aldA*	100 g/L glucose	86	0.86–86%	1.7 (max) *1.2* (*av.*)	(Wang et al., 2012)
Ethanol	*E. coli*	*pdc* adhB* cat** ev.	140 g/L xylose or glucose	63 or 61	*0.45*–88.5% or *0.43*–85%	*0.65* or *0.63* (*av.*)	(Yomano et al., 1998)
	S. cerevisiae (np)	*spt15*[m]	100 g/L glucose	*40*	*0.40–78*%[e]	2.03 (av.)	(Alper et al., 2006)
	L. plantarum	Δ[b]*ldh pdc**	40 g/L glucose	6.8	0.28–*55*%	nd	(Liu et al., 2006)
	L. lactis	Δ[c]*ldh* Δ*pta* Δ*adhE pdc* adhB**	2 g/L glucose	nd[f]	nd[f]	nd[f]	(Solem et al., 2013)
2,3-Butanediol[a]	*L. lactis* (S-BDO)	Δ[c]*ldh* Δ*pta* Δ*adhE* Δ*butBA* Δ*aldB* Δ*noxE* + chem.	18 g/L glucose (+hemin)	6.7	*0.41–82*%	*0.08* (*av.*)	(Liu et al., 2016)
	S. cerevisiae (meso-BDO)	Δ[e]*adh* Δ[b]*gpd alsS* bdh1* noxE**	glucose (fed-batch, aerated)	72.9	0.41–82%	1.43 (av.)	(Kim and Hahn, 2015)
	S. cerevisiae (meso-BDO)	Δ[b]*pdc alsS* alsD* bdh**	100 g/L glucose (aerated)	31.8	0.34–68%	0.26 (av.)	(Kim et al., 2013c)
	E. coli (meso-BDO)	*alsS* alsD* bdh**	57 g/L glucose (aerated)	24.6	*0.43–86*%	*1.45* (*av.*)	(Xu et al., 2014)
	K. oxytoca (np)	Δ*ldhA*	90 g/L glucose (aerated)	29.93	0.33–*66*%	0.98 (av.)	(Kim et al., 2013b)
1,3-Propanediol	*C. acetobutylicum*	*dhaB1* dhaB2 dhaT** ΔpSOL1 (*ctfAB, adc, aad*)	glycerol (fed-batch, anaerobic)	84.0	0.54–65%	1.77 (av.)	(González-Pajuelo et al., 2005)
	L. reuteri (np)	–	glycerol + glucose (repeated fed-batch)	65.3	0.80–97%	1.2 (av.)	(Jolly et al., 2014)
	L. diolivorans (np)	–	glycerol + glucose (fed-batch, anaerobic)	92	0.82–99%	1.74 (max) *0.56* (*av.*)	(Lindlbauer et al., 2017)
Butanol (without gas stripping)	*C. acetobutylicum*	Δ*buk* Δ*ptb*	29 g/L glucose	8.52	*0.30–73*%	nd	(Yoo et al., in press)
	E. coli	Δ*ldh* Δ*adh* Δ*frd* Δ*pta fdh* atoB* adhE2* crt* hbd**	glucose	15	0.36–88%	0.2 (av.)	(Shen et al., 2011)
	S. cerevisiae	*hbd* thi* bdh* adhE2**	20 g/L galactose	0.0025	nd	3.5×10^{-5} (*av.*)	(Steen et al., 2008)
	L. brevis	*thl* crt* bcd* etfAB* hbd**	20 g/L glucose	0.3	0.033	*0.004* (*av.*)	(Berezina et al., 2010)
Succinate	*Lb. Plantarum*	*pyc*↑ *pckA*↑	glucose	6.58 (22×)	*0.34–31*%	*0.14* (*av.*)	(Tsuji et al., 2013)
	E. coli	Δ*poxB* Δ*mgsA* Δ*ackA* Δ*adhE* Δ*ldhA* Δ(*focA-pflB*) Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*	100 g/L glucose	82.7	0.92–*82*%	0.88 (av.)	(Jantama et al., 2008)
	M. succiniciproducens (np)	Δ*ldhA* Δ*pflB* Δ*pta-ackA*	20 g/L glucose (anaerobic)	13.4	0.68–*61*%	*0.48* (*av.*)	(Lee et al., 2006)
Diacetyl[a]	*L. lactis* (S-acetoin)	Δ[c]*ldh* Δ*pta* Δ*adhE* Δ*butBA* Δ*aldB* Δ*noxE* + hemin	19.5 g/L glucose	8.2	*0.46–87*%	0.58 (av.)	(Liu et al., 2016)
	E. cloacae	Δ*aldb* Δ*dr*	glucose	1.45	*0.10–21*%	0.13 (av.)	(Zhang et al., 2015)
Acetoin[a]	*E. coli* (R-acetoin)	*budRAB* noxE**	100 g/L glucose (aerated)	40.3	*0.40–78*%	*1.68* (*av.*)	(Xu et al., 2015)
	L. lactis (R-acetoin)	Δ[c]*ldh* Δ*pta* Δ*adhE lac**	whey paste (70 g/L lactose) (aerated)	27	0.42–82%	0.64 (av.)	(Kandasamy et al., 2016)

This table does not aim at providing a complete overview of all known engineered strains per product, but rather a comparison of known engineered LAB to other organisms under similar conditions. The best-producing LAB strain was selected for each product. If this was *L. lactis*, also the best-known *Lactobacillus* or *Pediococcus* was added. The non-LAB organism was selected from available examples, where we aimed to select the best-producing strains under similar conditions. To this end, we mainly selected studies using simple glucose batch fermentations to make the comparison as direct and fair as possible, even though for several products and organisms higher yields, titers and productivities have been reached with fed-batch modes. Please note that process parameters such as medium, pH and temperature might differ, but these are not included here.

[a] Products for which no *Lactobacillus* or *Pediococcus* has been described.
[b] Asterisks indicate overexpression or heterologous expression. Ev.: evolved; [m]: mutated.
[c] Unless otherwise indicated, all fermentation are in batch mode and without any gas additions.
[d] Values in italics have been calculated from data from the original articles; all other values were directly provided in the original articles. th.: theoretical; av.: average; max.: maximum.
[e] The maximum yield used in the article was 0.41 g/g resulting in 98% of the theoretical maximum – here we used the standard 0.51 g/g maximum theoretical yield.
[f] Proof of concept showing the first homo-ethanologenic LAB, but no numbers available except end product fluxes in mmol/(gdw/h).

was shown that by keeping the consensus regions conserved and varying the areas in-between the consensus sequences, a 400-fold change in activity could be obtained (Jensen and Hammer, 1998). The method was subsequently used to modulate operon-based gene expression in *L. lactis* (Solem and Jensen, 2002). The same approach was later generalized and also applied to *L. plantarum* using *gusA* as a reporter gene (Rud et al., 2006). The consensus sequences for *L. plantarum* promoters were derived from 16S rRNA promoter alignments. These sequences were kept the same in the entire promoter library, whereas the space in-between the −10 and −35 consensus sites was varied. The resulting library covered 3–4 logs of expression levels and was shown to have similar activity in *L. casei*, suggesting the wider applicability of this system (Rud et al., 2006).

In addition to the development of promoters, the development of reporter genes is equally important, but whereas reporters such as *gusA* (Rud et al., 2006) and GFP (Guo et al., 2013) have been developed for *L. lactis*, examples for lactobacilli are more limited. In addition to the already mentioned use of mCherry in *L. plantarum* (Heiss et al., 2016), an elegant application was recently shown in *L. reuteri* (Karimi et al., 2016). Two strains of this probiotic species were labeled with beetle red luciferase (CBRluc) and mCherry to track them in vitro as well as in vivo in the GIT (Karimi et al., 2016) (Table 3).

3.2. Plasmid-based homologous recombination (HR) for creating clean gene deletions and insertions

In industrial fermentations, it is generally desired to integrate modifications into the genome in a clean or markerless way and not have plasmids inserted in the strain. Also, gene deletions are required for byproduct removal and for these reasons, gene integration and deletion systems based on targeted homologous recombination (HR) are needed. Several HR-based integration systems have been developed for LAB, including non-replicative or conditionally replicating vectors, as well as recombineering. Also the well-known Cre-*loxP* system has been developed for several species of LAB, but as this system leaves small lox sites it is not entirely clean (Banerjee and Biswas, 2008; Biswas et al., 1993; Lambert et al., 2007; Zhu et al., 2015). One of the oldest clean integration systems for LAB is the pORI-system, a plasmid system based on the broad host range lactococcal rolling circle plasmid pWV01. The gene encoding the replication protein repA was removed, creating a non-replicating integration vector when HR regions are cloned onto the plasmid (Leenhouts et al., 1996) (Table 4 Fig. 6A). To enable cloning of the constructs, cloning hosts have been constructed which provide a chromosomal copy of *repA in trans* (Law et al., 1995; Leenhouts et al., 1991).

An integration system based on a non-replicating vector can be used in species with sufficiently high transformation and integration efficiencies to allow for direct integration selection, but in many species these efficiencies are not high enough for this. Whereas the efficiencies for using non-replicative vectors in *L. lactis* are high enough, in lactobacilli transformation efficiencies are generally lower (Fang and O'Toole, 2009) and transformation with non-replicative integration vectors do not result in any transformed colonies. It has also been suggested that replication enhances recombination efficiencies, in particular the rolling circle method (Biswas et al., 1993; Morel-Deville and Ehrlich, 1996). To overcome a low transformation and/or recombination efficiency, the two events should be uncoupled (Russell and Klaenhammer, 2001), for which conditionally replicating vectors provide a solution. After transforming the target strain with a replicating vector under replication-permitting conditions, the strain is transferred to non-permissive but selective conditions, thereby disabling replication and selecting for integrated plasmids under the selective pressure (typically antibiotic resistance). Since lactobacilli and pediococci have a rather wide temperature growth range, an easy system for creating conditionally replicating plasmids is to use temperature-sensitive plasmids (Table 4, Fig. 6B). This is used in the pTRK-series of vectors, which are based on the pORI plasmids, with which a second plasmid is co-transformed encoding *repA*, which is unstable above 43 °C (Russell and Klaenhammer, 2001). After successful transformation and allowing the integration vector to recombine, the organism is placed in the non-replicative conditions, selecting for plasmids integrated via single crossover via the provided homologous region (Fig. 6B). Subsequently, a second crossover via a second provided homologous region leads to excision of the plasmid from the chromosome, resulting in either wild-type or mutant genotype, depending on whether the second crossover takes place via the same or via the other homologous region as the first crossover. A temperature-sensitive replicon in a single-plasmid based system was recently used for the engineering of *P. acidilactici*, which is the first example of genome engineering of this species (Yi et al., 2016). Another temperature-sensitive integration system for LAB is the pG$^+$host system (Biswas et al., 1993). Both pTRK and pG$^+$host are derived from lactococcal plasmid pGK12 (Kok et al., 1984). The pG$^+$host system uses a variant of *repA* with a lower permissive temperature than pTRK and has been used for successful recombination in poorly transformable lactococci, streptococci (Maguin et al., 1996), and *L. delbrueckii* (Serror et al., 2003).

3.3. Counter-selection methods

Whereas non-replicating or conditionally replicating plasmids enable easy single crossover selection, they cannot select for both the frequency and the type (via one or the other HR region) of the second crossover. This can lead to laborious screening procedures depending on recombination frequency and on whether there is a bias to revert to wild-type genotype. Therefore, several counter-selection systems have been developed to select for second crossover events. The most-frequently used system in lactobacilli, which has also been added to the pORI/pTRK-system, is counter-selection based on the uracil phosphoribosyltransferase gene *upp* (Goh et al., 2009) (Table 4, Fig. 6C). In *L. lactis*, instead of *upp*, the orotate transporter *oroP* is more frequently used (Defoor et al., 2007; Petersen et al., 2013). Both systems are based on genes in the purine and pyrimidine salvage pathways. The expression of *upp* or *oroP* from the integration vector causes sensitivity to 5-fluorouracil (5-FU) or 5′-fluoroorotic acid (FOA), respectively. This feature is used to select against colonies that still contain the plasmid and hence select for double crossover mutants that have excised the plasmid (Fig. 6C). In both cases, either *upp* or *oroP* first needs to be inactivated on the host's genome. Very recently, a counter-selection system based on bacteriocin sensitivity has been developed for *L. lactis*, which does not rely on temperature shifts, does not require the use of defined media and does not require initial knockout of the counter-selection gene (Wan et al., 2016).

The above-mentioned counter-selection methods select for plasmid excision and hence simplify the screening process for identifying double crossovers, but none of the methods is selective for mutants and the resulting strain can also be a wild-type revertant. Whereas in most cases there is an equal chance for the strain to become mutant or wild-type, for some genes there is a strong selective pressure to revert to wild-type. In these cases, a counter-selection system is also required to select against wild-type genotypes. The most powerful system currently available for counter-selection against wild-type revertants after bacterial genome editing is the CRISPR-Cas9 system (Jiang et al., 2013; Mougiakos et al., 2016) (Fig. 6D). It is based on the endonuclease activity of the Cas9 protein, which can be targeted to cleave a very specific DNA sequence in the wild-type gene. This system has been used extensively for eukaryotic genome editing, in which point mutations can be made by the error-prone Non-Homologous End-Joining (NHEJ) mechanism that repairs the dsDNA breaks made by Cas9. Most prokaryotes do not have a functional NHEJ and therefore Cas9-induced breaks are generally lethal. This principle enables the use of Cas9 as powerful counter-selection tool: when Cas9 is targeted against the wild-type gene of interest, only mutated cells escape Cas9-cleavage and are

being selected for (Jiang et al., 2013; Mougiakos et al., in press; Oh and van Pijkeren, 2014) (Fig. 6D). So far, among LAB this system has only been used in *L. reuteri* (Oh and van Pijkeren, 2014). In this species, the CRISPR-Cas9 system was used in combination with ssDNA recombineering (Fig. 6E). Further details about recombineering and how this can be applied in LAB are explained below in Section 3.4. In short, it is a recombination system based on phage-derived recombinases enabling recombination between short ssDNA or dsDNA oligos with the host chromosome. ssDNA recombineering enables the creation of precise, clean point mutations in the host chromosome without the need for antibiotic selection. A ssDNA recombineering system was established for *L. reuteri* and *L. lactis* and proof of principle of the method was also shown for *L. plantarum* and *Lactobacillus gasseri* (van Pijkeren and Britton, 2012). When ssDNA recombineering is used without Cas9-based counter-selection, the editing efficiency is maximum 50%, since the oligo is incorporated in only one strand of the chromosome during replication and is therefore present in only half of the DNA (Boyle et al., 2013). In case of multiple chromosomes, this number is brought further down and many other factors can create suboptimal recombineering conditions and bring the number even further down (Boyle et al., 2013). Hence, equal to the selection for mutant double crossovers described above, it can be laborious to screen for the desired mutations if no counter-selection is available. In *L. reuteri*, using Cas9 as counter-selection against wild-type, non-recombined, cells increased mutation efficiencies from 0.4–19% up to 100% (Oh and van Pijkeren, 2014). In order to obtain these efficiencies, cells had to be transformed according to a two-step procedure, in which recombineering was allowed to take place first before Cas9 was introduced and expressed to select against wild-type cells. If recombineering oligos and Cas9 were added simultaneously, 100-times fewer recombinants were obtained, which brought the number close to the detection limit (Oh and van Pijkeren, 2014). The use of Cas9 to select for recombinants enabled the deletion of up to 1 kb of chromosomal DNA using ssDNA oligos, which had not been previously shown in any *Firmicute* (Oh and van Pijkeren, 2014). The power of Cas9-counter-selection lies in its ability to select for mutations occurring at low frequencies and thus enables editing organisms with low transformation or recombination efficiencies, which is often the case for non-model industrial strains with limited genetic tools available (Mougiakos et al., 2016; Oh and van Pijkeren, 2014). In LAB it has so far only been used in combination with recombineering, and only for *L. reuteri*. However, it can also be used with the above-described plasmid-based HR methods, as has been shown for a number of other bacterial species, including several non-model organisms, such as several of species of *Clostridium* (Huang et al., 2016; Li et al., 2016; Nagaraju et al., 2016; Wang et al., 2015), *Streptomyces* (Cobb et al., 2015; Tong et al., 2015) and the facultative thermophile *Bacillus smithii* (Mougiakos et al., in press). These examples show that the CRISPR-Cas9-HR-tool can be adjusted to many different organisms, and this tool has been proven to greatly enhance engineering efficiencies in all these non-model organisms. Adjustment of this tool for other species of LAB would therefore be a desirable development for high-throughput engineering of these species.

3.4. Recombineering-based HR for creating clean chromosomal modifications

Whereas plasmid-based HR is well established in LAB, recombineering is less widely used (Table 4, Fig. 6E). The advantage of recombineering is that it increases throughput: DNA oligos or PCR products are directly added to the cells, thereby omitting cloning steps and saving time (Sharan et al., 2009). Recombineering (recombination-mediated genetic engineering) is based on phage-derived recombination genes from Lambda *red* (Exo, Beta, Gam) or the Rac prophage (RecET). Beta and RecT are ssDNA binding proteins that promote the recombination of the template with the host DNA, whereas Exo and RecE are exonucleases that are required in the case of dsDNA recombineering to create ssDNA overhangs for recombination (van Pijkeren and Britton, 2014). Gam can be added to repress host nucleases and is frequently used and shown to be beneficial when using dsDNA recombineering (Sharan et al., 2009) but is not essential (Datta et al., 2008). ssDNA recombineering is used for creating precise point mutations in the host chromosome, for which the inducible expression of recombinase *Beta* or *RecT* is required together with the ability of the host to take up small ssDNA oligos (van Pijkeren and Britton, 2014). Several factors need to be optimized in the oligo design to enable efficiencies high enough to detect mutant cells without antibiotic selection. These include creating sequences that avoid the mismatch repair system, use oligos matching the lagging strand of the target DNA, determining the optimal length and concentration of the oligos and identifying which sequences are not degraded by the host's exonucleases (van Pijkeren and Britton, 2014). The first step in establishing a recombineering system for a new host is the identification of a suitable *RecT* and its inducible expression, since high activity levels have been shown to reduce cell viability (van Pijkeren and Britton, 2014). Many bacterial genomes encode *RecT* homologs, suggesting that the system might be applicable to many species (Datta et al., 2008). Secondly, the transformation efficiency should be high enough to allow for ssDNA to enter the cell and enable selection of mutants without selective pressure. In the study using *L. lactis* and *L. reuteri*, a plasmid transformation efficiency of 10^5–10^6 colony forming units per μg of DNA was shown to be sufficient to enable ssDNA recombineering. Especially for lactobacilli and pediococci an optimization of transformation protocols will be required to use these methods (Landete et al., 2014; van Pijkeren and Britton, 2012; van Pijkeren and Britton, 2014) (Table 2).

Whereas ssDNA recombineering can only be used to create small mutations in the chromosome or in some cases also deletions, dsDNA recombineering (Table 4, Fig. 6E) can be used for both the insertion and deletion of very large DNA fragments flanked by HR regions generated by PCR (Sawitzke et al., 2007). Recently, dsDNA recombineering has been established for *L. plantarum* (Yang et al., 2015). ssDNA recombineering requires only expression of Beta or RecT, but for dsDNA recombineering also the corresponding exonucleases Exo and RecE are required and it might be necessary to add host nuclease inhibitors such as Gam. A prophage locus in the genome of *L. plantarum* WCFS1 was identified containing homologs of *Gam*, *Beta* and *Exo* in an operon. This set of genes was expressed in another *L. plantarum* strain, JDM1, which does not natively contain these genes and functional dsDNA recombineering was shown. The target gene was replaced with a chloramphenicol resistance gene (*cat*) flanked by *loxP* sites, which are in turn flanked by HR regions up- and downstream of the target gene. In a second step, the Cre recombinase was used to remove the *cat* cassette. When the strain was transformed with the *HR-lox-cat-lox-HR* cassette on a non-replicating plasmid, the result was 16 colonies, which were all single crossover. To obtain the double crossover, a laborious subcultivation and PCR-screening process was required since the recombination and plasmid excision rate was low – a problem observed more frequently as mentioned above in the counter-selection section. However, when using dsDNA recombineering with a linear DNA template, the selection is immediately for double crossover integrations, which significantly shortens the process. This was shown in the *L. plantarum* study, where 95% of the 30 obtained colonies after dsDNA recombineering had the correct double crossover genotype directly after transformation without further subculturing or PCR-screening (Yang et al., 2015). The downside of this study is that the *cat* gene was inserted, creating the need for Cre-mediated *cat* removal and leaving *loxP* scars. The *cat* selection was used to obtain the integrated cells, contrary to what was performed in *L. reuteri* ssDNA recombineering in which no selection was used, which is generally the case with recombineering and one of the advantages of the system. However, when the dsDNA recombineering was tested in the original strain *L. plantarum* WCFS1, a 50-times higher number of colonies was obtained compared to strain JDM1. The higher number is probably due to

electroporation efficiencies, which is around 10^3 CFU/μg DNA for JDM1 and 10^6 CFU/μg DNA for WCFS1. When using a non-replicating HR-plasmid instead of dsDNA recombineering, no colonies were obtained in this strain (Yang et al., 2015) and hence, the system is still a major improvement in integration efficiency and engineering efficiency even despite the two-step procedure for marker removal. The effect of transformation efficiency is in accordance with the observation made with ssDNA recombineering, in which the authors state that a plasmid transformation efficiency of 10^5–10^6 colony forming units per μg of DNA was shown to be sufficient to enable ssDNA recombineering (van Pijkeren and Britton, 2014). These numbers suggest that it might be possible to obtain dsDNA recombineering colonies without the need for marker insertion in *L. plantarum* WCFS1 as well, but this needs to be further investigated. It also highlights the need for electroporation protocol optimization to allow for the use of high-throughput techniques such as recombineering.

3.5. Future development of genetic tools for lactobacilli and pediococci

Altogether, a large number of gene expression and modification systems are available for several species of LAB (Tables 3 and 4). Several examples of successful metabolic engineering using these tools will be highlighted in the next section. Nevertheless, the number of highly engineered lactobacilli and pediococci for biotechnological production purposes is still relatively low (Table 5), and throughput of the engineering tools should be further increased to allow more extensive engineering. Further development of dsDNA-recombineering would improve the toolbox, as well as further developing it into high-throughput systems such as MAGE. The latter is especially powerful when combined with CRISPR-Cas9-counter-selection into CRMAGE, which has so far only been used in *E. coli* (Ronda et al., 2016). To establish the CRISPR-Cas9 system for more species than only *L. reuteri*, the adjustments of this system for other non-model organisms can be used as examples. In order to enable the use of recombineering strategies without marker insertion in more strains, electroporation protocols of industrially relevant strains need further optimization.

4. Current applications and metabolic engineering of lactobacilli and pediococci for biotechnological production purposes and comparisons to other organisms

As described earlier, lactobacilli and pediococci have many properties that make them interesting as platform organisms, and although genetic tools have been developed for some species, a lot of work still needs to be done to make these high-throughput and allow for acceleration of metabolic engineering. Hence, the number of highly edited lactobacilli and pediococci for bulk chemical and fuel production is not yet very high, but current studies show promising results that are encouraging for further development. Furthermore, LAB have the advantage that they can also be used for whole-cell applications such as vaccine or drug delivery, and as producers of antimicrobial compounds or as probiotics. Examples of biotechnological application of LAB via metabolic engineering for the production of industrially interesting compounds from a variety of substrates, as well as the use of whole cells for other applications will be discussed in the upcoming section. As several reviews deal with complete overviews of these items (Gaspar et al., 2013; Mazzoli et al., 2014; Papagianni, 2012), this section does not aim to provide an exhaustive overview but rather highlight the most recent developments, emphasize lactobacilli and pediococci and make a comparison of these organisms with other organisms to evaluate future perspectives of development (Table 5).

4.1. Production of bulk chemicals and fuels

As the name implies, LAB are mainly known for lactate production, which is historically why they have become interesting for the biotechnology industry. Lactic acid has traditionally been used as preservative and flavor-enhancer, but is increasingly used in cosmetics as emulsifier and moisturizer, in the tanning industry, as a solvent in the form of ethyl lactate, and as the bioplastic-precursor poly-lactic acid (PLA). PLA can be used as bulk packaging material, but also for high-value medical purposes such as suture (Castro-Aguirre et al., 2016). Lactate has two stereoisomers, the D- and the L-form. In the human body, only the L-form is naturally present and many people are allergic to the D-form, which is why the L-form is mainly used in food and cosmetics applications. For PLA, optically pure lactate is needed before polymerization, and different mixtures of the two forms give different properties to the plastic. For these reasons, the purity of the produced lactate is crucial. Whereas chemical synthesis always results in a mixture of D- and L-lactate, microbial production can result in either of the forms or both, depending on whether the organisms expresses L-*ldh*, D-*ldh*, or both (Fig. 3, Fig. S1). Hence, lactate production by microbes has benefits over chemical synthesis and 90% of the lactate world-wide is produced by microbial fermentation (Mazzoli et al., 2014; Sauer et al., 2008). The fact that LAB metabolism is relatively simple and strongly directed towards lactate production makes them efficient and suitable hosts for the production of this compound. Whereas some LAB contain only a single *ldh* and produce either D- or L-lactate, several species encode multiple copies and produce both stereoisomers. In such strains, metabolic engineering has been applied to obtain pure D- or L-lactate. In the case of homofermentative LAB, this could be achieved by simply knocking out all but one *ldh* (Kuo et al., 2015; Yi et al., 2016), whereas in *E. coli* the production of pure D-lactate as main product required far more extensive engineering, and lactate accumulation was found to be complicated by unexpected interconnectivities of metabolic routes(Kim et al., 2013a, Zhou, and Cui, W.-j., Liu, Z.-m., Zhou, Z.-m., 2016) (Table 5). In this respect, the small genome size of LAB (2 Mb) can generally be advantageous for engineering purposes since there is less interference of unexpected regulatory circuits or from competing metabolic pathways. Furthermore, LAB are capable of producing maximum lactate yield under fermentative conditions where no gas addition to the reactor is required. Fungi such as *Rhizopus oryzae* and several yeast strains are also considered for lactate production due to their lignocellulosic-degrading and low-pH fermentation capacities, respectively, but these organisms either require oxygen or yields are not yet optimal and need further engineering (Ilmén et al., 2013; Novy et al., 2017; Upadhyaya et al., 2014). Process parameters have a large influence on lactate production by LAB and are an important optimization factor. The effects of temperature, pH, sugar concentration, etc. have been well-studied (Hofvendahl, and Hahn–Hägerdal, B., 2000). Production of lactate at elevated temperatures has the benefits described earlier and has been shown in an efficient SSF process at pH values between 5.0 and 5.5 and temperatures of 48–50 °C with *P. acidilactici* (Zhao et al., 2013) and *B. coagulans* (Ou et al., 2011; van der Pol et al., 2016) and at 42 °C and pH ~5.5 by L. *delbrueckii* (Adsul et al., 2007). A different *L. delbrueckii* strain was used for the fermentation of beet molasses into lactic acid at 49 °C (Monteagudo et al., 1997).

In addition to lactate, LAB are capable of producing a range of other fuels and chemicals (Figs. 1 and 3, Table 5). The amount and type of end products mainly depends on the process conditions as described in Section 2.1 (Fig. 3). A big advantage of lactobacilli and pediococci in the production of many alcohols is their native tolerance towards high concentrations of these compounds. Their fermentative lifestyle is a strong advantage for the production of reduced compounds such as ethanol and 2,3-butanediol (2,3-BDO). The GRAS status of LAB is also important, as many 2,3-BDO production native producers, such as *Klebsiella pneumoniae* and *Enterobacter aerogenes*, are potential pathogens (Ji et al., 2011; Kim et al., 2013b). Although metabolic engineering has not yet been applied to such an extent that industrial titers have been reached, these characteristics make LAB potential production hosts for these compounds. Products for which LAB have traditionally

been used as whole cells (see next section) are food flavor compounds acetoin, acetaldehyde and diacetyl. However, these products are also of interest to be produced and purified on a larger scale and very recently, some major achievements have been made with these compounds in *L. lactis*, which was subsequently further engineered to produce the building block chemical 2,3-BDO (Table 5) (Liu et al., 2016).

Whether 2,3-BDO or any other product than lactate is the target product, in LAB the first step for engineering towards reduced compounds has to be the inactivation of the present *ldh* genes. When the main *ldh* is inactivated, its role is often taken over by secondary ones, which also need to be inactivated. In *L. lactis*, inactivation of three out of the four *ldh* genes led to some 2,3-BDO production but the main product was ethanol. The maximum theoretical yield of 0,67 g/g was achieved after overexpression of its native α-acetolactate synthase and acetoin reductase genes (Gaspar et al., 2011). 2,3-BDO exists in three different isomers; (*R*,*R*)-2,3-BDO, *meso*-2,3-BDO and (*S*,*S*)-2,3-BDO. Whereas the first two have been produced in large amounts by microbial fermentation, the direct formation of the third variant from glucose by fermentation was only recently shown for the first time. This was achieved in an *L. lactis* strain that had been engineered for the production of the highest diacetyl titers reported by a combination of system metabolic engineering including a modeling approach, optimizing respiratory conditions, and optimized chemical catalysis (Liu et al., 2016). Diacetyl formation includes the non-enzymatic conversion of α-acetolactate into diacetyl in the presence of oxygen. This step is rather inefficient and generally, most α-acetolactate is converted into acetoin (Fig. 3B) (Liu et al., 2016). To overcome redox problems and allow for respiration in a strain from which 8 genes for by-product pathways as well as NOX were deleted, hemin was added to the cell cultures. Respiration is possible in *L. lactis* when heme is added to the cultivation medium as the organism contains all the genes necessary for a functional electron transport chain, but not for hemin (Liu et al., 2016). When also Fe^{3+} or Cu^{2+} ions were added to the fermentation after the start of stationary phase, diacetyl production reached the highest reported levels (Table 5). The pathway was subsequently extended to optically pure (*S*,*S*)-2,3-BDO by adding a L-2,3-BDO dehydrogenase from *Brevibacterium saccharolyticum* (Liu et al., 2016).

Whereas 2,3-BDO is not a major native product in any LAB, 1,3-propanediol (1,3-PDO) is produced in large quantities already by some lactobacilli from glycerol (Fig. 3B). 1,3-PDO is a platform chemical that can be used as a building block in solvents, plastics, detergents, etc. Glycerol is a by-product of the biofuel industry and therefore an interesting second generation substrate and it is fermented by many LAB species naturally (Chen and Liu, 2016). Similar to 2,3-BDO producers, many 1,3-PDO producers are also potential pathogens, which is not the case for lactobacilli. *L. diolivorans* is close to pathogenic native producers like *Klebsiella* with titers up to 84,5 g/L (Pflügl et al., 2012). Subsequent development of genetic tools for this species, which was initially recalcitrant to transformation, will enable further improvement of production (Pflügl et al., 2013). The best characterized 1,3-PDO producer among LAB is *L. reuteri*, which naturally produces the compound already in substantial amounts and process optimization further increased yields (Dishisha et al., 2014; Jolly et al., 2014). Also *L. brevis* was shown to produce 1,3-PDO, but whereas this strain needed to be supplemented with vitamin B12 for the efficient action of vitamin B12-dependent glycerol dehydratase (Vivek et al., 2016), *L. reuteri* is a native producer of vitamin B12 and does not need additional B12 for 1,3-PDO production (Ricci et al., 2015; Santos, 2008; Santos et al., 2008). Another product that can be formed from glycerol via 3-hydroxypropionaldehyde (3-HPA, Fig. 3B) is 3-hydroxypropionic acid (3-HP), which yields one NADH and one ATP, compared to one NAD^+ for 1,3-PDO. Hence, redox balancing is important to determine the flux to either of these compounds. This was recently addressed via process optimization in *L. diolivorans*. By adjusting the feeding regime of glucose as electron donor and glycerol as electron acceptor, the ratio of 1,3-PDO to 3-HP could be modified, emphasizing the importance of process optimization (Lindlbauer et al., 2017). A complicating factor in the use of these pathways is the toxicity of 3-HPA and the compartmentalization of some of the intermediates, which was recently addressed in a metabolic flux analysis study in *L. reuteri* (Dishisha et al., 2014).

For ethanol production, yeast is generally used as production host. High titers and yields are obtained with yeast and the process is highly optimized, but many lactobacilli and pediococci have been described with higher ethanol tolerance than yeast and they are common contaminants of ethanol fermentations (Beckner et al., 2011; Geissler et al., 2016; Limayem et al., 2011; Roach et al., 2013), which is how pediococci were originally discovered and described (Pederson, 1949). Their ability to ferment sugars to ethanol at low pH combined with their high ethanol tolerance makes it a very robust potential process, which was shown with *L. plantarum* at pH 3.2 with 13% ethanol (G-Alegría et al., 2004). Also for ethanol production, all possible *ldh* genes need to be inactivated to prevent lactate production completely – if secondary *ldh* genes are not inactivated, often unstable strains are obtained that revert to lactate producers (Liu et al., 2006). Initial efforts did not result in highly producing strains, but this was most likely due to non-optimized expression of heterologous genes and recent efforts have created a homo-ethanologenic *L. lactis* (Solem et al., 2013). The authors indicate that the reason for using *L. lactis* for this engineering work was its genetic amenability as model species, but even though it is more ethanol-tolerant than *E. coli*, it is less ethanol tolerant than lactobacilli and therefore the latter might be more suitable for further development (Solem et al., 2013). A promising candidate for this might be a very recently described *Lactobacillus casei* strain, which was shown to be highly tolerant to several alcohols and other inhibitors such as lignotoxins and is currently being engineered for ethanol production (Vinay-Lara et al., 2016).

Also for the well-known solvent butanol, LAB might be interesting production hosts since they show high butanol tolerance, with several strains tolerating up to 3% (Berezina et al., 2010; Knoshaug and Zhang, 2009; Li et al., 2010). This is at least 3 times higher than in most solventogenic *Clostridia* and *E. coli*, although for these organisms efficient in situ gas stripping technologies have been developed to prevent toxicity and produce titers above the toxicity level (Ezeji et al., 2003; Jensen et al., 2012; Shen et al., 2011). *L. lactis* and *L. buchneri* produced butanol when the *C. beijerincki* P260 thiolase was expressed to reroute acetyl-CoA to butanol (Liu et al., 2010). *L. brevis* natively contains part of the pathway and was engineered with a shortened version of the clostridial pathway for butanol production, (Table 5). Further engineering of this strain via for example removal of by-product pathways is necessary to obtain higher production (Berezina et al., 2010).

Making use of the wide range of available (partial) pathways of LAB was also employed for succinate production with *L. plantarum* NCIMB 8826. This strain has an incomplete TCA cycle and naturally produces small amounts of succinate (Tsuji et al., 2013). Overexpression of pyruvate carboxylase (*pyc*) was found to be the critical step, and together with overexpression of phosphoenolpyruvate carboxylase (*pckA*), the mutant produced 22-fold more succinate than its wild-type parent strain. Production needs to be further optimized by metabolic engineering and process optimization, which has been extensively done for several native producers as well as for engineered *E. coli* (Table 5) (Ahn et al., 2016).

Other food ingredients that can be produced by LAB are polyols such as xylitol, mannitol and sorbitol (Table 5). These are low-calorie sweeteners with possible health-promoting properties, but mannitol also has applications in medicine and in the chemical industry (Park et al., 2016). The current commercial production is based on chemical catalysis that requires very pure substrates and expensive down-stream processing and therefore, microbial production is an economically attractive option (Gaspar et al., 2013; Papagianni and Legiša, 2014). Xylitol is most frequently produced by natural xylitol-producing yeast,

but sorbitol and mannitol are mostly produced using LAB (Park et al., 2016). Whereas chemical synthesis yields mannitol and sorbitol that are hard to separate, heterofermentative LAB produce mannitol and no sorbitol from fructose or fructose-glucose mixtures in a single enzymatic step from fructose via mannitol dehydrogenase. Homofermentative LAB use mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase to convert fructose-6-P into mannitol in two steps (Park et al., 2016). However, other by-products such as lactate still need to be eliminated via metabolic engineering. High mannitol yields in homolactic *L. lactis* have been achieved after removing mannitol uptake systems and removal of several *ldh* genes (Gaspar et al., 2011; Gaspar et al., 2004) or by *ldh*-removal combined with heterologous expression of mannitol 1-phosphatase and mannitol 1-phosphate dehydrogenase genes (Wisselink et al., 2005). In heterofermentative *L. reuteri* ATCC 55730, the heterologous expression of *pfk* ensured higher flux through the EMP instead of the PKP pathway. This resulted in a 5.6-times increase in mannitol production, most likely because the increased glycolytic flux resulted in higher NADH availability, which is necessary for mannitol dehydrogenase activity and mannitol production (Papagianni and Legiša, 2014). The strain still produced acetate and lactate and further engineering is required to reduce byproducts, but the strategy to shift the main pathways can be applied for other species and products as well. LAB, namely *L. plantarum* and *L. casei*, have also been engineered for sorbitol production using similar strategies as for mannitol, via overexpression of production pathways, preventing re-utilization, eliminating by-products and improving redox balance (De Boeck et al., 2010; Ladero et al., 2007; Park et al., 2016).

A more recent addition to the specialty LAB product portfolio are plant secondary metabolites such as isoprenoids and polyphenols, which have applications as antimicrobials, antioxidants, cardiovascular drugs, and as flavoring compounds (Pandey et al., 2016). Extraction of these metabolites from plants is typically not highly profitable due to low productivities and high down-stream processing costs. The common platform organisms *E. coli* and yeast have been rather well-established for the production of plant metabolites, but LAB and other Gram positive organisms such as *Corynebacterium glutamicum* are currently being explored as alternative hosts with improved product resistance (Dudnik et al., submitted; Gaspar et al., 2013; Kallscheuer et al., 2016; Pandey et al., 2016; Song et al., 2014b).

4.2. Engineering substrate utilization

Initial bio-based production processes mainly utilized purified sugars from food resources – mainly glucose and sucrose, which are readily fermented by most LAB and other microorganisms. Current second generation processes aim at utilizing raw substrates, or sugars derived from non-food resources. This is especially important for the production of bulk products such as those described above for plastics, nylons, etc. These second generation substrates often contain different sugars, of which not all are readily fermented by most organisms, requiring the selection of strains that naturally do so, or use metabolic engineering to expand the substrate range. It is frequently mentioned that LAB, like yeast, are unable to utilize pentoses (Lu et al., 2016), but as shown in the first section of this work this highly depends on the species that is used and many lactobacilli and pediococci are well able to utilize a wide range of sugars. In some cases, they even carry out mixed sugar fermentations without carbon catabolite repression. For example, *L. brevis* co-utilized xylose and glucose from lignocellulose into lactate without carbon catabolite repression (Guo et al., 2014; Mazzoli et al., 2014). Several other lactobacilli have been shown to utilize different carbon sources such as whey or molasses, either naturally or after metabolic engineering. An extensive overview of this has recently been provided (Mazzoli et al., 2014). Most LAB use the PKP pathway for pentose utilization (Fig. 3A), but *L. plantarum* was engineered to become homolactic via the PPP by heterologous expression of a transketolase for arabinose utilization (Okano et al., 2009a) and after the addition of *xylAB* the strain also fermented xylose in a homolactic way via this pathway (Okano et al., 2009b). The same strain was later shown to be able to co-utilize xylose and glucose during SSF on lignocellulosic substrates (Hama et al., 2015; Zhang et al., 2016). A recent study showed high L-lactate yields (96%) on lignocellulosic hydrolysates using *L. paracasei* with a disrupted D-*ldh* gene (Kuo et al., 2015). In another study, *L. pentosus* was used in an SSF process using corn stover without sterilization (Hu et al., 2016), resulting in a robust and cost-efficient process. A similar SSF-process for lactic acid production from corn stover was shown for *P. acidilactici* (Yi et al., 2016). Whereas non-engineered lactobacilli and pediococci that use both pentoses and hexoses can readily be used in SSF processes and many of them can utilize cellobiose, they generally cannot ferment longer polymers and there are few examples for direct polymer fermentation. These include *L. plantarum* with an endoglucanase from *C. thermocellum* for cellulose conversion (Okano et al., 2010), and *L. brevis* with a xylanase from a metagenomics sample for xylan utilization (Hu et al., 2011). Also *B. subtilis* has been engineered to directly convert xylan to fermentation products (Rhee et al., 2016) and several thermophilic organisms that naturally utilize xylan or cellulose are available as interesting potential hosts for lactate and ethanol production such as *Thermoanaerobacterium aotearoense* (Yang et al., 2013), *G. thermodenitrificans* (Daas et al., 2016) and *C. thermocellum* (Tian et al., 2016). However, for all the mentioned organisms, engineering as such, as well as the expression of (hemi)cellulolytic enzymes and understanding of both organism physiology and enzyme mechanisms and expression is still challenging (Bosma et al., 2013; Mazzoli et al., 2012; Olson et al., 2015; Taylor et al., 2011; Yamada et al., 2013). For these natural hosts as well as for LAB, *B. subtilis* and others, extensive optimization of both substrate utilization and product formation is required before final conclusions about the optimal hosts can be drawn.

4.3. Specialty chemical production and whole-cell applications of LAB: food ingredients, antimicrobials, probiotics and drug delivery

One of the major advantages of LAB over other potential platform organisms is their ability to be used not only for excreted products, but also as whole cells – either when added to food to produce flavor compounds such as diacetyl or acetoin (for which the production as bulk products has been described above), but also for many other purposes in which secondary metabolites are the products of interest, or the entire cell is used, for example as a delivery vehicle for drugs. Such options are hard to engineer in non-native organisms due to the complexity of the pathways and the use of whole cells.

Several LAB-derived secondary metabolites have applications as specialty chemicals within medicine or nutraceuticals (food additives to enhance health benefits). Examples include vitamins or γ-aminobutyric acid (GABA). Most of these compounds are currently preferably added indirectly via probiotics producing such compounds, for which LAB are often considered (Johnson and Klaenhammer, 2014), but several lines of research also investigate the production of these compounds, so they can be added as separate chemicals. Bioactive amines such as GABA are produced by humans and LAB by the decarboxylation of amino acids and serves as an energy source as well as protection mechanism in acidic environments, making LAB very suitable hosts for this product (Diana et al., 2014; Mazzoli et al., 2010). Several processes for GABA-production have been developed for different species of lactobacilli (Diana et al., 2014). Almost all of these focus on the optimization of environmental factors such as pH, but a recent study focused on metabolic engineering of *L. brevis*. GABA-production by this strain was made less dependent on environmental conditions by creating a FoF1-ATPase deficient and glutamate decarboxylase overexpressing strain (Lyu et al., 2017).

The rise of antibiotic-resistant bacteria and subsequent untreatable infections has accelerated research in new antimicrobial compounds. Several alternatives to antibiotics are currently already used in cases

where the use of antibiotics is prohibited, such as in the livestock industry, where probiotic bacteriocins are added to animal nutrition (Ma et al., 2016). Bacteriocins are small, ribosomally synthesized peptides that are secreted by many different bacteria to inhibit the growth of other bacteria, as well as fungi and some parasites (López-Cuellar et al., 2016). The bacteriocins are classified in different subclasses based on characteristics including post-translational modifications, size, thermostability, etc. (Alvarez-Sieiro et al., 2016). They have a wide variety of applications ranging from food preservation to improving health in the GI-tract (López-Cuellar et al., 2016) and they are possible candidates as antibiotic-replacers (Cotter et al., 2013). Many LAB naturally produce bacteriocins, such as nisin by *L. lactis*, sakacin by *L. sakei* or pediocins by pediococci (Alvarez-Sieiro et al., 2016). Metabolic engineering has been used to increase production, such as of pediocin in *P. acidilactici* (Eom et al., 2012) or to broaden antimicrobial activities by adding multiple bacteriocins into one strain, such as microcin V and pediocin PA-1 in *L. plantarum* (Ma et al., 2016). Bacteriocins can be used by adding a producing culture or by adding only the produced and separated compound. In both cases, but especially in the first scenario, LAB are highly preferred because they are food-grade with a long history of safe use in food, and many products derived from LAB strains have received GRAS status (Alvarez-Sieiro et al., 2016).

Another application for which the safety of LAB is important and for which the entire organism is used, is their application as delivery vehicles for drugs targeted at the GI-tract or as in situ vaccine production hosts (Michon et al., 2016). lactobacilli have been shown effective as oral or nasal vaccines against several bacterial and viral infections, and clinical trials with an *L. casei*-based vaccine against human papillomavirus were successful and might become the first approved LAB-based therapy (Rosales-Mendoza et al., 2016). The use of LAB for the delivery of drugs has been evaluated for diseases such as Crohn's disease, diabetes and colorectal cancer (Cano-Garrido et al., 2015). Several drugs can be produced by the same delivery microorganisms and in many cases, side-effects were shown to be reduced compared to traditionally administered medicine (Cano-Garrido et al., 2015). Importantly, by using one organism for production and delivery, expensive purification of the vaccine or drug is prevented, increasing the economic feasibility of these systems (Cano-Garrido et al., 2015; Rosales-Mendoza et al., 2016).

5. Conclusions and future directions

To realize a bio-based economy, it is important that the most suitable production organism is used for each process. Whereas in some cases it is best to use one specific organism for a certain product, it is generally desired to use a platform organism with which several products can be produced. However, this does not need to be a one-organism-fits-all solution and a set of platform organisms each producing a set of products can be envisioned. Economic feasibility analysis will be important to evaluate which process parameters are most crucial for each set, such as medium composition, pH, final titer and productivity, aeration conditions, temperature, contamination risk, etc. In general, it is important to have a flexible and robust organism, and in that respect lactobacilli and pediococci are very suitable candidates: they perform well under a wide range of temperatures, pH values and are generally highly tolerant to high concentrations of several products and stresses. Their small genome size can be of advantage when engineering due to less interference of unexpected regulatory circuits or competing metabolic pathways. Another major advantage of LAB over other organisms is their wide number of possible applications: they are not only potentially suitable as platform organism for bulk products, but also for food applications and specialty products, antimicrobials and medical applications. One could thus envision a LAB-based refinery in which all these applications come together. However, in order to compete with highly engineered species such as *E. coli* and *S. cerevisiae* as platform organisms, the development of high-throughput genetic tools for lactobacilli and pediococci has to be advanced. Whereas they cannot compete with the highly engineered production organisms as of yet, further studies will be very promising as lactobacilli and pediococci show higher tolerance to acid stress and alcohols, as well as faster (facultatively) anaerobic growth and fermentation than many current production hosts. Development of highly engineered strains as well as process optimization, combined with medium optimization by using cheap nutrient sources will likely lead to the development of lactobacilli and pediococci into competitive cell factories with application in biorefineries.

Acknowledgements

Thanks to Kristin Schoppel for excellent technical assistance. This work was supported by Marine Biotechnology ERA-NET “ThermoFactories” under grant number 5178-00003B and the Novo Nordisk Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.biotechadv.2017.04.002.

References

Abdel-Rahman, M.A., Sonomoto, K., 2016. Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. J. Biotechnol. 236, 176–192.

Adsul, M., Khire, J., Bastawde, K., Gokhale, D., 2007. Production of lactic acid from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. Appl. Environ. Microbiol. 73 (15), 5055–5057.

Ahn, J.H., Jang, Y.-S., Lee, S.Y., 2016. Production of succinic acid by metabolically engineered microorganisms. Curr. Opin. Biotechnol. 42, 54–66.

Alegre, M.T., Rodríguez, M.C., Mesas, J.M., 2005. Nucleotide sequence, structural organization and host range of pRS4, a small cryptic *Pediococcus pentosaceus* plasmid that contains two cassettes commonly found in other lactic acid bacteria. FEMS Microbiol. Lett. 250 (1), 151–156.

Allain, T., Aubry, C., Natividad, J.M., Chatel, J.-M., Langella, P., Bermúdez-Humarán, L.G., 2015. Engineering Lactic Acid Bacteria and Bifidobacteria for Mucosal Delivery of Health Molecules, Biotechnology of Lactic Acid Bacteria. John Wiley & Sons, Ltd, pp. 170–190.

Alper, H., Moxley, J., Nevoigt, E., Fink, G.R., Stephanopoulos, G., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science 314 (5805), 1565–1568.

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D., Kuipers, O.P., 2016. Bacteriocins of lactic acid bacteria: extending the family. Appl. Microbiol. Biotechnol. 100 (7), 2939–2951.

Årsköld, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Rådström, P., van Niel, E.W.J., 2008. Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis. J. Bacteriol. 190 (1), 206–212.

Aukrust, T., Blom, H., 1992. Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res. Int. 25 (4), 253–261.

Axelsson, L., Lindstad, G., Naterstad, K., 2003. Development of an inducible gene expression system for *Lactobacillus sakei*. Lett. Appl. Microbiol. 37 (2), 115–120.

Banerjee, A., Biswas, I., 2008. Markerless multiple-gene-deletion system for *Streptococcus mutans*. Appl. Environ. Microbiol. 74 (7), 2037–2042.

Beckner, M., Ivey, M., Phister, T., 2011. Microbial contamination of fuel ethanol fermentations. Lett. Appl. Microbiol. 53 (4), 387–394.

Benachour, A., Flahaut, S., Frère, J., Novel, G., 1996. Plasmid transfer by electroporation and conjugation in *Tetragenococcus* and *Pediococcus* genera and evidence of plasmid-linked metabolic traits. Curr. Microbiol. 32 (4), 188–194.

Benbouziane, B., Ribelles, P., Aubry, C., Martin, R., Kharrat, P., Riazi, A., Langella, P., Bermúdez-Humarán, L.G., 2013. Development of a Stress-Inducible Controlled Expression (SICE) system in *Lactococcus lactis* for the production and delivery of therapeutic molecules at mucosal surfaces. J. Biotechnol. 168 (2), 120–129.

Berezina, O.V., Zakharova, N.V., Brandt, A., Yarotsky, S.V., Schwarz, W.H., Zverlov, V.V., 2010. Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. 87 (2), 635–646.

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K.M., Sani, R.K., 2013. Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. Bioresour. Technol. 128, 751–759.

Biswas, I., Gruss, A., Ehrlich, S.D., Maguin, E., 1993. High-efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. 175 (11), 3628–3635.

Boguta, A.M., Bringel, F., Martinussen, J., Jensen, P.R., 2014. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. Microb. Cell Fact. 13 (1), 97.

Bosma, E.F., van der Oost, J., de Vos, W.M., van Kranenburg, R., 2013. Sustainable production of bio-based chemicals by extremophiles. Curr. Biotechnol. 2 (4), 360–379.

Bosma, E.F., van de Weijer, A.H.P., Daas, M.J.A., van der Oost, J., de Vos, W.M., van Kranenburg, R., 2015. Isolation and screening of thermophilic bacilli from compost for electrotransformation and fermentation: characterization of *Bacillus smithii* ET 138 as a new biocatalyst. Appl. Environ. Microbiol. 81 (5), 1874–1883.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J.C., Gerds, M.L., Hammes, W.P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I.B., Prajapati, J.B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., Hansen, E.B., 2012. Food fermentations: microorganisms with technological beneficial use. Int. J. Food Microbiol. 154 (3), 87–97.

Boyle, N.R., Reynolds, T.S., Evans, R., Lynch, M., Gill, R.T., 2013. Recombineering to homogeneity: extension of multiplex recombineering to large-scale genome editing. Biotechnol. J. 8 (5), 515–522.

Budzianowski, W.M., 2017. High-value low-volume bioproducts coupled to bioenergies with potential to enhance business development of sustainable biorefineries. Renew. Sust. Energ. Rev. 70, 793–804.

Budzianowski, W.M., Postawa, K., 2016. Total chain integration of sustainable biorefinery systems. Appl. Energy 184, 1432–1446.

Burgé, G., Saulou-Bérion, C., Moussa, M., Allais, F., Athes, V., Spinnler, H.-E., 2015. Relationships between the use of Embden Meyerhof pathway (EMP) or phosphoketolase pathway (PKP) and lactate production capabilities of diverse *Lactobacillus reuteri* strains. J. Microbiol. 53 (10), 702–710.

Caggianiello, G., Kleerebezem, M., Spano, G., 2016. Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. Appl. Microbiol. Biotechnol. 100 (9), 3877–3886.

Caldwell, S.L., McMahon, D.J., Oberg, C.J., Broadbent, J.R., 1996. Development and characterization of lactose-positive *Pediococcus* species for milk fermentation. Appl. Environ. Microbiol. 62 (3), 936–941.

Cano-Garrido, O., Seras-Franzoso, J., Garcia-Fruitós, E., 2015. Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microb. Cell Fact. 14 (1), 137.

Castro-Aguirre, E., Iñiguez-Franco, F., Samsudin, H., Fang, X., Auras, R., 2016. Poly(lactic acid)—mass production, processing, industrial applications, and end of life. Adv. Drug Deliv. Rev. 107, 333–366.

Chassy, B.M., Murphy, C.M., 1993. *Lactococcus* and *Lactobacillus*, *Bacillus subtilis* and other Gram-positive bacteria. American Society of Microbiology.

Chen, Z., Liu, D., 2016. Toward glycerol biorefinery: metabolic engineering for the production of biofuels and chemicals from glycerol. Biotechnol. Biofuels 9 (1), 205.

Chen, J., Shen, J., Ingvar Hellgren, L., Ruhdal Jensen, P., Solem, C., 2015. Adaptation of *Lactococcus lactis* to high growth temperature leads to a dramatic increase in acidification rate. Sci. Rep. 5, 14199.

Chikindas, M.L., Venema, K., Ledeboer, A.M., Venema, G., Kok, J., 1995. Expression of lactococcin A and pediocin PA-1 in heterologous hosts. Lett. Appl. Microbiol. 21 (3), 183–189.

Cobb, R.E., Wang, Y., Zhao, H., 2015. High-efficiency multiplex genome editing of *Streptomyces* species using an engineered CRISPR/Cas system. ACS Synth. Biol. 4 (6), 723–728.

Cotter, P.D., Ross, R.P., Hill, C., 2013. Bacteriocins: a viable alternative to antibiotics? Nat. Rev. Microbiol. 11 (2), 95–105.

Daas, M.J.A., van de Weijer, A.H.P., de Vos, W.M., van der Oost, J., van Kranenburg, R., 2016. Isolation of a genetically accessible thermophilic xylan degrading bacterium from compost. Biotechnol. Biofuels 9, 210.

Datta, S., Costantino, N., Zhou, X., Court, D.L., 2008. Identification and analysis of recombineering functions from gram-negative and gram-positive bacteria and their phages. Proc. Natl. Acad. Sci. U. S. A. 105 (5), 1626–1631.

De Boeck, R., Sarmiento-Rubiano, L.A., Nadal, I., Monedero, V., Pérez-Martínez, G., Yebra, M.J., 2010. Sorbitol production from lactose by engineered *Lactobacillus casei* deficient in sorbitol transport system and mannitol-1-phosphate dehydrogenase. Appl. Microbiol. Biotechnol. 85 (6), 1915–1922.

Defoor, E., Kryger, M.-B., Martinussen, J., 2007. The orotate transporter encoded by *oroP* from *Lactococcus lactis* is required for orotate utilization and has utility as a food-grade selectable marker. Microbiology 153 (11), 3645–3659.

Deguchi, Y., Morishita, T., 1992. Nutritional requirements in multiple auxotrophic lactic acid bacteria: genetic lesions affecting amino acid biosynthetic pathways in *Lactococcus lactis*, *Enterococcus faecium*, and *Pediococcus acidilactici*. Biosci. Biotechnol. Biochem. 56 (6), 913–918.

Diana, M., Quílez, J., Rafecas, M., 2014. Gamma-aminobutyric acid as a bioactive compound in foods: a review. J. Funct. Foods 10, 407–420.

Dishisha, T., Pereyra, L.P., Pyo, S.-H., Britton, R.A., Hatti-Kaul, R., 2014. Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. Microb. Cell Fact. 13 (1), 76.

Dobson, C.M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S., Ziola, B., 2002. Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. Int. J. Syst. Evol. Microbiol. 52 (6), 2003–2010.

Dudnik, A., Andrade, R., Avila, B., Bañados, P., Barbay, D., Bassard, J.E., Benkoulouche, M., Bott, M., Braga, A., Breitel, D., Brennan, R., Bulteau, L., Chanforan, C., Costa, I., Costa, R.S., Doostmohammadi, M., Faria, N., Feng, C., Fernandes, A., Ferreira, P., Ferro, R., Foito, A., Freitag, S., Gaspar, P., Godinho-Pereira, J., Hamberger, B., Hartmann, A., Heider, H., Jardim, C., Julien-Laferriere, A., Kallscheuer, N., Kerbe, W., Kuipers, O.P., Lacroix, V., Li, S., Love, N., Marchetti-Spaccamela, A., Marienhagen, J., Martin, C., Mary, A., Mazurek, V., Meinhart, C., Sevillano, D.M., Menezes, R., Naesby, M., Nørholm, M., Okkels, F.T., Oliveira, J., Ottens, M., Parrot, D., Pei, L., Pereira, J., Rocha, I., Rosado-Ramos, R., Rousseau, C., Sagot, M.F., 2016. BacHBerry: BACterial Hosts for production of Bioactive phenolics from bERRY fruits (submitted).

Endo, A., Dicks, L.M., 2014. Physiology of the LAB, Lactic Acid Bacteria: Biodiversity and Taxonomy. pp. 13–30.

Eom, J.-E., Park, J.-Y., Moon, G.-S., 2012. Increased bacteriocin activity of a recombinant *Pediococcus acidilactici*. Food Sci. Biotechnol. 21 (6), 1781–1784.

Ezeji, T.C., Qureshi, N., Blaschek, H.P., 2003. Production of acetone, butanol and ethanol by *Clostridium beijerinckii* BA101 and in situ recovery by gas stripping. World J. Microbiol. Biotechnol. 19 (6), 595–603.

Fang, F., O'Toole, P.W., 2009. Genetic tools for investigating the biology of commensal lactobacilli. Front. Biosci. 14 (8), 3111–3127.

Flahaut, N.A.L., de Vos, W.M., 2015. 8 - systems biology and metabolic engineering of lactic acid bacteria for improved fermented foods A2 - Holzapfel, Wilhelm. In: Advances in Fermented Foods and Beverages Woodhead Publishing, pp. 177–196.

Franz, C.M.A.P., Endo, A., Abriouel, H., Reenen, C.A.V., Gálvez, A., Dicks, L.M.T., 2014. The Genus *Pediococcus*, Lactic Acid Bacteria. John Wiley & Sons, Ltd.pp. 359–376.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., Ruiz-Larrea, F., 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. 230 (1), 53–61.

Gaspar, P., Neves, A.R., Ramos, A., Gasson, M.J., Shearman, C.A., Santos, H., 2004. Engineering *Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system. Appl. Environ. Microbiol. 70 (3), 1466–1474.

Gaspar, P., Neves, A.R., Gasson, M.J., Shearman, C.A., Santos, H., 2011. High yields of 2,3-butanediol and mannitol in *Lactococcus lactis* through engineering of NAD + cofactor recycling. Appl. Environ. Microbiol. 77 (19), 6826–6835.

Gaspar, P., Carvalho, A.L., Vinga, S., Santos, H., Neves, A.R., 2013. From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria. Biotechnol. Adv. 31 (6), 764–788.

Geissler, A.J., Behr, J., von Kamp, K., Vogel, R.F., 2016. Metabolic strategies of beer spoilage lactic acid bacteria in beer. Int. J. Food Microbiol. 216, 60–68.

Goel, A., Wortel, M.T., Molenaar, D., Teusink, B., 2012. Metabolic shifts: a fitness perspective for microbial cell factories. Biotechnol. Lett. 34 (12), 2147–2160.

Goh, Y.J., Azcárate-Peril, M.A., O'Flaherty, S., Durmaz, E., Valence, F., Jardin, J., Lortal, S., Klaenhammer, T.R., 2009. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. 75 (10), 3093–3105.

González-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J.C., Vasconcelos, I., Soucaille, P., 2005. Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab. Eng. 7 (5–6), 329–336.

Guo, T., Hu, S., Kong, J., 2013. Functional analysis and randomization of the nisin-inducible promoter for tuning gene expression in *Lactococcus lactis*. Curr. Microbiol. 66 (6), 548–554.

Guo, W., He, R., Ma, L., Jia, W., Li, D., Chen, S., 2014. Construction of a constitutively expressed homo-fermentative pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. 98 (15), 6641–6650.

Hama, S., Mizuno, S., Kihara, M., Tanaka, T., Ogino, C., Noda, H., Kondo, A., 2015. Production of D-lactic acid from hardwood pulp by mechanical milling followed by simultaneous saccharification and fermentation using metabolically engineered *Lactobacillus plantarum*. Bioresour. Technol. 187, 167–172.

Hartke, A., Bouche, S., Gansel, X., Boutibonnes, P., Auffray, Y., 1994. Starvation-induced stress resistance in *Lactococcus lactis* subsp. *lactis* IL1403. Appl. Environ. Microbiol. 60 (9), 3474–3478.

Heiss, S., Hörmann, A., Tauer, C., Sonnleitner, M., Egger, E., Grabherr, R., Heinl, S., 2016. Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in *Lactobacillus plantarum*. Microb. Cell Fact. 15 (1), 50.

Henard, C.A., Freed, E.F., Guarnieri, M.T., 2015. Phosphoketolase pathway engineering for carbon-efficient biocatalysis. Curr. Opin. Biotechnol. 36, 183–188.

Hofvendahl, K., Hahn–Hägerdal, B., 2000. Factors affecting the fermentative lactic acid production from renewable resources. Enzyme Microb. Technol. 26 (2–4), 87–107.

Hu, C.Y., Chi, D.J., Chen, S.S., Chen, Y.C., 2011. The direct conversion of xylan to lactic acid by *Lactobacillus brevis* transformed with a xylanase gene. Green Chem. 13 (7), 1729–1734.

Hu, J., Lin, Y., Zhang, Z., Xiang, T., Mei, Y., Zhao, S., Liang, Y., Peng, N., 2016. High-titer lactic acid production by *Lactobacillus pentosus* FL0421 from corn stover using fed-batch simultaneous saccharification and fermentation. Bioresour. Technol. 214, 74–80.

Huang, H., Chai, C., Li, N., Rowe, P., Minton, N.P., Yang, S., Jiang, W., Gu, Y., 2016. CRISPR/Cas9-based efficient genome editing in *Clostridium ljungdahlii*, an autotrophic gas-fermenting bacterium. ACS Synth. Biol. 5 (12), 1355–1361.

Huys, G., Leisner, J., Björkroth, J., 2012. The lesser LAB gods: Pediococcus, Leuconostoc, Weissella, Carnobacterium, and affiliated genera. In: Lactic Acid Bacteria: Microbiological and Functional Aspectspp. 93–121.

Ilmén, M., Koivuranta, K., Ruohonen, L., Rajgarhia, V., Suominen, P., Penttilä, M., 2013. Production of L-lactic acid by the yeast *Candida sonorensis* expressing heterologous bacterial and fungal lactate dehydrogenases. Microb. Cell Fact. 12 (1), 53.

Jantama, K., Zhang, X., Moore, J.C., Shanmugam, K.T., Svoronos, S.A., Ingram, L.O., 2008. Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. Biotechnol. Bioeng. 101 (5), 881–893.

Jensen, P.R., Hammer, K., 1998. The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. Appl. Environ. Microbiol. 64 (1), 82–87.

Jensen, T.Ø., Kvist, T., Mikkelsen, M.J., Christensen, P.V., Westermann, P., 2012.

Fermentation of crude glycerol from biodiesel production by *Clostridium pasteurianum*. J. Ind. Microbiol. Biotechnol. 39 (5), 709–717.
Ji, X.-J., Huang, H., Ouyang, P.-K., 2011. Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnol. Adv. 29 (3), 351–364.
Jiang, W., Bikard, D., Cox, D., Zhang, F., Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol. 31 (3), 233–239.
Jiang, R., Ingle, K.N., Golberg, A., 2016. Macroalgae (seaweed) for liquid transportation biofuel production: what is next? Algal Res. 14, 48–57.
Jiménez, J.J., Diep, D.B., Borrero, J., Gútiez, L., Arbulu, S., Nes, I.F., Herranz, C., Cintas, L.M., Hernández, P.E., 2015. Cloning strategies for heterologous expression of the bacteriocin enterocin A by *Lactobacillus sakei* Lb790, *Lb. plantarum* NC8 and *Lb. casei* CECT475. Microb. Cell Fact. 14 (1), 166.
Johnson, B.R., Klaenhammer, T.R., 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antonie Van Leeuwenhoek 106 (1), 141–156.
Jolly, J., Hitzmann, B., Ramalingam, S., Ramachandran, K.B., 2014. Biosynthesis of 1,3-propanediol from glycerol with *Lactobacillus reuteri*: effect of operating variables. J. Biosci. Bioeng. 118 (2), 188–194.
Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103–112.
Kallscheuer, N., Vogt, M., Stenzel, A., Gätgens, J., Bott, M., Marienhagen, J., 2016. Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2S)-flavanones. Metab. Eng. 38, 47–55.
Kandasamy, V., Liu, J., Dantoft, S.H., Solem, C., Jensen, P.R., 2016. Synthesis of (3R)-acetoin and 2,3-butanediol isomers by metabolically engineered *Lactococcus lactis*. Sci. Rep. 6, 36769.
Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 49 (3), 209–224.
Karimi, S., Ahl, D., Vågesjö, E., Holm, L., Phillipson, M., Jonsson, H., Roos, S., 2016. In vivo and in vitro detection of luminescent and fluorescent *Lactobacillus reuteri* and application of red fluorescent mCherry for assessing plasmid persistence. PLoS One 11 (3), e0151969.
Kawai, S., Murata, K., 2016. Biofuel production based on carbohydrates from both brown and red macroalgae: recent developments in key biotechnologies. Int. J. Mol. Sci. 17 (2), 145.
Kelbert, M., Romaní, A., Coelho, E., Pereira, F.B., Teixeira, J.A., Domingues, L., 2015. Lignocellulosic bioethanol production with revalorization of low-cost agroindustrial by-products as nutritional supplements. Ind. Crop. Prod. 64, 16–24.
Kim, S., Hahn, J.-S., 2015. Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing. Metab. Eng. 31, 94–101.
Kim, J.-H., Shoemaker, S.P., Mills, D.A., 2009. Relaxed control of sugar utilization in *Lactobacillus brevis*. Microbiology 155 (4), 1351–1359.
Kim, H.J., Hou, B.K., Lee, S.G., Kim, J.S., Lee, D.-W., Lee, S.J., 2013a. Genome-wide analysis of redox reactions reveals metabolic engineering targets for d-lactate overproduction in *Escherichia coli*. Metab. Eng. 18, 44–52.
Kim, D.-K., Rathnasingh, C., Song, H., Lee, H.J., Seung, D., Chang, Y.K., 2013b. Metabolic engineering of a novel *Klebsiella oxytoca* strain for enhanced 2,3-butanediol production. J. Biosci. Bioeng. 116 (2), 186–192.
Kim, S.-J., Seo, S.-O., Jin, Y.-S., Seo, J.-H., 2013c. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. Bioresour. Technol. 146, 274–281.
Kleerebezem, M., Beerthuyzen, M.M., Vaughan, E.E., de Vos, W.M., Kuipers, O.P., 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl. Environ. Microbiol. 63 (11), 4581–4584.
Knoshaug, E.P., Zhang, M., 2009. Butanol tolerance in a selection of microorganisms. Appl. Biochem. Biotechnol. 153 (1), 13–20.
Kok, J., van der Vossen, J.M., Venema, G., 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. 48 (4), 726–731.
Kuipers, O.P., de Ruyter, P.G.G.A., Kleerebezem, M., de Vos, W.M., 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J. Biotechnol. 64 (1), 15–21.
Kuo, Y.-C., Yuan, S.-F., Wang, C.-A., Huang, Y.-J., Guo, G.-L., Hwang, W.-S., 2015. Production of optically pure L-lactic acid from lignocellulosic hydrolysate by using a newly isolated and D-lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain. Bioresour. Technol. 198, 651–657.
Kylä-Nikkilä, K., Hujanen, M., Leisola, M., Palva, A., 2000. Metabolic engineering of *Lactobacillus helveticus* CNRZ32 for production of pure l-(+)-lactic acid. Appl. Environ. Microbiol. 66 (9), 3835–3841.
Ladero, V., Ramos, A., Wiersma, A., Goffin, P., Schanck, A., Kleerebezem, M., Hugenholtz, J., Smid, E.J., Hols, P., 2007. High-level production of the low-calorie sugar sorbitol by *Lactobacillus plantarum* through metabolic engineering. Appl. Environ. Microbiol. 73 (6), 1864–1872.
Lambert, J.M., Bongers, R.S., Kleerebezem, M., 2007. Cre-*lox*-based system for multiple gene deletions and selectable-marker removal in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 73 (4), 1126–1135.
Landete, J.M., 2016. A review of food-grade vectors in lactic acid bacteria: from the laboratory to their application. Crit. Rev. Biotechnol. 1–13.
Landete, J.M., Arqués, J.L., Peirotén, Á., Langa, S., Medina, M., 2014. An improved method for the electrotransformation of lactic acid bacteria: a comparative survey. J. Microbiol. Methods 105, 130–133.
Law, J., Buist, G., Haandrikman, A., Kok, J., Venema, G., Leenhouts, K., 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J. Bacteriol. 177 (24), 7011–7018.
Lazzi, C., Meli, F., Lambertini, F., Bottesini, C., Nikolaev, I., Gatti, M., Sforza, S., Koroleva, O., Popov, V., Neviani, E., Dossena, A., 2013. Growth promotion of *Bifidobacterium* and *Lactobacillus* species by proteinaceous hydrolysates derived from poultry processing leftovers. Int. J. Food Sci. Technol. 48 (2), 341–349.
Lee, S.J., Song, H., Lee, S.Y., 2006. Genome-based metabolic engineering of *Mannheimia succiniciproducens* for succinic acid production. Appl. Environ. Microbiol. 72 (3), 1939–1948.
Leenhouts, K.J., Kok, J., Venema, G., 1991. Lactococcal plasmid pWV01 as an integration vector for *Lactococci*. Appl. Environ. Microbiol. 57 (9), 2562–2567.
Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G., Kok, J., 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. 253 (1), 217–224.
Li, J., Zhao, J.B., Zhao, M., Yang, Y.L., Jiang, W.H., Yang, S., 2010. Screening and characterization of butanol-tolerant micro-organisms. Lett. Appl. Microbiol. 50 (4), 373–379.
Li, Q., Chen, J., Minton, N.P., Zhang, Y., Wen, Z., Liu, J., Yang, H., Zeng, Z., Ren, X., Yang, J., Gu, Y., Jiang, W., Jiang, Y., Yang, S., 2016. CRISPR-based genome editing and expression control systems in *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Biotechnol. J. 11 (7), 961–972.
Limayem, A., Hanning, I.B., Muthaiyan, A., Illeghems, K., Kim, J.W., Crandall, P.G., O'Bryan, C.A., Ricke, S.C., 2011. Alternative antimicrobial compounds to control potential *Lactobacillus* contamination in bioethanol fermentations. J. Environ. Sci. Health B 46 (8), 709–714.
Lin, L., Xu, J., 2013. Dissecting and engineering metabolic and regulatory networks of thermophilic bacteria for biofuel production. Biotechnol. Adv. 31 (6), 827–837.
Lindlbauer, K.A., Marx, H., Sauer, M., 2017. Effect of carbon pulsing on the redox household of *Lactobacillus diolivorans* in order to enhance 1,3-propanediol production. New Biotechnol. 34, 32–39.
Liu, S., Nichols, N.N., Dien, B.S., Cotta, M.A., 2006. Metabolic engineering of a *Lactobacillus plantarum* double *ldh* knockout strain for enhanced ethanol production. J. Ind. Microbiol. Biotechnol. 33 (1), 1–7.
Liu, S., Skinner-Nemec, K.A., Leathers, T.D., 2008. *Lactobacillus buchneri* strain NRRL B-30929 converts a concentrated mixture of xylose and glucose into ethanol and other products. J. Ind. Microbiol. Biotechnol. 35 (2), 75–81.
Liu, S., Bischoff, K.M., Qureshi, N., Hughes, S.R., Rich, J.O., 2010. Functional expression of the thiolase gene *thl* from *Clostridium beijerinckii* P260 in *Lactococcus lactis* and *Lactobacillus buchneri*. New Biotechnol. 27 (4), 283–288.
Liu, J., Chan, S.H.J., Brock-Nannestad, T., Chen, J., Lee, S.Y., Solem, C., Jensen, P.R., 2016. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. Metab. Eng. 36, 57–67.
Lopez de Felipe, F., Starrenburg, M.J.C., Hugenholtz, J., 1997. The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis* subsp. *lactis* MG1363. FEMS Microbiol. Lett. 156 (1), 15–19.
Lopez de Felipe, F., Kleerebezem, M., de Vos, W.M., Hugenholtz, J., 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. J. Bacteriol. 180 (15), 3804–3808.
López-Cuellar, M.d.R., Rodríguez-Hernández, A.-I., Chavarría-Hernández, N., 2016. LAB bacteriocin applications in the last decade. Biotechnol. Biotechnol. Equip. 30 (6), 1039–1050.
Lu, H., Zhao, X., Wang, Y., Ding, X., Wang, J., Garza, E., Manow, R., Iverson, A., Zhou, S., 2016. Enhancement of D-lactic acid production from a mixed glucose and xylose substrate by the *Escherichia coli* strain JH15 devoid of the glucose effect. BMC Biotechnol. 16 (1), 19.
Lyu, C.-J., Zhao, W.-r., Hu, S., Huang, J., Lu, T., Jin, Z.-h., Mei, L.-h., Yao, S.-j., 2017. Physiology-oriented engineering strategy to improve gamma-aminobutyrate production in *Lactobacillus brevis*. J. Agric. Food Chem.
Ma, X., Wang, G., Li, D., Hao, Y., 2016. Microcin V production in *Lactobacillus plantarum* LB-B1 using heterologous leader peptide from pediocin PA-1. Curr. Microbiol. 72 (3), 357–362.
Madsen, S.M., Arnau, J., Vrang, A., Givskov, M., Israelsen, H., 1999. Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of *Lactococcus lactis*. Mol. Microbiol. 32 (1), 75–87.
Maguin, E., Prévost, H., Ehrlich, S.D., Gruss, A., 1996. Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. J. Bacteriol. 178 (3), 931–935.
Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-H., Díaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. U. S. A. 103 (42), 15611–15616.
Mazzoli, R., Pessione, E., Dufour, M., Laroute, V., Giuffrida, M.G., Giunta, C., Cocaign-Bousquet, M., Loubière, P., 2010. Glutamate-induced metabolic changes in *Lactococcus lactis* NCDO 2118 during GABA production: combined transcriptomic and proteomic analysis. Amino Acids 39 (3), 727–737.
Mazzoli, R., Lamberti, C., Pessione, E., 2012. Engineering new metabolic capabilities in bacteria: lessons from recombinant cellulolytic strategies. Trends Biotechnol. 30 (2), 111–119.
Mazzoli, R., Bosco, F., Mizrahi, I., Bayer, E.A., Pessione, E., 2014. Towards lactic acid bacteria-based biorefineries. Biotechnol. Adv. 32 (7), 1216–1236.
Michon, C., Langella, P., Eijsink, V.G.H., Mathiesen, G., Chatel, J.M., 2016. Display of recombinant proteins at the surface of lactic acid bacteria: strategies and applications. Microb. Cell Fact. 15 (1), 70.

Mierau, I., Kleerebezem, M., 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl. Microbiol. Biotechnol. 68 (6), 705–717.
Monteagudo, J.M., Rodríguez, L., Rincón, J., Fuertes, J., 1997. Kinetics of lactic acid fermentation by *Lactobacillus delbrueckii* grown on beet molasses. J. Chem. Technol. Biotechnol. 68 (3), 271–276.
Morel-Deville, F., Ehrlich, S.D., 1996. Theta-type DNA replication stimulates homologous recombination in the *Bacillus subtilis* chromosome. Mol. Microbiol. 19 (3), 587–598.
Morello, E., Bermúdez-Humarán, L.G., Llull, D., Solé, V., Miraglio, N., Langella, P., Poquet, I., 2008. *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion. J. Mol. Microbiol. Biotechnol. 14 (1–3), 48–58.
Mougiakos, I., Bosma, E.F., de Vos, W.M., van Kranenburg, R., van der Oost, J., 2016. Next generation prokaryotic engineering: the CRISPR-Cas toolkit. Trends Biotechnol. 34 (7), 575–587.
Mougiakos, I., Bosma, E.F., Weenink, K., Vossen, E.M., Goijvaerts, K., Van Der Oost, J., van Kranenburg, R. Efficient genome editing of a facultative thermophile using the mesophilic spCas9. ACS Synth. Biol., (in press), http://dx.doi.org/10.1021/acssynbio.6b00339.
Nagaraju, S., Davies, N.K., Walker, D.J.F., Köpke, M., Simpson, S.D., 2016. Genome editing of *Clostridium autoethanogenum* using CRISPR/Cas9. Biotechnol. Biofuels 9 (1), 219.
Nakagawa, A., Kitahara, K., 1959. Taxonomic studies on the genus *Pediococcus*. J. Gen. Appl. Microbiol. 5 (3), 95–126.
Nguyen, T.-T., Nguyen, H.-M., Geiger, B., Mathiesen, G., Eijsink, V.G., Peterbauer, C.K., Haltrich, D., Nguyen, T.-H., 2015. Heterologous expression of a recombinant lactobacillal β-galactosidase in *Lactobacillus plantarum*: effect of different parameters on the sakacin P-based expression system. Microb. Cell Fact. 14 (1), 30.
Novy, V., Brunner, B., Müller, G., Nidetzky, B., 2017. Toward "homolactic" fermentation of glucose and xylose by engineered *Saccharomyces cerevisiae* harboring a kinetically efficient L-lactate dehydrogenase within pdc1-pdc5 deletion background. Biotechnol. Bioeng. 114 (1), 163–171.
O'Donnell, M.M., O'Toole, P.W., Ross, R.P., 2013. Catabolic flexibility of mammalian-associated lactobacilli. Microb. Cell Factories 12 (1), 1–11.
Oh, J.-H., van Pijkeren, J.-P., 2014. CRISPR–Cas9-assisted recombineering in *Lactobacillus reuteri*. Nucleic Acids Res. 42 (17), e131.
Okano, K., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2009a. Homo-D-lactic acid fermentation from arabinose by redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. Appl. Environ. Microbiol. 75 (15), 5175–5178.
Okano, K., Yoshida, S., Yamada, R., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2009b. Improved production of homo-d-lactic acid via xylose fermentation by introduction of xylose assimilation genes and redirection of the phosphoketolase pathway to the pentose phosphate pathway in l-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. Appl. Environ. Microbiol. 75 (24), 7858–7861.
Okano, K., Zhang, Q., Yoshida, S., Tanaka, T., Ogino, C., Fukuda, H., Kondo, A., 2010. D-lactic acid production from cellooligosaccharides and β-glucan using l-LDH gene-deficient and endoglucanase-secreting *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 85 (3), 643–650.
Olofsson, K., Bertilsson, M., Lidén, G., 2008. A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol. Biofuels 1 (1), 7.
Olson, D.G., Sparling, R., Lynd, L.R., 2015. Ethanol production by engineered thermophiles. Curr. Opin. Biotechnol. 33, 130–141.
Ou, M.S., Mohammed, N., Ingram, L.O., Shanmugam, K.T., 2009. Thermophilic *Bacillus coagulans* requires less cellulases for simultaneous saccharification and fermentation of cellulose to products than mesophilic microbial biocatalysts. Appl. Biochem. Biotechnol. 155 (1), 76–82.
Ou, M.S., Ingram, L.O., Shanmugam, K.T., 2011. L(+)-lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*. J. Ind. Microbiol. Biotechnol. 38 (5), 599–605.
Overbeck, T., Steele, J.L., Broadbent, J.R., 2016. Fermentation of de-oiled algal biomass by *Lactobacillus casei* for production of lactic acid. Bioprocess Biosyst. Eng. 39 (12), 1817–1823.
Özcelik, S., Kuley, E., Özogul, F., 2016. Formation of lactic, acetic, succinic, propionic, formic and butyric acid by lactic acid bacteria. LWT - Food Sci. Technol. 73, 536–542.
Pandey, R.P., Parajuli, P., Koffas, M.A.G., Sohng, J.K., 2016. Microbial production of natural and non-natural flavonoids: pathway engineering, directed evolution and systems/synthetic biology. Biotechnol. Adv. 34 (5), 634–662.
Papagianni, M., 2012. Metabolic engineering of lactic acid bacteria for the production of industrially important compounds. Comput. Struct. Biotechnol. J. 3 (4), 1–8.
Papagianni, M., Legiša, M., 2014. Increased mannitol production in *Lactobacillus reuteri* ATCC 55730 production strain with a modified 6-phosphofructo-1-kinase. J. Biotechnol. 181, 20–26.
Park, Y.-C., Oh, E.J., Jo, J.-H., Jin, Y.-S., Seo, J.-H., 2016. Recent advances in biological production of sugar alcohols. Curr. Opin. Biotechnol. 37, 105–113.
Patel, M.A., Ou, M.S., Harbrucker, R., Aldrich, H.C., Buszko, M.L., Ingram, L.O., Shanmugam, K.T., 2006. Isolation and characterization of acid-tolerant, thermophilic bacteria for effective fermentation of biomass-derived sugars to lactic acid. Appl. Environ. Microbiol. 72 (5), 3228–3235.
Patnaik, R., Louie, S., Gavrilovic, V., Perry, K., Stemmer, W.P.C., Ryan, C.M., del Cardayre, S., 2002. Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat. Biotechnol. 20 (7), 707–712.
Pavan, S., Hols, P., Delcour, J., Geoffroy, M.-C., Grangette, C., Kleerebezem, M., Mercenier, A., 2000. Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study *in vivo* biological effects. Appl. Environ. Microbiol. 66 (10), 4427–4432.
Pederson, C.S., 1949. The genus *Pediococcus*. Bacteriol. Rev. 13 (4), 225–232.
Petersen, K.V., Martinussen, J., Jensen, P.R., Solem, C., 2013. Repetitive, marker-free, site-specific integration as a novel tool for multiple chromosomal integration of DNA. Appl. Environ. Microbiol. 79 (12), 3563–3569.
Pflügl, S., Marx, H., Mattanovich, D., Sauer, M., 2012. 1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*. Bioresour. Technol. 119, 133–140.
Pflügl, S., Marx, H., Mattanovich, D., Sauer, M., 2013. Genetic engineering of *Lactobacillus diolivorans*. FEMS Microbiol. Lett. 344 (2), 152–158.
van Pijkeren, J.-P., Britton, R.A., 2012. High efficiency recombineering in lactic acid bacteria. Nucleic Acids Res. 40 (10), e76.
van Pijkeren, J.P., Britton, R.A., 2014. Precision genome engineering in lactic acid bacteria. Microb. Cell Factories 13 (1), 1–10.
Pittet, V., Phister, T.G., Ziola, B., 2013. Transcriptome sequence and plasmid copy number analysis of the brewery isolate *Pediococcus claussenii* ATCC BAA-344^T during growth in beer. PLoS One 8 (9), e73627.
van der Pol, E.C., Eggink, G., Weusthuis, R.A., 2016. Production of L(+)-lactic acid from acid pretreated sugarcane bagasse using *Bacillus coagulans* DSM2314 in a simultaneous saccharification and fermentation strategy. Biotechnol. Biofuels 9 (1), 248.
Pot, B., Felis, G.E., Bruyne, K.D., Tsakalidou, E., Papadimitriou, K., Leisner, J., Vandamme, P., 2014. The Genus *Lactobacillus*, Lactic Acid Bacteria. John Wiley & Sons, Ltd.pp. 249–353.
Rallu, F., Gruss, A., Maguin, E., 1996. *Lactococcus lactis* and stress. Antonie Van Leeuwenhoek 70 (2), 243–251.
Rhee, M.S., Wei, L., Sawhney, N., Kim, Y.S., Rice, J.D., Preston, J.F., 2016. Metabolic potential of *Bacillus subtilis* 168 for the direct conversion of xylans to fermentation products. Appl. Microbiol. Biotechnol. 100 (3), 1501–1510.
Ricci, M.A., Russo, A., Pisano, I., Palmieri, L., de Angelis, M., Agrimi, G., 2015. Improved 1,3-propanediol synthesis from glycerol by the robust *Lactobacillus reuteri* strain DSM 20016. J. Microbiol. Biotechnol. 25 (6), 893–902.
Roach, D.R., Khatibi, P.A., Bischoff, K.M., Hughes, S.R., Donovan, D.M., 2013. Bacteriophage-encoded lytic enzymes control growth of contaminating *Lactobacillus* found in fuel ethanol fermentations. Biotechnol. Biofuels 6 (1), 20.
Rodríguez, M.C., Alegre, M.T., Mesas, J.M., 2007. Optimization of technical conditions for the transformation of *Pediococcus acidilactici* P60 by electroporation. Plasmid 58 (1), 44–50.
Ronda, C., Pedersen, L.E., Sommer, M.O.A., Nielsen, A.T., 2016. CRMAGE: CRISPR optimized MAGE recombineering. Sci. Rep. 6, 19452.
Rosales-Mendoza, S., Angulo, C., Meza, B., 2016. Food-grade organisms as vaccine biofactories and oral delivery vehicles. Trends Biotechnol. 34 (2), 124–136.
Rud, I., Jensen, P.R., Naterstad, K., Axelsson, L., 2006. A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*. Microbiology 152 (4), 1011–1019.
Russell, W.M., Klaenhammer, T.R., 2001. Efficient system for directed integration into the *Lactobacillus acidophilus* and *Lactobacillus gasseri* chromosomes via homologous recombination. Appl. Environ. Microbiol. 67 (9), 4361–4364.
de Ruyter, P.G., Kuipers, O.P., Beerthuyzen, M.M., van Alen-Boerrigter, I., de Vos, W.M., 1996a. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. 178 (12), 3434–3439.
de Ruyter, P.G., Kuipers, O.P., de Vos, W.M., 1996b. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. 62 (10), 3662–3667.
Ryssel, M., Hviid, A.-M.M., Dawish, M.S., Haaber, J., Hammer, K., Martinussen, J., Kilstrup, M., 2014. Multi-stress resistance in *Lactococcus lactis* is actually escape from purine-induced stress sensitivity. Microbiology 160 (11), 2551–2559.
Salgado, J.M., Rodríguez, N., Cortés, S., Domínguez, J.M., 2009. Development of cost-effective media to increase the economic potential for larger-scale bioproduction of natural food additives by *Lactobacillus rhamnosus*, *Debaryomyces hansenii*, and *Aspergillus niger*. J. Agric. Food Chem. 57 (21), 10414–10428.
Sanoja, R.R., Morlon-Guyot, J., Guyot, J.P., 1999. Electrotransformation of *Lactobacillus manihotivorans* LMG 18010T and LMG 18011. J. Appl. Microbiol. 87 (1), 99–107.
Santos, F., 2008. Vitamin B12 Synthesis in *Lactobacillus reuteri*. (PhD thesis) Wageningen University.
Santos, F., Vera, J.L., van der Heijden, R., Valdez, G., de Vos, W.M., Sesma, F., Hugenholtz, J., 2008. The complete coenzyme B12 biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. Microbiology 154 (1), 81–93.
Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26 (2), 100–108.
Saulnier, D.M., Santos, F., Roos, S., Mistretta, T.-A., Spinler, J.K., Molenaar, D., Teusink, B., Versalovic, J., 2011. Exploring metabolic pathway reconstruction and genome-wide expression profiling in *Lactobacillus reuteri* to define functional probiotic features. PLoS One 6 (4), e18783.
Sawitzke, J.A., Thomason, L.C., Costantino, N., Bubunenko, M., Datta, S., Court, D.L., 2007. Recombineering: *In Vivo* Genetic Engineering in *E. coli*, *S. enterica*, and Beyond, Methods in Enzymology. Academic Presspp. 171–199.
Seidl, P.R., Goulart, A.K., 2016. Pretreatment processes for lignocellulosic biomass conversion to biofuels and bioproducts. Curr. Opin. Green Sustain. Chem. 2, 48–53.
Serror, P., Ilami, G., Chouayekh, H., Ehrlich, S.D., Maguin, E., 2003. Transposition in *Lactobacillus delbrueckii* subsp. *bulgaricus*: identification of two thermosensitive replicons and two functional insertion sequences. Microbiology 149 (6), 1503–1511.
Sharan, S.K., Thomason, L.C., Kuznetsov, S.G., Court, D.L., 2009. Recombineering: a homologous recombination-based method of genetic engineering. Nat. Protoc. 4 (2), 206–223.
Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M., Liao, J.C., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl. Environ. Microbiol. 77 (9), 2905–2915.
Solem, C., Jensen, P.R., 2002. Modulation of gene expression made easy. Appl. Environ.

Microbiol. 68 (5), 2397–2403.
Solem, C., Dehli, T., Jensen, P.R., 2013. Rewiring *Lactococcus lactis* for ethanol production. Appl. Environ. Microbiol. 79 (8), 2512–2518.
Song, L., Cui, H., Tang, L., Qiao, X., Liu, M., Jiang, Y., Cui, W., Li, Y., 2014a. Construction of *upp* deletion mutant strains of *Lactobacillus casei* and *Lactococcus lactis* based on counterselective system using temperature-sensitive plasmid. J. Microbiol. Methods 102, 37–44.
Song, A.A., Ong Abdullah, J., Abdullah, M.P., Shafee, N., Othman, R., Noor, N.M., Rahim, R.A., 2014b. Engineering the lactococcal mevalonate pathway for increased sesquiterpene production. FEMS Microbiol. Lett. 355 (2), 177–184.
Sørvig, E., Mathiesen, G., Naterstad, K., Eijsink, V.G.H., Axelsson, L., 2005. High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. Microbiology 151 (7), 2439–2449.
Steen, E.J., Chan, R., Prasad, N., Myers, S., Petzold, C.J., Redding, A., Ouellet, M., Keasling, J.D., 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. Microb. Cell Fact. 7 (1), 36.
Sun, Z., Harris, H.M.B., McCann, A., Guo, C., Argimón, S., Zhang, W., Yang, X., Jeffery, I.B., Cooney, J.C., Kagawa, T.F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M.C., O'Sullivan, O., Ritari, J., Douillard, F.P., Paul Ross, R., Yang, R., Briner, A.E., Felis, G.E., de Vos, W.M., Barrangou, R., Klaenhammer, T.R., Caufield, P.W., Cui, Y., Zhang, H., O'Toole, P.W., 2015. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. Nat. Commun. 6, 8322.
Tanaka, K., Komiyama, A., Sonomoto, K., Ishizaki, A., Hall, S., Stanbury, P., 2002. Two different pathways for D-xylose metabolism and the effect of xylose concentration on the yield coefficient of L-lactate in mixed-acid fermentation by the lactic acid bacterium *Lactococcus lactis* IO-1. Appl. Microbiol. Biotechnol. 60 (1), 160–167.
Tauer, C., Heinl, S., Egger, E., Heiss, S., Grabherr, R., 2014. Tuning constitutive recombinant gene expression in *Lactobacillus plantarum*. Microb. Cell Fact. 13 (1), 150.
Taylor, M., van Zyl, L., Tuffin, I., Leak, D., Cowan, D., 2011. Genetic tool development underpins recent advances in thermophilic whole-cell biocatalysts. Microb. Biotechnol. 4 (4), 438–448.
Tian, L., Papanek, B., Olson, D.G., Rydzak, T., Holwerda, E.K., Zheng, T., Zhou, J., Maloney, M., Jiang, N., Giannone, R.J., Hettich, R.L., Guss, A.M., Lynd, L.R., 2016. Simultaneous achievement of high ethanol yield and titer in *Clostridium thermocellum*. Biotechnol. Biofuels 9 (1).
Tong, Y., Charusanti, P., Zhang, L., Weber, T., Lee, S.Y., 2015. CRISPR-Cas9 based engineering of Actinomycetal genomes. ACS Synth. Biol. 4 (9), 1020–1029.
Tsuji, A., Okada, S., Hols, P., Satoh, E., 2013. Metabolic engineering of *Lactobacillus plantarum* for succinic acid production through activation of the reductive branch of the tricarboxylic acid cycle. Enzym. Microb. Technol. 53 (2), 97–103.
U.S. Food and Drug Administration, 2017. GRAS Notices. http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices (Accessed 08.02. 17).
Upadhyaya, B.P., DeVeaux, L.C., Christopher, L.P., 2014. Metabolic engineering as a tool for enhanced lactic acid production. Trends Biotechnol. 32 (12), 637–644.
Vázquez, J.A., Murado, M.A., 2008. Mathematical tools for objective comparison of microbial cultures: application to evaluation of 15 peptones for lactic acid bacteria productions. Biochem. Eng. J. 39 (2), 276–287.
Vinay-Lara, E., Wang, S., Bai, L., Phrommao, E., Broadbent, J.R., Steele, J.L., 2016. *Lactobacillus casei* as a biocatalyst for biofuel production. J. Ind. Microbiol. Biotechnol. 43 (9), 1205–1213.
Vivek, N., Pandey, A., Binod, P., 2016. Biological valorization of pure and crude glycerol into 1,3-propanediol using a novel isolate *Lactobacillus brevis* N1E9.3.3. Bioresour. Technol. 213, 222–230.
de Vos, W.M., 1996. Metabolic engineering of sugar catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 70 (2), 223–242.
de Vos, W.M., 2011. Systems solutions by lactic acid bacteria: from paradigms to practice. Microb. Cell Fact. 10 (1), S2.
de Vos, W.M., Hugenholtz, J., 2004. Engineering metabolic highways in lactococci and other lactic acid bacteria. Trends Biotechnol. 22 (2), 72–79.
Wan, X., Usvalampi, A.M., Saris, P.E.J., Takala, T.M., 2016. A counterselection method for *Lactococcus lactis* genome editing based on class IIa bacteriocin sensitivity. Appl. Microbiol. Biotechnol. 100 (22), 9661–9669.
Wang, Y., Li, Y., Pei, X., Yu, L., Feng, Y., 2007. Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. J. Biotechnol. 129 (3), 510–515.
Wang, Q., Ingram, L.O., Shanmugam, K.T., 2011. Evolution of D-lactate dehydrogenase activity from glycerol dehydrogenase and its utility for D-lactate production from lignocellulose. Proc. Natl. Acad. Sci. U. S. A. 108 (47), 18920–18925.
Wang, Y., Tian, T., Zhao, J., Wang, J., Yan, T., Xu, L., Liu, Z., Garza, E., Iverson, A., Manow, R., Finan, C., Zhou, S., 2012. Homofermentative production of D-lactic acid from sucrose by a metabolically engineered *Escherichia coli*. Biotechnol. Lett. 34 (11), 2069–2075.
Wang, Y., Li, K., Huang, F., Wang, J., Zhao, J., Zhao, X., Garza, E., Manow, R., Grayburn, S., Zhou, S., 2013. Engineering and adaptive evolution of *Escherichia coli* W for L-lactic acid fermentation from molasses and corn steep liquor without additional nutrients. Bioresour. Technol. 148, 394–400.
Wang, Y., Zhang, Z.-T., Seo, S.-O., Choi, K., Lu, T., Jin, Y.-S., Blaschek, H.P., 2015. Markerless chromosomal gene deletion in *Clostridium beijerinckii* using CRISPR/Cas9 system. J. Biotechnol. 200, 1–5.
Weusthuis, R.A., Lamot, I., van der Oost, J., Sanders, J.P.M., 2011. Microbial production of bulk chemicals: development of anaerobic processes. Trends Biotechnol. 29 (4), 153–158.
Wisselink, H.W., Moers, A.P.H.A., Mars, A.E., Hoefnagel, M.H.N., de Vos, W.M., Hugenholtz, J., 2005. Overproduction of heterologous mannitol 1-phosphatase: a key factor for engineering mannitol production by *Lactococcus lactis*. Appl. Environ. Microbiol. 71 (3), 1507–1514.
Xu, Y., Chu, H., Gao, C., Tao, F., Zhou, Z., Li, K., Li, L., Ma, C., Xu, P., 2014. Systematic metabolic engineering of *Escherichia coli* for high-yield production of fuel biochemical 2,3-butanediol. Metab. Eng. 23, 22–33.
Xu, Q., Xie, L., Li, Y., Lin, H., Sun, S., Guan, X., Hu, K., Shen, Y., Zhang, L., 2015. Metabolic engineering of *Escherichia coli* for efficient production of (3R)-acetoin. J. Chem. Technol. Biotechnol. 90 (1), 93–100.
Yamada, R., Hasunuma, T., Kondo, A., 2013. Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. Biotechnol. Adv. 31 (6), 754–763.
Yang, X., Lai, Z., Lai, C., Zhu, M., Li, S., Wang, J., Wang, X., 2013. Efficient production of L-lactic acid by an engineered *Thermoanaerobacterium aotearoense* with broad substrate specificity. Biotechnol. Biofuels 6 (1), 124.
Yang, P., Wang, J., Qi, Q., 2015. Prophage recombinases-mediated genome engineering in *Lactobacillus plantarum*. Microb. Cell Fact. 14 (1), 154.
Ye, L., Zhao, H., Li, Z., Wu, J.C., 2013. Improved acid tolerance of *Lactobacillus pentosus* by error-prone whole genome amplification. Bioresour. Technol. 135, 459–463.
Yi, X., Zhang, P., Sun, J., Tu, Y., Gao, Q., Zhang, J., Bao, J., 2016. Engineering wild-type robust *Pediococcus acidilactici* strain for high titer L- and D-lactic acid production from corn stover feedstock. J. Biotechnol. 217, 112–121.
Yomano, L.P., York, S.W., Ingram, L.O., 1998. Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. J. Ind. Microbiol. Biotechnol. 20 (2), 132–138.
Yoo, M., Croux, C., Meynial-Salles, I., Soucaille, P., 2017. Metabolic flexibility of a butyrate pathway mutant of Clostridium acetobutylicum. Metab. Eng. 40, 138–147.
Zhang, L., Zhang, Y., Liu, Q., Meng, L., Hu, M., Lv, M., Li, K., Gao, C., Xu, P., Ma, C., 2015. Production of diacetyl by metabolically engineered *Enterobacter cloacae*. Sci. Rep. 5, 9033.
Zhang, Y., Vadlani, P.V., Kumar, A., Hardwidge, P.R., Govind, R., Tanaka, T., Kondo, A., 2016. Enhanced D-lactic acid production from renewable resources using engineered *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 100 (1), 279–288.
Zhao, K., Qiao, Q., Chu, D., Gu, H., Dao, T.H., Zhang, J., Bao, J., 2013. Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2. Bioresour. Technol. 135, 481–489.
Zheng, J., Ruan, L., Sun, M., Gänzle, M., 2015. A genomic view of *Lactobacilli* and *Pediococci* demonstrates that phylogeny matches ecology and physiology. Appl. Environ. Microbiol. 81 (20), 7233–7243.
Zhou, L., Cui, W.-j., Liu, Z.-m., Zhou, Z.-m., 2016. Metabolic engineering strategies for D-lactate over production in *Escherichia coli*. J. Chem. Technol. Biotechnol. 91 (3), 576–584.
Zhou, X., Ye, L., Wu, J., 2013. Efficient production of L-lactic acid by newly isolated thermophilic *Bacillus coagulans* WCP10-4 with high glucose tolerance. Appl. Microbiol. Biotechnol. 97 (10), 4309–4314.
Zhu, D., Zhao, K., Xu, H., Zhang, X., Bai, Y., Saris, P.E.J., Qiao, M., 2015. Construction of *thyA* deficient *Lactococcus lactis* using the Cre-*loxP* recombination system. Ann. Microbiol. 65 (3), 1659–1665.