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Food grade microbial synthesis of the butter aroma compound butanedione using engineered and non-engineered Lactococcus lactis

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ABSTRACT

The design-build-test-learn (DBTL) cycle has been implemented in metabolic engineering processes for optimizing the production of valuable compounds, including food ingredients. However, the use of recombinant microorganisms for producing food ingredients is associated with different challenges, e.g., in the EU, a content of more than 0.9% of such ingredients requires to be labeled. Therefore, we propose to expand the DBTL cycle and use the "learn" module to guide the development of non-engineered strains for clean label production. Here, we demonstrate how this approach can be used to generate engineered and natural cell factories able to produce the valuable food flavor compound - butanedione (diacetyl). Through comprehensive rerouting of the metabolism of *Lactococcus lactis* MG1363 and re-installment of the capacity to metabolize lactose and dairy protein, we managed to achieve a high titer of diacetyl (6.7 g/L) in pure dairy waste. Based on learnings from the engineering efforts, we successfully achieved the production of diacetyl without using recombinant DNA technology. We accomplish the latter by process optimization and by relying on high-throughput screening using a microfluidic system. Our results demonstrate the great potential that lies in combining metabolic engineering and natural approaches for achieving efficient production of food ingredients.

CRediT authorship contribution statement

J.L., P.J., C.S. designed and performed experiments, analyzed data and wrote the manuscript. L.C. carried out experiments on plasmid construction and strain design.

1. Introduction

Metabolic engineering efforts often involve four highly interdependent modules: Design, Build, Test and Learn (DBTL)(Nielsen and Keasling, 2016). The DBTL cycle focuses on designing efficient metabolic pathways, building robust and productive biological systems, testing the desired function(s), and learning how to further improve performance (Fig. 1). DBTL has been successfully used for engineering microorganisms into efficient cell factories for valuable food ingredients, such as heme (Zhao et al., 2018), vitamin B_{12} (Fang et al., 2018), omega-3 fatty acids (Xue et al., 2013) and flavor compounds (Koma et al., 2012; Liu et al., 2016a). These genetically modified microorganisms (GMOs), despite their impressive performance, have some potential drawbacks. In EU, food ingredients made from GMOs need to be labeled if they constitute more than 0.9% (Bruetschy, 2019), and the use of GMOs in food is heavily regulated (Wasmer, 2019). It thus appears that for food applications, there is a need for expanding the DBTL cycle. More specifically, the knowledge about cellular metabolism, metabolic regulations and flux limitations learned from the development of engineered strains, can be used to guide the design of non-engineered strains with improved performance (Fig. 1).

We took the C4 compound butanedione as an example. Butanedione, also called diacetyl, is a food ingredient with high value that contributes to the buttery aroma of many fermented foods, including cheese and butter. There have been several metabolic engineering efforts to produce diacetyl, e.g., using *Enterobacter cloacae* (Zhang et al., 2015), *Bacillus* spp. (Hao et al., 2017) and *L. lactis* (Liu et al., 2016a; Hugenholtz et al., 2000). However, in all these studies, either potentially pathogenic microorganisms or expensive rich growth substrates were used, which is not compatible with industrial production. There are also reports describing diacetyl production using non-engineered organisms, in particular dairy *L. lactis* strains (Boumerdassi et al., 1997; Monnet et al.,

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2000), but in all cases, rather low titers or an unstable production phenotype was observed. *L. lactis* is an obvious microbial candidate for producing diacetyl for many reasons. First, *L. lactis* has a long record of safe use for the production of fermented food products. Its ability to prolong shelf-life, affect texture and enhance flavor in combination with its safe status has secured it an important role in the food industry. Secondly, some *L. lactis* strains naturally produce diacetyl and can be included in starter cultures used in food fermentations to provide butter flavor. Moreover, the dairy industry generates large waste streams. It is reported that a total of 40 × 10^6 tons/year whey is produced in EU (Sebastián-Nicolás et al., 2020), and in the process to make whey-derived products, including whey protein, lactose and dry whey powder, large amounts of waste are produced. *L. lactis* has a great inherent capacity to convert these waste materials into valuable food ingredients (Liu et al., 2020).

Other features of dairy *L. lactis* include their flexible metabolism and their many critical plasmid-encoded traits, enabling them to thrive in a dairy environment, e.g., ability to metabolize lactose, degrade proteins, utilize citrate and their collective resistance to bacteriophages. It is widely accepted that *L. lactis* originated from a plant-associated niche and acquired plasmid-encoded traits in their adaptation to the nutrient-rich milk environment (Wels et al., 2019). Genome sequences of 16 *L. lactis* strains revealed the presence of 83 plasmids (Kelleher et al., 2019) and for instance, *L. lactis* subsp. *lactis* biovar diacetylactis FM03P has a large plasmidome consisting of 12 plasmids (van Mastrigt et al., 2018). Most of these plasmids rely on theta-type replication and are stably maintained in the presence of selection pressure (i.e., in a dairy environment). *L. lactis* MG1363 is a well-characterized model strain, which is a plasmid-free derivative of the dairy isolate NCDO712 (Tarazanova et al., 2016). Due to its well-developed genetic toolbox, including synthetic promoter libraries and convenient gene integration or knockout strategies, MG1363 is an excellent chassis for developing food-grade microbial cell factories. Although MG1363 lacks the plasmids that enable growth in milk, these can be re-introduced after genetic manipulation. By studying the performance of the engineered strains in media composed of readily available dairy waste, knowledge can be acquired that can be used for optimizing industrial strains in a natural (non-recombinant) manner.

In this study, we successfully engineered MG1363 into producing high amounts of diacetyl by enhancing the native metabolic flux and by knocking out competitive pathways (Fig. 2). The introduction of lactose and protease plasmids enabled the growth and production of diacetyl on pure dairy waste. Diacetyl can be purified and used as a flavor enhancer in foods or serve other applications. From the engineering efforts, we learned the importance of inactivating lactate dehydrogenase or lower its expression for diverting the metabolic flux towards α-acetolactate (AL), the precursor for diacetyl. We furthermore realized that the conversion of AL to diacetyl occurs slowly and that heme, which can activate respiratory metabolism in *L. lactis*, is able to stimulate diacetyl production. We managed to harness these findings and develop non-engineered *L. lactis* strains able to produce diacetyl naturally and efficiently.

2. Materials and methods

2.1. Strains and plasmids

All bacterial strains and plasmids used or constructed in this study are listed in Table 1. The plasmid-free strain *L. lactis* subsp. *cremoris* MG1363 or derivatives were used as chassis for metabolic engineering. *L. lactis* subsp. *lactis* biovar diacetylactis RD1M5, a natural mutant of industrial strain SD96 (Dorau et al., 2020), has an *ldh* gene frameshift mutation with a duplicated region "CCGTCAAG" between T464 and C465 (Liu et al., 2020). *Escherichia coli* ABLE C (*E. coli* C lac (LacZ$^-$) [Kan^r McrA$^-$McrCB$^-$ McrF$^-$Mrr$^-$HsdR ($r_K^-m_K^-$)] [F' proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]) was used for cloning purposes. The plasmid pCS1966 (Solem et al., 2008) was used for deleting *aldB* gene encoding acetolactate decarboxylase. The plasmid pTD6 (Liu et al., 2016a) was used for expressing ALS (acetolactate synthase) and NOX (NADH oxidase) from different sources.

2.2. Growth medium and conditions

E. coli strains were grown aerobically at 30°C in Luria-Bertani broth. Dia001 and the SLC strains were grown in M17 broth (Oxoid) supplemented with 2% glucose with shaking at 200 rpm. The fermentation

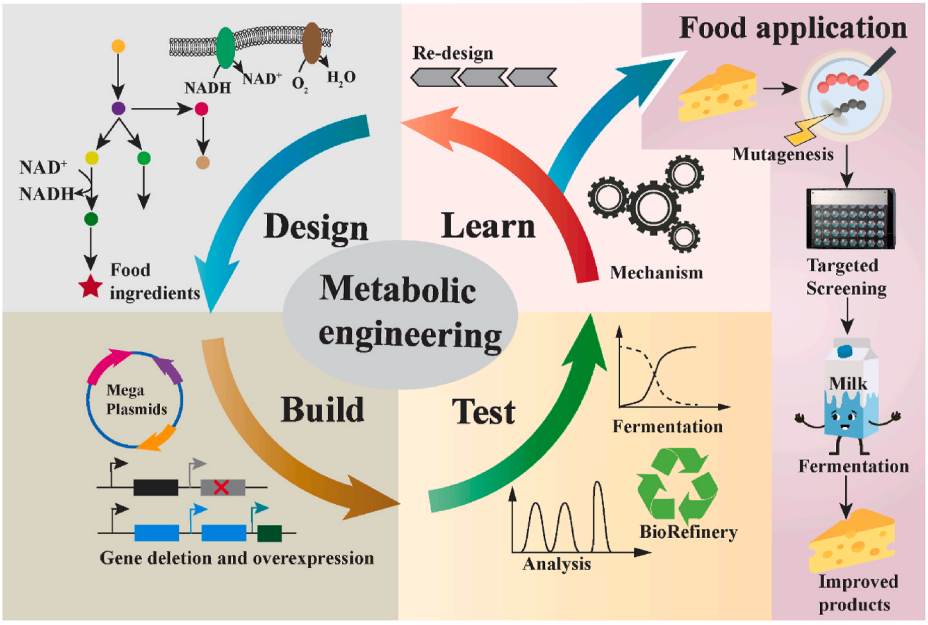

Fig. 1. The design-build-test-learn (DBTL) cycle for metabolic engineering as well as for improving food microorganisms using non-engineered approaches. Through the iterative DBTL cycle, learned knowledge can be harnessed and used for optimizing food microorganisms in a natural manner, i.e., without using recombinant DNA technology. Such naturally optimized microorganisms have a great potential for direct use in foods.

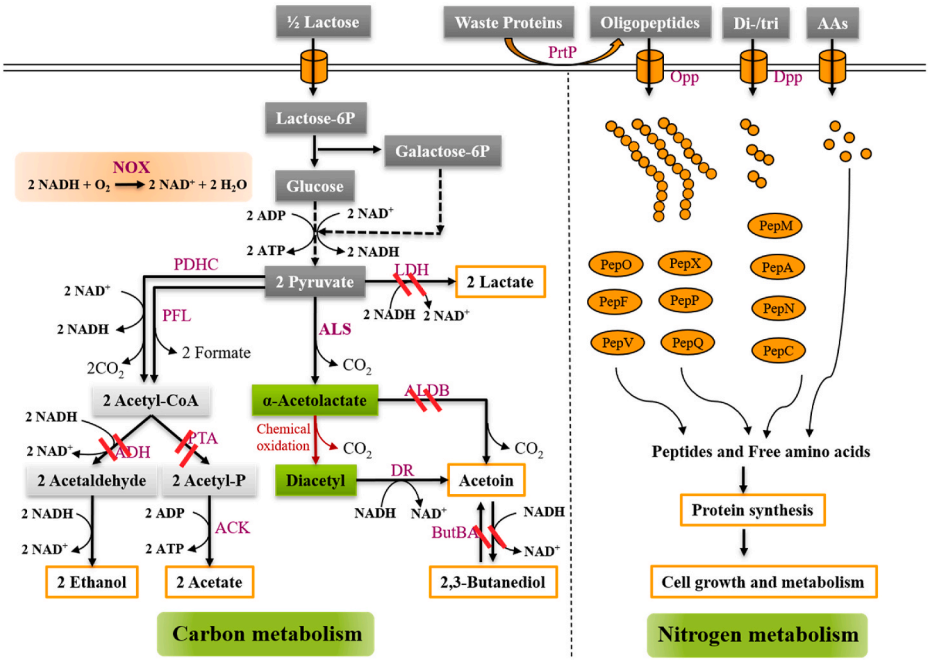

Fig. 2. Cellular metabolism of *L. lactis*. *L. lactis* can metabolize lactose and produce diacetyl. The proteolytic machinery of *L. lactis* enables it to obtain amino acids from the proteins contained in milk and dairy waste. The "\" lines indicate inactivated competing pathways. Abbreviation: LDH, lactate dehydrogenase; ALS, α-acetolactate synthase; ALDB, α-acetolactate decarboxylase; DR: diacetyl reductase; ButBA, butanediol dehydrogenase; PDHC, pyruvate dehydrogenase complex; PFL, pyruvate formate lyase; PTA, phosphotransacetylase; ADH, alcohol dehydrogenase; ACK, acetate kinase; NOX, NADH oxidase. PepM-A-N-C, aminopeptidase. PepX-P-Q, proline-specific peptidases. PepO-F-N, endopeptidases. Opp, oligopeptide permease. Dpp, di (tri) peptides permease. AAs, amino acids. A further description of the proteolytic system of *L. lactis* can be found here (Guédon et al., 2001).

Table 1
Strains and plasmids.

Designation	Genotype or description	Reference
***L. lactis* strains**		
CS4234	MG1363 Δ[3]*ldh*Δ*pta*	Liu et al., 2016
GM001	MG1363 Δ[3]*ldh*Δ*pta*Δ*aldB*	This work
Ace001	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*	Liu et al., 2016
Ace002	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA pCS4564*	This work
Dia001M	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB pCS4564*	This work
Dia001	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB*	This work
SLC0	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pTD6	This work
SLC1	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC1	This work
SLC2	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC2	This work
SLC3	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC3	This work
SLC17	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC17	This work
SLC4	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC4	This work
SLC5	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC5	This work
SLC6	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC6	This work
SLC18	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC18	This work
LacDia	MG1363 Δ[3]*ldh*Δ*pta*Δ*adhE*Δ*butBA*Δ*aldB* pLC18 pLasmids	This work
RD1M5	Natural mutant from dairy *L. lactis* subsp. *lactis* biovar diacetylactis	Liu et al., 2020
F101	Natural isolate from an aromatic mesophilic starter	This work
SENSOR	*E. coli*::GFP Δ*ilvBIG*	This work
Plasmids		
pCS4564	pG[+]host8::*ldhA* (*E. coli*)	Liu et al., 2016
pLasmids	Lactococcal plasmids extracted from RD1M5	This work
pLC1	pTD6::*Efals* (from *Enterococcus faecalis* ATCC 29212)	This work
pLC2	pTD6::*Blals* (from *Bacillus licheniformis* WX-02)	This work
pLC3	pTD6::*Lrals* (from *Lactobacillus rhamnosus*)	This work
pLC17	pTD6::*als* (from *Lactococcus lactis* MG1363)	This work
pLC4	pTD6::*noxE* (from *L. lactis* MG1363)	This work
pLC5	pTD6::*LrnoxE* (from *L. rhamnosus* DSM, 20021)	This work
pLC6	pTD6::*EfnoxE1* (from *E. faecalis* ATCC 29212)	This work
pLC18	pTD6::*Efals*::*noxE*	This work

samples were taken after 24 h to evaluate the effects of overexpression of ALS and NOX activities. When required, antibiotics were added in the following concentrations: erythromycin: 200 μg/mL for *E. coli* and 5 μg/mL for *L. lactis*, tetracycline: 8 μg/mL for *E. coli* and 5 μg/mL for *L. lactis*. Regarding the use of waste streams, residual whey permeate (RWP) is.

the residue obtained after recovery of lactose from whey permeate. Its composition is shown in Table S1. Protein waste (PW) is a by-product in the whey protein production process and has a protein content of 70 g/L (Table S2). Both RWP and PW were provided by Arla Food Ingredients (Viby, Denmark). The combination of RWP and PW can serve as a complete fermentation medium for *L. lactis*. PW has a milky appearance and for easy monitoring of bacterial growth, PW was pretreated with HCl (20 mL 32% HCl in 1 L PW) for 10 min. The supernatant obtained after centrifugation termed PWF was used as an alternative nitrogen source.

2.3. DNA techniques and strain construction

The lactose plasmid (49.6 kb, GenBank accession number: CP043524) and the protease plasmid (64.9 kb, GenBank accession number: CP043526) were extracted from RD1M5 based on the method of Andersen (Anderson and McKay, 1983). Chromosomal DNA from *L. lactis* was isolated using the method described for *E. coli* with the modification that cells were treated with 20 μg/mL lysozyme for 2 h. Cells of *E. coli* were transformed using electroporation. Cells of L. *lactis* were made electrocompetent by growing in GM17 medium containing 1% glycine with the modifications that 20 μg/mL ampicillin was added at an optical density of 0.5 (OD_{600}) and was allowed to act for 0.5 h prior to harvesting the cells. The plasmid pCS4564 (Liu et al., 2017), which carries a *ldh* gene from *E. coli* and a thermosensitive replicon, was introduced into Ace001 and obtained strain Ace002 that can grow anaerobically. Then we knocked out the *aldB* gene in Ace002 and the resulting strain was Dia001 (MG1363 Δ[3]*ldh* Δ*pta* Δ*adhE* Δ*butBA* Δ*aldB*), whereas pCS4564 was lost by incubation at 36°C.

For overexpression of ALS, we cloned different *als* genes, including *Efals* from *Enterococcus faecalis* ATCC 29212, *Blals* from *Bacillus licheniformis* WX-02, *Lrals* from *Lactobacillus rhamnosus* DSM 20021 and *als* from *L. lactis* MG1363. These *als* gene fragments were inserted into plasmid pTD6 under the driven of P8 promoter (Zhu et al., 2015). For overexpression of NOX, we inserted *noxE* from *L. lactis* MG1363, *LrnoxE* from *L. rhamnosus* DSM 20021 and *EfnoxE* from *E. faecalis* ATCC 29212 into plasmid pTD6, respectively, using the native *noxE* promoter. For

co-expression of ALS and NOX, the *noxE* gene with its own promoter was inserted into pLC1 that contains *Efals* gene. All the primers used were shown in Table S3.

2.4. Analytical methods

Cell growth was frequently monitored by measuring the cell density OD600 and quantification of lactose, glucose and acetoin were carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with a Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). For pyruvate detection, the DAD-3000 diode array detector (Dionex, Sunnyvale, USA) was used. The column oven temperature was set at 60°C and the mobile phase consisted of 5 mM H2SO4, at a flow rate of 0.5 ml/min α-Acetolactate was measured colorimetrically using a method developed by Westerfeld (Westerfield, 1945) with modifications by Dorau et al. (Dorau et al., 2019). Diacetyl concentration was determined as described by Benson et al. (1996).

2.5. Diacetyl production from waste streams

We mixed 15% (v/v) RWP with 20% PW as the fermentation medium (FM) and then adjusted the pH to 7.0. *L. lactis* LacDia and RD1M5 were grown on 20 mL medium in 250-mL flasks under aerobic conditions with shaking speed at 200 rpm. When required, different concentrations of heme were added into the medium. When the fermentation was finished at 24 h, 20 mM $FeCl_3$ was added into the medium. For testing and confirming the growth of *L. lactis* on waste streams, PWF was used to replace PW at the same concentration.

2.6. Measurement of plasmid stabilities

To measure lactose plasmid stability without the selection pressure, LacDia was grown in 5 mL M17 medium with 1% (*w/v*) glucose as a carbon source, and culture was transferred every day into the same medium. At different times, culture was spread on agar-plates (same medium as the liquid) and 10 colonies were randomly picked up to assess the ability to grow on modified M17 medium with 1% (*w/v*) lactose, which indicated the presence of lactose plasmids. The modified M17 medium was prepared by growing *L. lactis* MG1363 in M17 for 12 h, and then subsequently removing cells by centrifugation and filtration. M17 (Oxoid) medium can support growth of *L. lactis* to an optical density at 600 nm (OD_{600}) of around 0.6 without adding additional fermentable sugars, and by pre-growing the medium with MG1363, depletion of the contained fermentable sugars can be accomplished. To assess the stability of the protease plasmid, we grew LacDia in lactose-supplemented M17 medium, and the presence of protease plasmid was determined as ability to grow on FM.

2.7. Cell catalysis

RD1M5 was cultivated on 100 mL 15% RWP with 20% PWF in 1 L flask for 20 h. The cells were harvested by centrifugation and re-suspended in UHT milk to a cell density of 10 (OD_{600}) and the cell catalysis was performed at shaking conditions at 200 rpm. Similarly, the cells were also re-suspended in pure RWP medium with 10 mM $FeCl_3$ using the same conditions as mentioned above. The samples were analyzed for lactose, AL and diacetyl.

2.8. E. coli biosensor strain development

In *E. coli* DY330 (Yu et al., 2000), the three genes *ilvB*, *ilvG* and *ilvI*, encoding ALS isozymes catalyzing the first step of the parallel isoleucine-valine biosynthetic pathway, were inactivated using the CRISPR-Cas9 system (Jiang et al., 2015). In the resulting strain, *E. coli ΔilvBIG*, a *gfp* gene expressed from the J23100 promoter, was introduced, whereby the SENSOR strain was obtained. More details can be found in Fig. S2. The primers used are listed in Table S3. The growth of the SENSOR strain was tested on SA^- medium without (with) all the three branched-chain amino acid (leucine, valine and isoleucine) and SA^- medium with 100 mg/L isoleucine plus AL. AL is not stable and hence was prepared fresh by hydrolyzing ethyl-2-acetoxy-2-methylacetoacetate using an equimolar amount of NaOH for 1 h (Dorau et al., 2019).

2.9. Isolation of a dairy strain from a commercial culture

An L. *lactis* strain prototrophic for valine and leucine was isolated from an aromatic undefined mesophilic starter provided by Arla Foods, using a modified solid synthetic amino acid medium (SA) (Jensen and Hammer, 1993) lacking valine and leucine. To identify the strains isolated, 16s rRNA gene was amplified using the two universal primers Y1 (TGGCTCAGGACGAACGCTGGCGGC) and Y2 (CCTACTGCTGCCTCCCGTAGGAGT).

2.10. Microfludic screening

The experimental setup of the microfluidic devices was described by Chen et al., 2017 (Chen et al., 2017). The mutant library was constructed based on the isolated strain F101 using ethyl methanesulfonate (EMS; Sigma, St. Louis, MO, USA) mutagenesis (Chen et al., 2017). The library and *E. coli* SENSOR strain were diluted in SA medium to a final concentration of 10^7 cells/mL, and subsequently mixed. The oil-containing surfactant (Dolomite Pico-Surf 2.5%, Royston, United Kindom) and the aqueous culture were set to 10 μl/min inflow rates. A 1-ml syringe was connected to the outlet to collect the emulsions and incubated 12 h at 30°C. For sorting the droplets containing the cells, the content of the syringe was injected into the sorting chip at a rate of 1 μl/min, with the oil flow at 10 μl/min. The cells were sorted based on fluorescence intensity of the preset threshold level (Chen et al., 2017). The collected emulsions were centrifuged at 100 g for 30 s. The oil layer was carefully removed and 300 μl of PFOH (1H,1H,2H,2H-Perfluoro-1-octanol) (Sigma-Aldrich, St. Louis, MO, United States) was added to disrupt the droplets and release the cells into the aqueous layer. The cells were then plated onto SA-agar plates. Fifty colonies were randomly picked up and grown in UHT milk, and the amount of diacetyl produced was quantified.

3. Results and discussion

3.1. Inactivation of all the competitive pathways for diacetyl production

Previously, we constructed an *L. lactis* strain, Ace001, devoid of lactate dehydrogenase (*Δldh ΔldhB* and *ΔldhX*), butanediol dehydrogenase (*ΔbutBA*), phosphotransacetylase (*Δpta*) and alcohol dehydrogenase (*ΔadhE*) activities (Liu et al., 2016b) (Fig. 2). Ace001 is able to produce large amounts of acetoin, which is a valuable butter aroma compound. Diacetyl, another desirable butter aroma compound, is approximately 100-fold more potent than acetoin (Bars and Yvon, 2008). These two compounds are derived from the same precursor AL, although diacetyl is formed from AL in a non-enzyme catalyzed manner. In order to increase the pool of AL and produce high amounts of diacetyl, we decided to further delete the *aldB* gene encoding ALDB. The final strain was designated Dia001 and its genotype can be seen in Table 1. Dia001 can only grow under aerobic conditions, as it requires NADH oxidase (NOX) for the regeneration of NAD^+ (Fig. 2). In the presence of 110 mM glucose in M17 medium, the final cell density of Dia001 reached an OD_{600} of 4.1. We observed the accumulation of 35.8 mM AL and 4.1 mM diacetyl after 24 h fermentation, and during the process 61.9 mM glucose was consumed and acetoin biosynthesis was successfully blocked (Fig. 3A). We also noticed the co-production of 18.5 mM pyruvate.

In normal conditions, the conversion from AL to diacetyl, which is a spontaneous non-enzymatic decarboxylation, is slow as witnessed by the

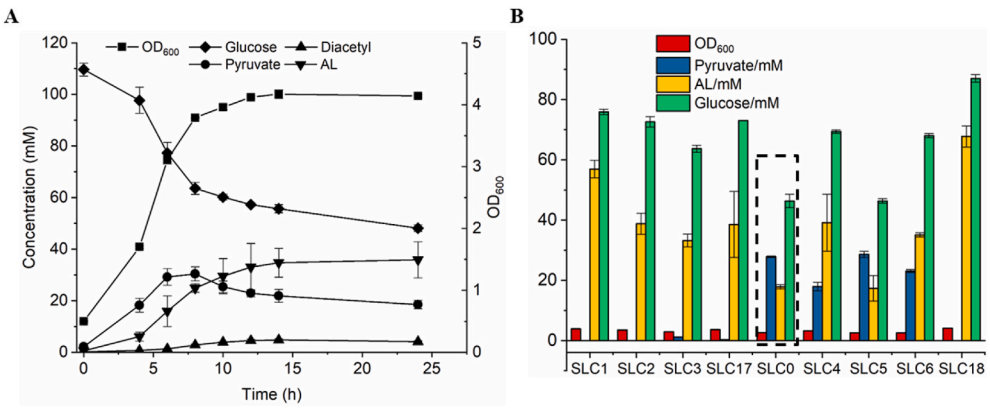

Fig. 3. Biosynthesis of diacetyl using strain Dia001 and derivatives overexpressing α-acetolactate synthase (ALS), NADH oxidase (NOX) or both enzymes. **A**. Growth and product formation for Dia001 using M17 medium with 110 mM glucose under aerated conditions. **B**. Effects of overexpressing ALS and NOX on growth and product formation. Glucose consumption is indicated by the green bar. SLC0 (enclosed) is the control strain with an empty plasmid. ALS from different bacteria were also tested: *Enterococcus faecalis* (SLC1), *Bacillus licheniformis* (SLC2), *L. rhamnosus* (SLC3), *L. lactis* (SLC17). We also expressed NOX from different bacteria in Ace001: *L. lactis* (SLC4), *L. rhamnosus* (SCL5), and *E. faecalis* (SLC6). SLC18 overexpresses the ALS from *E. faecalis* and the NOX from *L. lactis*. Experiments were conducted in duplicate independently and error bars indicate standard deviations.

accumulation of AL in our fermentation broth. Diacetyl is a highly toxic compound, and as little as 2 mM can significantly repress growth of *L. lactis* (Liu et al., 2016a). The build-up of pyruvate is a consequence of the weak affinity of ALS for pyruvate (*Km* = 50 mM)(Bars and Yvon, 2008), which is a bottleneck in the AL synthetic pathway. We also speculated that accumulation of pyruvate could have a negative effect on the glucose consumption flux and growth, which was quite slow.

3.2. Overexpression of ALS and NOX

To reduce pyruvate accumulation and enhance formation of AL, we expressed ALS from various microorganisms in Dia001, including EfAls from *E. faecalis* (*Km* = 1.37 mM)(Huo et al., 2018), BlAls from *B. licheniformis* (*Km* = 3.96 mM), LrAls from *L. rhamnosus* (*Km* = 10 mM), and we also overexpressed the native ALS. As shown in Fig. 3B, it is possible to reduce pyruvate accumulation and increase AL production by overexpressing ALS. SLC1 with EfAls displayed the best performance in terms of AL production and glucose consumption.

It has been reported that diacetyl can inhibit the NOX activity (Hugenholtz et al., 2000) and overexpression of NOX was found to be beneficial for diacetyl production (Guo et al., 2012). Therefore, we investigated the effect of expressing different NOX enzymes in Ace001 – the native NoxE, LrNoxE from *L. rhamnosus* and EfNoxE from *E. faecalis*. Fig. 3B shows that strain SLC4, which overexpresses the native NoxE, consumed more glucose and produced the highest amount of AL. In contrast to the strains with elevated ALS activity, the strains with higher NOX activity still produced significant amounts of pyruvate. An additive effect was seen, when both the *E. faecalis* ALS and the *L. lactis* NoxE were expressed in Ace001 (SLC18), and this strain consumed the highest amount of glucose (87.0 mM glucose) and produced 67.7 mM AL (with the co-production of 8.1 mM diacetyl).

These engineering efforts clearly demonstrated that there are several bottlenecks in the diacetyl production pathway. 1) The low ALS activity, as overexpression of this enzyme significantly stimulated sugar consumption and AL (diacetyl) production. 2) Cofactor regeneration, as overexpression of the NOX activity had a beneficial effect on sugar consumption, AL production and slightly decreased pyruvate accumulation. The effect of NOX overexpression indicates that the $NADH/NAD^+$ ratio is high in Dia001, and previously it has been found that a high $NADH/NAD^+$ ratio can hamper the glycolytic flux (Garrigues et al., 1997). 3) High lactate production, as LDH has a high enzymatic activity towards pyruvate (*Km* = 1.7 mM)(Gaspar et al., 2007), and more than 80% of the pyruvate flux goes to lactate when it is active (Gaspar et al., 2007). Therefore, the inactivation of LDH is essential, if the pyruvate flux is to be redirected towards AL. Under aerobic conditions, the pyruvate formate lyase (PFL) is inactive in the presence of oxygen and the pyruvate dehydrogenase (PDHC) activity is very low as well. To confirm that the flux to acetyl-CoA indeed was low, we constructed a strain where the native alcohol dehydrogenase (ADH) was kept active, and the performance of this strain GM001 was compared to that of Dia001 under aerated conditions. We observed that the performance of GM001 and Dia001 was very similar (Fig. S1), which indicated that the flux to acetyl-CoA indeed was small under these conditions. 4) Another critical target for diacetyl production is ALDB, the activity of which needs to be reduced or eliminated. We successfully knocked out the *aldB* gene in Dia001, which drove the flux from acetoin to the accumulation of AL and diacetyl production.

3.3. Introduction of plasmidome into the diacetyl strain

The sustainable and cost-effective production of green compounds calls for cheap and renewable feedstocks. Many efforts have been made to use non-food plant biomass and derived carbohydrates as feedstocks, but significant challenges are associated with this (Kawaguchi et al., 2016). Side streams from the food industry could be a better alternative. They often are generated in large amounts, and usually contain readily fermentable carbohydrates and other nutrients that microorganisms need. One example is RWP, obtained after extracting proteins and lactose from whey, and PW, a leftover from the whey-protein production. RWP is rich in lactose, vitamins and minerals, whereas PW is rich in proteins. Together, RWP and PW could serve as a complete medium for *L. lactis*. We decided to explore this using our engineered *L. lactis* SLC18 strain, which has elevated ALD and NOX activities. However, since SCL18 lacks the lactose and protease plasmids, we first re-installed these to enable growth on a RWP/PW based medium (Fig. 2).

Previously, we have characterized *L. lactis* subsp. *lactis* biovar diacetylactis RD1M5, a strain that grows efficiently on dairy waste (Liu et al., 2020). We found that RD1M5 contains 5 plasmids: the lactose plasmid (49.6 kb) containing a lactose operon (Fig. 4A), the protease plasmid (64.9 kb) containing the proteinase PrtP (Fig. 4B), a citrate plasmid (8.3 kb), a phage defense plasmid (7.3 kb) and a plasmid with unknown function (2.7 kb). The lactose plasmid also contains an *opp* operon (*oppD*, *oppF*, *oppB*, *oppC* and *oppA*) responsible for oligopeptide transport and one gene encoding an endopeptidase (*pepO*). We extracted a plasmid mixture from RD1M5 and subsequently transformed this into SLC18, thereby obtaining the strain LacDia, which has both the lactose and the protease plasmid (verified by PCR, data not shown). LacDia was unable to grow in RWP, however, grew well in RWP supplemented with YE (Fig. 5A and B). By replacing YE with PW, similar growth was attained, although the biomass yield was slightly reduced (OD_{600} = 3.8 compared with 4.3 in the presence of YE). The parent strain SLC18 was unable to grow in this medium. After growing LacDia for 24 h in the RWP/PW

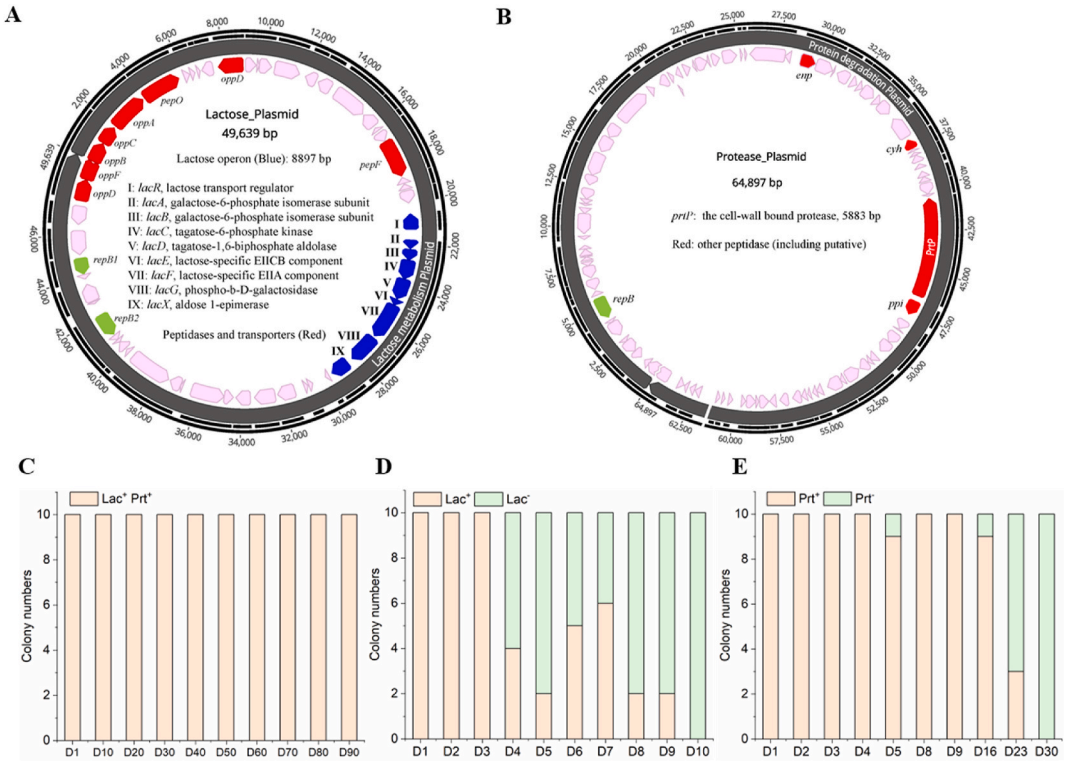

Fig. 4. The lactose and protease plasmids from *L. lactis* subsp *lactis* biovar diacetylactis RD1M5 and their stabilities. **A, B**. The lactose operon is shown in blue and genes encoding the cell envelope protease, peptidases and oligopeptide transporters are shown in red. The sequence and detailed annotation of lactose plasmid and protease plasmid are deposited in Genbank under accession numbers CP043524 and CP043526. **C**. Lactose plasmid persistence in the dairy waste based medium FM. LacDia was grown in 5 mL FM and the bacteria were transferred every day into the same fresh medium. Every ten days samples were streaked on solid FM medium, and 10 randomly picked colonies were tested for ability to metabolize lactose on M17 supplemented with lactose. **D**. Persistence of the lactose plasmid in the absence of selection pressure. The same approach as in **C** was used, however, LacDia was grown in M17 medium supplemented with glucose. To evaluate persistence of the lactose plasmid, daily samples were streaked on GM17 plates, and 10 random colonies from this plate were assessed for their abilities to grow on lactose-supplemented M17. **E**. Persistence of the protease plasmid. LacDia was grown in lactose supplemented (1% (w/v)) M17 and the presence of protease plasmid was determined by ability to grow on FM medium.

substrate, 5.3 mM diacetyl and 50.4 mM AL were generated, with the consumption of 37.0 mM lactose (Fig. 5C). Since the conversion from AL to diacetyl can be accelerated by metal ion catalysis (Liu et al., 2016a; Mohr et al., 1997), 20 mM Fe^{3+} was added at 24 h and finally 56 mM (4.8 g/L) diacetyl was achieved with a yield of 75% of the theoretical maximum. Our results demonstrated that LacDia was an efficient cell factory for diacetyl production from pure dairy wastes.

Despite of the good performance of LacDia, we found that 18 mM of lactose was left unmetabolized in the fermentation broth (Fig. 5C). One reason for this could be the toxic effect of diacetyl and we have previously found that 1.2 mM diacetyl can inhibit 70% of the NOX activity (Hugenholtz et al., 2000), which is essential for cofactor regeneration. Respiration was found to be superior for regenerating NAD^+ for a diacetyl-producing strain grown in rich M17 medium (Liu et al., 2016a). *L. lactis* normally does not respire, since it is unable to synthesize heme, an essential component of the cytochrome oxidase in the respiratory chain (Blank et al., 2001; Lechardeur et al., 2011) (Fig. 5D). However, when exogenous hemin is available, respiration can be activated (Liu et al., 2017). We explored the addition of hemin and noticed that all the lactose could be completely consumed within 24 h, and that 9.3 mM diacetyl and 71.8 mM AL could be produced (Fig. 5E). After adding 20 mM of Fe^{3+}, 76 mM (6.5 g/L) of diacetyl was formed in 4 h with a yield of 70% of the theoretical maximum.

Dairy L. *lactis* strains typically have various plasmids encoding important traits, such as lactose metabolism and casein protein degradation. They acquired these plasmids while adapting from a plant to a milk niche (Wels et al., 2019). These plasmids replicate using either a theta-type replication or by rolling-circle replication. The two RepB proteins (locus_tag: FTN78_p050052 and FTN78_p050047) encoded by the lactose plasmid are 100% identity in amino acid sequence to RepB1 and RepB2, encoded by plasmid pLd8 found in *L. lactis* FM03P (van Mastrigt et al., 2018). The Rep protein (locus_tag: FTN78_p020008) encoded by the protease plasmid is 100% identical to RepB in plasmid pIL7 of *L. lactis* IL594 (Górecki et al., 2011). Both plasmids replicate using theta-type replication and it is common that a single *L. lactis* cell can contain multiple theta-replicating plasmids, which belong to a highly related family that comprises different members (Seegers et al., 1994; Kiewiet et al., 1993). We tested the stability and compatibility of the lactose/protease plasmids in LacDia and found that all the plasmids were stably maintained in the presence of selection pressure (Fig. 4C), where lactose and casein proteins are needed for maintaining the lactose and the protease plasmid, respectively. Without selection pressure, the lactose plasmid can be quickly lost, and only after 10 days of growth in M17 with glucose, we found that 10 randomly selected colonies were unable to grow with lactose as the sole carbon source (Fig. 4D). Similarly, we found that the protease plasmid was unstably maintained on M17 and in 30 days 10 randomly selected colonies all had lost the ability to grow on FM, for which the protease is essential (Fig. 4E).

Establishing natural production of diacetyl based on learnings from the DBTL process.

As mentioned, the food industry has a strong preference for microorganisms that are considered natural. The knowledge gained from studying recombinant microorganisms can be used for developing more natural analogues that can be used in clean-label production. From the recombinant diacetyl producing strain, we learned some important lessons. As a starting point, it is important to select a food-approved

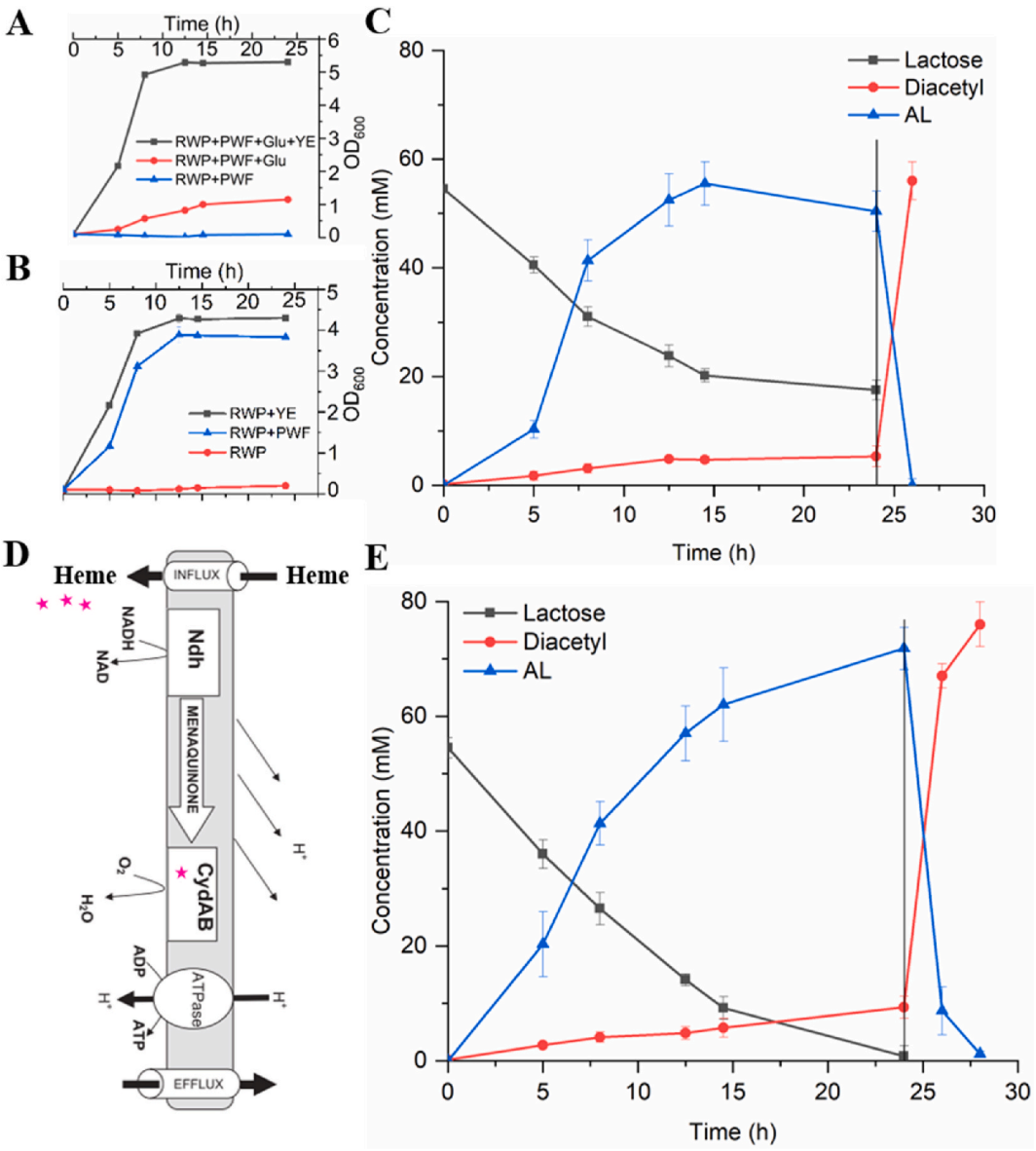

Fig. 5. Diacetyl production from dairy waste. **A**. The growth of strain SLC18 on RWP with PWF was negligible. By adding glucose and YE growth could be stimulated. **B**. Growth of strain LacDia on RWP with PWF was similar to growth on RWP supplemented with YE. **C**. Growth of LacDia on FM (RWP supplemented with PW). 20 mM Fe^{3+} was added at 24 h to accelerate the conversion of AL to diacetyl. **D**. The electron transport chain of *L. lactis*. *L. lactis* can not synthesize heme, an essential cofactor of cytochrome oxidase (CydAB), and its exogenous supplement can activate a respiratory metabolism. **E**. Growth of LacDia on RWP and PW with 5 μg/mL heme. 20 mM Fe^{3+} was added at 24 h to speed up the conversion of AL to diacetyl. Two independent biological experiments were carried out in 20 mL medium/250-mL shake flasks under aerobic conditions.

lactic acid bacterium, which naturally can metabolize lactose and grow on dairy waste. Our aim is to create an efficient diacetyl-producer from a dairy *L. lactis* industrial strain without using recombinant technologies, which claims as a top-down approach (De Lorenzo and Galperin, 2009). It is important to inactivate LDH to redirect the metabolic flux from lactate to AL production. Furthermore, we know that the conversion of AL to diacetyl is slow and constitutes a bottleneck and that a respiratory metabolism is beneficial for diacetyl production.

Example 1. Tailoring of a natural industrial strain

We turned our attention to *L. lactis* RD1M5, a natural derivative of industrial strain SD96 (Liu et al., 2020). This strain was obtained by chemical mutagenesis and has an insertion mutation in the *ldh* gene. RD1M5 was found to be an excellent cell factory for transforming dairy waste into acetoin (Liu et al., 2020). RD1M5 has a functional ALD enzyme, which catalyzes decarboxylation of AL into acetoin (Fig. 6A), and in order to achieve diacetyl production, this activity needs to be reduced or eliminated. Chemical mutagenesis has been used for this purpose, however, the ALD deficient strains obtained were reported to be unstable and the diacetyl-producing phenotype was easily lost (Monnet et al., 2000; Hernandez-Valdes et al., 2020). Strains lacking ALD are bound to generate some diacetyl during growth, and since diacetyl is toxic, there is a strong selection pressure for mutants that stop producing diacetyl. We decided to explore a different approach to uncouple growth and diacetyl production. It is known that ALD is sensitive to a low pH and has a pH optimum at around 6.7 (Godtfredsen et al., 1984). We thus decided to investigate the effect of using a low pH for producing diacetyl. An added benefit of this is that a low pH can stimulate the non-enzymatic conversion of AL to diacetyl (Haukeli and Lie, 1978). Because our non-engineered strain RD1M5 grew well in milk, we first investigated if we could enhance diacetyl formation in milk by lowering the pH. This was indeed possible and 2.8 mM (0.2 g/L) diacetyl

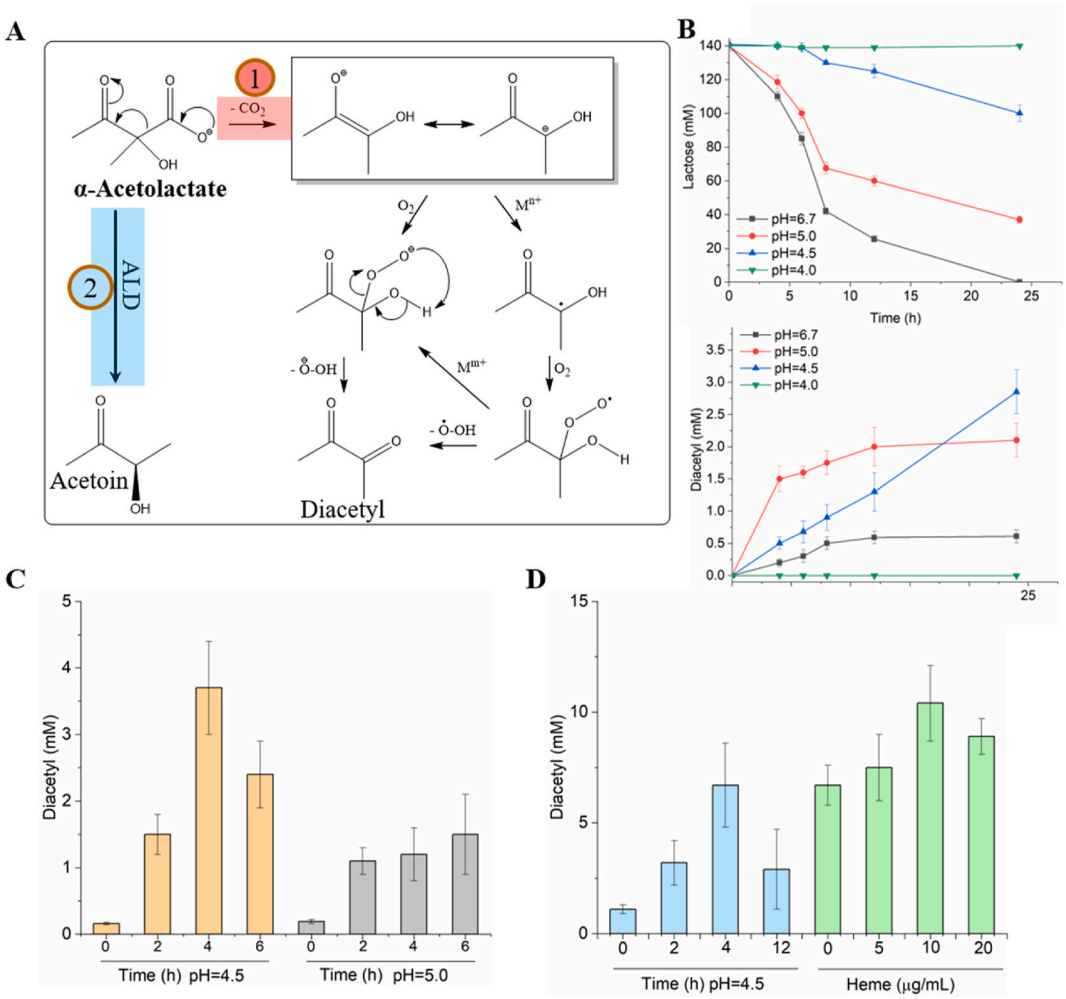

Fig. 6. Fermentation of a natural strain RD1M5. Milk contains 140 mM (47.9 g/L) lactose and has a pH of 6.7. The initial cell density was 1.0 (OD_{600}). **A**. Both diacetyl and acetoin share the same precursor AL. The conversion of AL to diacetyl (1) is spontaneous and starts with non-enzymatic oxidative decarboxylation, resulting in the formation of an enolate anion and its carbanion tautomer, which can react with oxygen and divalent metal ions and ultimately result in formation of diacetyl. **B**. Lactose consumption and production of diacetyl during the fermentation process under different pH in milk. **C**. Cells with a high cell density (OD_{600} = 10) were resuspended in 20 mL milk under pH 4.5 or 5.0. The catalysis was carried out at 200 rpm and 30°C. **D**. Cells with a high cell density (OD_{600} = 10) were resuspended in 20 mL pure 15% (w/v) RWP in the presence of 10 mM Fe^{3+} at pH 4.5. Additionally, we tested the same conditions with different concentrations of heme and after 4 h, diacetyl was measured. Two biologically independent experiments were performed to calculate the standard deviation.

was generated at pH 4.5 after 24 h fermentation (Fig. 6B). At pH 5 in just 4 h, 1.5 mM diacetyl could be formed, whereas in non-pH adjusted milk (pH 6.7), only 0.5 mM diacetyl was formed. In the latter case, acetoin dominated as the fermentation product with a high concentration of 262 mM (23 g/L) (Fig. S4), which is similar to what we found in a previous study (Liu et al., 2020). At pH 4.0, lactose was not metabolized, which is not surprising as it is well-known that the cellular metabolism of *L. lactis* halts at such a low pH (Sánchez et al., 2008). For these experiments, we did not rely on a catalyst to enhance diacetyl formation, e.g. Fe^{3+}, since such additions are generally not allowed in foods. Clark et al. summarized the measured levels of diacetyl in selected foods (Clark and Winter 2015) and found that cheddar cheese has less than 1 ppm diacetyl. Therefore, the diacetyl concentration (0.2 g/L) in our fermented milk is more than 200 times higher than in cheddar cheese, which demonstrates the great potential of RD1M5 as a butter aroma producer in butter and cheese manufacturing.

The low pH needed for diacetyl formation has a negative effect on cell growth and diacetyl productivity. In an attempt to overcome these potential bottlenecks, we explored a cell catalysis approach for producing diacetyl. First, we grew RD1M5 in RWP medium with PWF and then resuspended the cells in milk to a high cell density (OD_{600} = 10). At pH 4.5, we achieved the highest concentration of diacetyl (3.7 mM/0.3 g/L) in just 4 h of biocatalysis (Fig. 6C). After 4 h, a drop in diacetyl concentration was observed, which most likely is due to the diacetyl reductase that can reduce diacetyl to acetoin. When the pH was 5.0, only 1.5 mM of diacetyl was formed. In addition to milk, we tested pure dairy waste as the substrate with 10 mM Fe^{3+} and achieved more than doubled the diacetyl titer (6.7 mM/0.6 g/L) in the same period. Inspired by the results from the recombinant diacetyl strain, the effect of different concentrations of heme was measured and we found that 10 μg/mL heme could further increase the diacetyl concentration to 10.4 mM (0.9 g/L) (Fig. 6D). The food-grade status of strain RD1M5 makes it possible to use the described process for clean label production of diacetyl with many potential food applications. In this example, we focused on the optimization of pH. There are other parameters (or combinations), such as temperature (Fig. S5A), O_2 availability, cell density, metal ions and fermentation optimization (diacetyl removal during the process) that will affect diacetyl productions. There are great possibilities to further enhance the diacetyl titer and yield in the following studies.

Example 2. High-throughput screening to select a non-engineered and diacetyl-producing dairy strain

As mentioned, the conversion of AL to diacetyl is slow, and strains

deficient in LDH and ALD accumulate AL in the fermentation broth. We speculated whether it would be possible to develop an *E. coli* based biosensor that could detect AL, as such a biosensor would be valuable when screening for strains with high AL producing capacities. First, we created a mutant that relied on AL for growth. AL is a precursor for valine and leucine and *E. coli* has three genes *ilvB*, *ilvI* and *ilvG* that encode ALS isozymes, which we knocked out (Fig. 7A and B). The resulting strain, *E. coli ΔBIG* requires the three branched-chain amino acids valine, leucine and isoleucine in order to grow. As we had hoped, AL was able to substitute valine and leucine and support growth on SA^- medium with isoleucine (Fig. 7C). We subsequently introduced a gene encoding a green fluorescent protein (GFP) into its chromosome, which was expressed from the strong J23100 promoter, thus obtaining the SENSOR strain. In parallel, we isolated a natural dairy strain F101 auxotrophic for valine and leucine, from a mesophilic starter culture provided by Arla Foods. Based on 16S rRNA gene sequencing analysis, it was identified as *Lactococcus lactis* (Table S4). We generated a mutant library of F101 using EMS mutagenesis and mixed an equal amount of the mutant library and the *E. coli* SENSOR strain in SA medium (SA^- plus isoleucine). The number of cells in droplets can be estimated using the Poisson distribution (Mazutis et al., 2013), and it can be calculated that there is a 13.5% probability for co-encapsulation of two different cell types, when the two cell types are mixed in a 1:1 ratio. The size of the droplets used was 50-pL and the cell density in the aqueous phase was approximately 2×10^7 cells/mL.

We generated droplets at about 1 kHz using a droplet generator and approximately 10^7 droplets were collected in a syringe. After 12 h incubation, the droplet containing emulsion was reinjected into the microfluidic sorting device. Droplets were screened for the presence of GFP fluorescence, which can be excited at 490 nm and detected at 509 nm. The sorting was performed at about 0.3 kHz and approximately 100 of the most fluorescent droplets were collected. We repeated the droplet generation and sorting several times and collected all the droplets together. The cells in the collected droplets were released from the emulsions and plated on SA^--agar medium to form single colonies (only *L. lactis* can grow in the plates). We picked 50 colonies and grew them in milk aerobically for 12 h. The diacetyl concentration was measured and it was shown that several colonies were able to produce more than 1.5 mM diacetyl (0.1 g/L, Fig. 7E), which was more than 10 times the amount of diacetyl produced by the wild type strain (around 0.1 mM). The concentration of diacetyl was further enhanced to 3.4 mM (0.3 g/L) using the best mutant strain F101-A on milk fermentation, when heme was provided at 10 μg/mL (Fig. S5B). Thus high-throughput screening in combination with the AL biosensor is an excellent approach for obtaining superior diacetyl-producing variants, but one potential bottleneck in our developed platform is the prototrophy requirement for valine and leucine in the selected strains. It is not compatible to use this microfluidic approach for RD1M5 (Example 1) or the metabolically engineered strains (MG1363 derived), which is auxotrophic for these two amino acids (Jensen and Hammer, 1993).

In principle, the production of diacetyl can be further enhanced by additional rounds of screening based on the selected mutants. However, diacetyl is highly toxic and 2–4 mM diacetyl can significantly inhibit growth of *L. lactis* (Liu et al., 2016a). It might be a good approach to enhance diacetyl tolerance of the mutants first by adaptive evolution and then proceed further with mutagenesis and screening. Classical mutagenesis, when used together with engineered biosensors and high-throughput microfluidic screening systems, clearly is a powerful approach for generating useful and safe microorganisms for use in foods, especially when guided by knowledge obtained via the DBTL cycle.

In conclusion, we propose that the DBTL cycle implemented in metabolic engineering optimization processes can be used to guide the development of non-engineered strains with improved performance, and this approach is particularly well suited for food applications. Here we metabolically engineered the model organism of LAB, *L. lactis*, and achieved high amounts of diacetyl (6.7 g/L) from a virtually free dairy waste-based substrate. The diacetyl can be easily concentrated and purified by vacuum distillation prior to use in foods or could be used as a precursor for making other valuable compounds e.g. 2,3-butanediol. We subsequently transferred the lessons learned from the metabolic engineering efforts into two successful examples for the development of non-engineered strains. One lies in the optimization of a real industrial strain and development of sustainable processes for clean-label production of diacetyl in both milk and pure wastes, and the final concentration was 0.6 g/L and 0.9 g/L, respectively. The other focuses on the isolation of natural strains with the aid of high-throughput microfluidic screening approach and coculture with biosensors. The combination of metabolic engineering and non-GMO techniques for strain improvements opens up

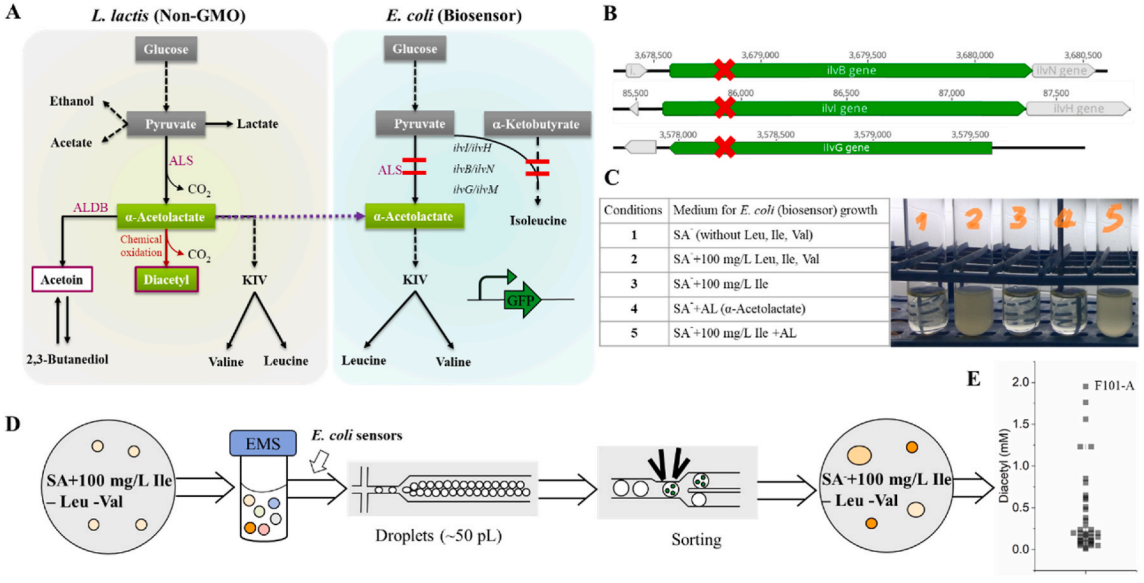

Fig. 7. The use of microfluidic system to isolate strains with superior diacetyl-producing capacity. **A**. *E. coli*:GFP *ΔilvBIG* (SENSOR) was constructed as a biosensor strain to reflect the production of AL produced by a dairy isolate *L. lactis*. **B**. Three genes *ilvB*, *ilvI*, *ilvG* encoding ALS isozymes were knocked out in *E. coli*. **C**. Characterization of SENSOR growth. **D**. The simplified workflow for high-throughput screening approach. **E**. The diacetyl production from 50 selected colonies in UHT milk.

more possibilities for their exploitations in food applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2021.08.006.

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