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Research Article

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Pyruvate stimulates transamination of leucine into α-ketoisocaproic acid and supports 3-methylbutanal production by *Lactococcus lactis*

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Abstract

Aim: To investigate the effect of pyruvate and glucose on leucine transamination and 3-methylbutanal production by *Lactococcus lactis*, including the comparison with cells possessing glutamate dehydrogenase (GDH) activity.

Methods and results: *Lactococcus lactis* cells were incubated in chemically defined medium (CDM) with the pH controlled at 5.2 to mimic cheese conditions. Pyruvate supplementation stimulated the production of the key flavour compound 3-methylbutanal by 3–4 times after 72 h of incubation. Concurrently, alanine production increased, demonstrating the involvement of pyruvate in transamination reactions. Glucose-metabolizing cells excreted α-ketoisocaproic acid and produced even 3 times more 3-methylbutanal after 24 h than pyruvate-supplemented cells. Conjugal transfer technique was used to transfer the plasmid pGdh442 carrying the *gdh* gene encoding for GDH to *L. lactis*. Introducing GDH did not stimulate the excretion of α-ketoisocaproic acid and the production of 3-methylbutanal.

Conclusions: These results demonstrate that *Lactococcus* uses pyruvate to transaminate leucine into α-ketoisocaproic acid which supports 3-methylbutanal production. Surprisingly, GDH activity did not stimulate leucine transamination and 3-methylbutanal production.

Impact Statement

Our findings suggest that increasing the residual sugar content during cheese ripening can support flavour production. We propose to increase the concentration of galactose, which is metabolized relatively slowly by *Lactococcus* compared to lactose and helps to control post-acidification during ripening. We discuss the design criteria for such a starter culture, which will allow manufacturers to improve the cost efficiency of cheese production.

Keywords: lactococcus; flavour; cheese; amino acid catabolism; branched-chain amino acids

Introduction

Flavour development in hard and semi-hard type cheeses, including Cheddar and Gouda, is a time-consuming process. A number of methods have been proposed to stimulate the flavour development, such as increased cheese ripening temperatures, the use of cheese ripening enzymes, and applying specific adjunct cultures (Fox et al. 1996, Khattab et al. 2019). We hypothesize that the most straightforward way to stimulate flavour development is by using fast acidifying starter cultures with strong flavour-forming capabilities. The starter cultures used for production of these types of cheese predominantly contain *Lactococcus lactis* and *Lactococcus cremoris* strains, which have the capacity to play an important role in the conversion of milk proteins to flavour compounds. Intracellular enzymes mediate the degradation of amino acids into volatile compounds such as aldehydes and alcohols, which contribute to the cheese flavour profile and intensity. Transamination is the first step in the amino acid degradation, which is catalysed by transaminases that require pyridoxal-5-phosphate and an α-ketoacid as amino group acceptor (Yvon et al. 1997) (Fig. 1). It is preferable to select *Lactococcus* strains that rapidly transaminate amino acids into their corresponding α-ketoacids. Under cheese ripening conditions, the rate of transamination is assumed to be limited by the availability of α-ketoglutarate, which is regarded as the preferred amino group acceptor (Yvon et al. 1997, 1998, 2000, Engels et al. 2000, Banks et al. 2001, Tanous et al. 2002, Williams et al. 2004). One method of increasing the availability of α-ketoglutarate is to select *Lactococcus* strains that can produce this compound. *Lactococcus lactis* strains that express glutamate dehydrogenase (GDH) activity, which converts glutamate into α-ketoglutarate, were developed. Amino acid breakdown was indeed stimulated in these strains (Rijnen et al. 2000, Tanous et al. 2006).

In a previous study, we proposed that pyruvate might also act as an amino group acceptor in transamination reactions (Brandsma et al. 2021). However, these *Lactococcus* transaminases appear to exhibit a much higher affinity for α-ketoglutarate compared to other α-ketoacids such as pyruvate and oxaloacetate (Engels et al. 2000, Yvon et al. 2000). On the other hand, the availability of pyruvate might be significantly higher when cells are metabolizing sugars. In the second step

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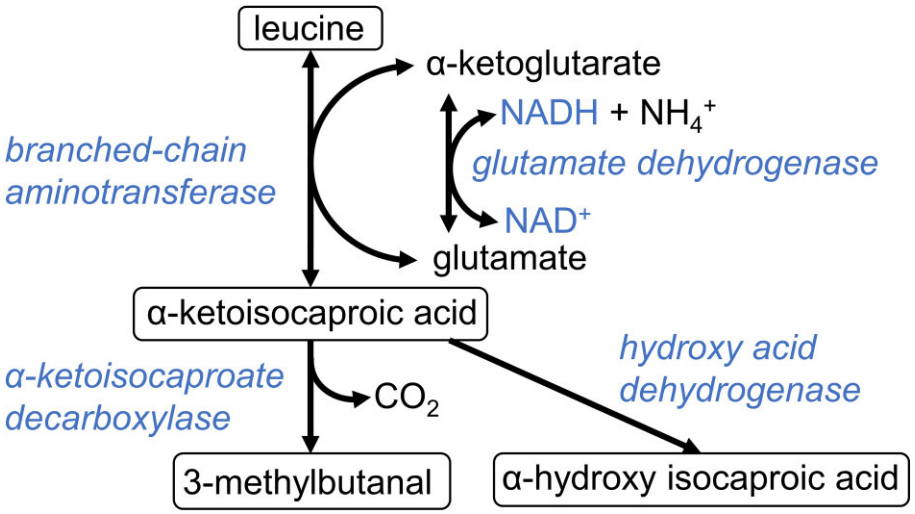

Figure 1. Simplified metabolic pathway for the production of 3-methylbutanal, adapted from Yvon and Rijnen (2001).

of the amino acid degradation, α-ketoacids are converted into volatile compounds, by enzymes such as dehydrogenases and decarboxylases. This is especially the case for the decarboxylation of α-ketoacids derived from branched-chain amino acids, which results in the formation of several characteristic cheese key flavours, including 3-methylbutanal and 2-methylbutanal (Smit et al. 2005b, Jo et al. 2018, Chen et al. 2020). Dehydrogenases convert α-ketoacids into hydroxy acids, which are presumed not to contribute to the cheese flavour. Strains with this activity are thus not preferred in cheese starter cultures (Chambellon et al. 2009).

Lactococcus strains with relatively high α-ketoacid decarboxylase activity have to date not been studied in relation to transamination rate and/or limited availability of amino group acceptors such as α-ketoglutarate and pyruvate. This study focused on ways to stimulate the transamination of branched-chain amino acids in a *L. lactis* strain with a relatively high α-ketoacid decarboxylase activity. *Lactococcus lactis* DS84445 was selected, and conjugational transfer was used to develop a derivative strain containing the plasmid pGdh442, which carries the gene encoding for GDH. *In vitro* studies were carried out to compare the stimulatory effect of pyruvate, with and without the presence of GDH activity, on the transamination of leucine into α-ketoisocaproic acid, which is further decarboxylated into the cheese key-flavour 3-methylbutanal. Our findings were used to discuss a novel design for a Gouda-type starter culture with enhanced flavour-forming properties during cheese ripening.

Material and methods

Strains and chemicals

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* DS84445 is a wild-type strain isolated from an artisanal produced dairy product and selected for its high capacity to produce 3-methylbutanal in milk. Strains DS84445, DS84445-lac^{neg}, DS84445-lac^{neg}-pGdh442, and TIL504 were stored in cryovials containing M17 broth with 10% (vol/vol) glycerol and with 10 g l^{-1} lactose (LM17) or glucose (GM17). Strains were incubated at 30°C for 18 h and used to inoculate 1% (vol/vol) precultures in LM17 or GM17 broth (Oxoid, Hampshire, UK). Chemicals used in this study were purchased from Sigma–Aldrich (Steinheim, Germany) unless otherwise stated.

Development of the lactose-negative DS84445 strain

Plasmid curation of strain DS84445 was performed by incubating cells in GM17 with 1.25 mg l^{-1} ethidium bromide at 30°C. After 18 h of incubation, cells were harvested by centrifugation. Subsequently, cells were resuspended in GM17 and maintained for 4 h at 30°C to facilitate recovery. Fluorescence-activated cell sorting was used to sort cells into the wells of 384 microtiter plates. The sorting was conducted based on the forward and sideward scattering properties. The microtiter plates contained 180 μl of LM17 broth. Following the addition of single cells to wells, the microtiter plates were incubated at 30°C. After 8 h of incubation, the OD_{600nm} was measured. Cells that reached a relatively low OD_{600nm} were expected to have lost the ability to grow on lactose. Cells were therefore plated on LM17 agar containing 40 mg ml^{-1} bromocresol purple. On these plates, white colonies were considered lactose-negative and were purified by three times streaking on GM17 agar and stored in GM17 with glycerol at –40°C, until further analysis.

Conjugational transfer of pGdh442

The donor strain TIL504 and the recipient strains DS84445 and DS84445-lac^{neg} were pre-incubated for 18 h at 30°C in M17 with 10 g l^{-1} xylose (XM17) (for recipient strain) or GM17 (for donor strain). In the next step, 50 ml precultures (1% v/v inoculum) were again prepared in XM17 and GM17 and grown for 4.5 h at 30°C to reach the beginning of the exponential phase. Conjugational transfer was initiated by mixing donor and recipient strains in a 1:1 volume-ratio, giving a total volume of 3 ml. The donor-recipient mixture was filtered through a sterile glass vacuum funnel with a 0.45 μm cellulose filter paper (white gridded, 47 mm, from Merck Millipore, Massachusetts, USA). The filter papers were placed in the centre of GM17 agar plates (Oxoid, Hampshire, UK) and incubated for 20 h at 30°C. The filter papers were then removed from the GM17 agar plates and placed in a 5 ml peptone physiological salt (PPS) solution (Tritium Microbiologie, Eindhoven, The Netherlands). Selective M17 medium was prepared by first growing the donor strain on

Table 1. Strains used in this study.

Strains or plasmid	Characteristics	Source or reference
L. lactis DS84445	Xylose positive, does not comprise *gdh* encoding gene	Royal DSM N.V. (Delft, The Netherlands)
L. lactis DS84445-lac^{neg}	Xylose positive, and lactose negative	This study
L. lactis DS84445-lac^{neg}-pGdh442	Xylose positive, lactose negative, and carrying plasmid pGdh442	This study
L. lactis TIL504	*L. lactis* MG1363 containing plasmid pGdh442, cadmium resistant, xylose negative. MG1363 is a plasmid cured derivative of NCDO 712.	Tanous et al. (2006)

liquid M17 medium without sugar for 18 h at 30°C, allowing the natural energy sources present in M17 to be depleted. Subsequently, the donor strain was removed by filtration. Selective compounds: 0.2 mmol l^{-1} cadmium chloride, 2% (wt/wt) xylose, 0.4% (wt/vol) of the pH indicator bromocresol purple, and agar were added to the filtrated M17 medium. The selective XM17 medium was sterilized by heating (121°C for 15 min), then cooled to 46°C and poured in Petri dishes. The plasmid pGdh442 carries the cadA and cadC genes encoding for cadmium resistance, which was used to select for cells containing this plasmid (Tanous et al. 2007). Xylose was the sole energy source in the selective XM17, which permitted the growth of only the recipient strains DS84445 and DS84445-lac^{neg}. The cells of the recipient strain that received the plasmid pGdh442 were capable of utilizing xylose and were resistant to cadmium, which was used as a selective trait for the isolation of strain DS84445-lac^{neg}-pGdh442. The PPS solution obtained from the conjugational transfer test was plated out at various dilutions on the selective XM17 agar plates and incubated at 30°C for 2–4 days. The xylose-metabolizing DS84445-lac^{neg}-pGdh442 cells produced lactic acid, and when the pH of the colony was below 5.2, bromocresol purple turned yellow. The selected yellow colonies were purified by streaking three times on selective XM17 agar plates and stored in GM17 with glycerol at –40°C until further analysis.

Molecular analysis

Genome sequencing of strains

Full grown cultures (2 ml with an OD_{600nm} between 1 and 3) of strains DS84445, DS84445-lac^{neg}, DS84445-lac^{neg}-pGdh442, and TIL504 were pelleted and lysed in 200 µl of 0.9% (vol/vol) physiological salt (Xebios Diagnostics, Düsseldorf, Germany) supplemented with 2 µl RNAse (Qiagen, Hilden, Germany) and 20 mg ml^{-1} lysozyme. The mixture was incubated for 60 min at 37°C. Next, 200 µl of 2X cell lysis solution (0.05 mol l^{-1} EDTA, 4% SDS) was added and mixed. Then, 10 µl proteinase K (Qiagen, Hilden, Germany) was added, and the mixture was incubated at 55°C for 10 min. After cooling, the mixture was kept on ice, and 168 µl of protein precipitation solution (10 mol l^{-1} NH_4Ac) was added. Proteins were precipitated by centrifugation at 20 000 *g* for 10 min at 4°C. The DNA in the supernatant was precipitated with an equal volume of isopropanol and centrifuged at 16 000 *g* for 2 min at room temperature. The DNA pellet was washed with 70% (vol/vol) ethanol. The ethanol was discarded, and the pellet was dried and then dissolved in MilliQ water. The isolated total DNA was quantified using Qubit (Thermofisher Scientific, Waltham, MA, USA) and Nanodrop (Thermofisher Scientific, Waltham, MA, USA), purified using the Zymo Research gDNA Clean and Concentrator Kit, and sequenced using the Oxford Nanopore Technologies Rapid DNA Ligation Kit (SQK-RAD004) with Rapid Barcoding Kit 1-12 (SQK-RBK004) according to manufacturer's instructions on a GridION device (FLOW-MIN106 flow cell). Genomes were assembled and analysed using Geneious software (Biomatters, Auckland, New Zealand). Comparative genomic analysis showed that strains DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 shared the same genetic backbone as strain DS84445 and that the plasmid encoded *gdh* gene was completely transferred from the donor strain TIL504 to the recipient strain DS84445-lac^{neg}-pGdh442.

Stability of pGdh442 plasmid

The stability of the plasmid in growing TIL504 and DS84445-lac^{neg}-pGdh442 cells was determined by comparing the specific GDH activity of cells pre-incubated and incubated in GM17 with 1% (wt/vol) glucose with and without 0.2 mmol l^{-1} cadmium chloride. In addition, the presence of the pGdh442 plasmid in sugar-deprived cells of strain DS84445-lac^{neg}-pGdh442 was determined by colony PCR according to Tanous et al. (2007). Samples were plated on GM17 agar and incubated at 30°C for 3 days. Ten colonies were picked for PCR analysis using primers CTA AAT GGC TTT AGC GGT GGG and CAC AAT CAA TTG GTG CTT CCC to detect the presence of the *gdh* gene. The strain DS84445-lac^{neg} was used as a control and was negative for the *gdh* gene.

Microbial analysis

Viable cell counts

Appropriate dilutions of samples taken from chemically defined medium (CDM) incubations were made in PPS, streaked onto GM17 agar plates, and incubated in an aerobic jar at 30°C for 5 days.

Live/dead cell counts

The number of cells with a non-compromised cell membrane (live) and cells with a compromised cell membrane (damaged or dead) was determined using the LIVE/DEAD Baclight Bacterial Viability Kit (Thermo Fisher Scientific, MA, USA) and analysed using a CytoFLEX flow cytometer (Beckman Coulter, CA, USA). The CDM samples were diluted 100 or 1000 times with PPS, and 199 µl of each diluted sample was added to a 96-well F-bottom microtiter plate (Greiner, Frickenhausen, Germany). A staining mixture of 4.5 µl SYTO 9, 4.5 µl propidium iodide (PI), and 141 µl demi-water was prepared. Cells were stained by adding 1 µl of the staining mixture to each 96-well F-bottom microtiter plate, which was stored in the dark for 15 min at room temperature. The green fluorescent SYTO 9 labelled the nuclei of live cells, while the red fluorescent PI labelled dead cells or cells with a compromised cell membrane. The number of live, damaged, and dead cells was counted with the flow cytometer at 488 nm to a maximum of 10 000 events. In all measurements, the number of

damaged cells was <1% of the number of live cells. These counts are not discussed in the results section.

Chemical analysis

Volatile compounds

The α-ketoisocaproic acid and 3-methylbutanal levels were determined by headspace solid-phase micro extraction gas chromatography-mass spectrometry (HS-GC-MS) using a Trace 1300 gas chromatograph with a TriPlus RSH autosampler, and an ISQ QD mass spectrometer (Thermo Fisher Scientific, MA, USA). The air-tight sealed vials were maintained at −1°C throughout the total run. Samples were incubated at 60°C for 20 min, followed by an extraction at 60°C for 20 min with a divinylbenzene/carboxene/polymethylsiloxane fibre (DVB/Car/PDMS) (Supelco, Pennsylvania, USA). Volatiles were desorbed from the fibre to the injector for 2 min on a Stabilwax-DA-Crossband-Carbowax-polyethyleneglycol column (30 m length, 0.25 mm ID, 0.5 μm df). The gas chromatograph was operated in split mode with a ratio of 1:5 at 250°C. The initial oven temperature was set at 35°C for 2 min and then gradually increased at a rate of 10°C per minute until it reached 240°C, where it was maintained for 5 min. The total analysis time was 27.5 min. Helium was used as the carrier gas at a constant flow rate of 1.2 ml per minute. Mass spectral data were collected over the range *m/z* 33–250 in full scan mode at 3.0 scans per second. The data obtained were analysed using Chromeleon 7.2. Peak integration was performed using the ICIS algorithm, and the NIST14 main library was used to match the mass spectral profiles with the profiles to NIST14. Peak areas were calculated using the MS quantification peak area (highest *m/z* peak per compound) and then corrected with blanks containing the same medium and incubated under the same conditions but without the addition of strains. The corrections were <3% of the total peak areas of the samples.

Alanine concentration

Amino acids were extracted from 400 mg sample with 25 ml of 0.1 mol l^{-1} HCl. After centrifugation at 20 000 *g* for 5 min, clear supernatants were obtained. 100 μl of the supernatants were mixed with 100 μl of an internal standard mixture, containing ^{13}C, ^{15}N isotopically labelled alanine at a concentration of ∼1 mg ml^{-1}. Samples were analysed using the AccQ•Tag Ultra method developed by Waters Corporation (Milford, MA, USA). Data analysis was performed using the Waters MassLynx and QuanLynx software. The lowest detectable concentration was 2.6 μg g^{-1}.

Enzyme activity measurements

Samples (100 ml) were taken from incubation experiments, and cells were harvested by centrifugation at 10 670 *g* for 10 min at 5°C. The concentrated cells were washed three times with 50 mmol l^{-1} sodium phosphate buffer (pH 7.0) and resuspended in 2 ml of the same buffer. Then 1 ml of the washed biomass was added to a 2 ml beat tube containing 1–1.5 g zirconia beads. Cell-free extract (CFE) was prepared by beating the tubes at 4 m s^{-1} in a FastPrep®-24 (MP Biomedicals, Solon, USA) for 30 s, followed immediately by cooling on melting ice for 5 min. The beating and cooling steps were repeated four times. Cell debris and supernatant were separated by centrifugation at 10 670 *g* for 10 min at 5°C. The supernatants were collected as CFE and stored on melting ice until enzyme activities and protein concentrations were measured. Protein concentrations of CFE were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Leucine aminotransferase activity measurement was previously described by Brandsma et al. (2008). Briefly, the reaction mixture contained 25 mmol l^{-1} phosphate buffer (pH 7.5), 0.05 mmol l^{-1} pyridoxal-5′-phosphate, 50 mmol l^{-1} ammonium sulphate, 0.35 mmol l^{-1} NADH, 10 mmol l^{-1} α-ketoglutarate, 10 mmol l^{-1} L-leucine as substrate, and 100 μl CFE in a final volume of 1 ml. Leucine aminotransferase activities were determined by the maximum reduction rate of NADH measured at OD_{340nm} for 15 min at 30°C. Enzyme activities were corrected for blanks containing the same reaction medium but without the addition of CFE. The protein concentrations of CFE expressed in μg ml^{-1} were used to calculate the specific enzyme activity (nmol min^{-1} mg^{-1}).

GDH activity was measured using the GDH Kit (KA0879, Abnova, Taipei, Taiwan). Enzyme activities were corrected for blanks containing the same reaction medium but without the addition of CFE, and the positive control from the GDH Kit was as expected (see Supplementary Material). The protein concentrations of CFE expressed in μg ml^{-1} were used to calculate the specific enzyme activity (nmol min^{-1} mg^{-1}).

α-Ketoisocaproic acid decarboxylase activity. CFE of strain DS84445 grown in GM17 for 18 h at 30°C was prepared as described here above. α-Ketoisocaproic acid decarboxylase activity was measured according to the method described by Smit et al. (2005a). Briefly, CFE was incubated in 3 ml reaction mixture containing 50 mmol l^{-1} McIlvaine buffer at pH of 6.3, 100 μmol l^{-1} thiamine pyrophosphate, 500 μmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} α-ketoisocaproic acid for 3 h at 30°C in air-tight sealed 10 ml vials. The reaction was stopped by lowering the pH to a value between 2 and 3 with 6 mol l^{-1} HCl. The concentration of 3-methylbutanal was quantified by static headspace GC by using a calibration curve in the reaction mixture. The protein concentrations of CFE, expressed in μg ml^{-1}, were used to calculate the specific enzyme activity in nmol 3-methylbutanal min^{-1} mg^{-1}.

Incubation experiments

Inoculum of DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 strains for the incubation experiments was prepared from precultures. Cells were harvested from full-grown GM17 precultures by centrifugation at 10 670 *g* for 5 min at 4°C and washed twice with 50 mM sodium phosphate buffer (pH 6.5 ± 0.1). Incubation media were prepared based on CDM described by Otto et al. (1983), with the following modifications. Salt and vitamin-free casein hydrolysate were omitted from the recipe. Depending on the experiment, CDM was prepared either with potassium phosphate buffer (39 mmol l^{-1}, pH 6.5 ± 0.1) or with citrate phosphate buffer containing 50 mmol l^{-1} citric acid and 100 mmol l^{-1} disodium hydrogen phosphate (pH 5.2 ± 0.1). CDM media with an initial pH of 6.5 and 5.2 were inoculated with concentrated cell suspensions obtained from the precultures to a final cell count of 2×10^7 or 2×10^8 per ml, respectively (determined by flow cytometry). Cell counts were measured using the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) and flow cytometry, from which the inoculation rate was calculated. Immediately after inoculation, the 15 ml CDM was divided into two portions. A portion

of 13 ml was transferred to a 15 ml conical tube (Greiner, Pleidelsheim, Germany) for cell counting and pH measurement, and a portion of 2 ml was transferred to a 10 ml air-tight sealed vial (Crimp-nek N20, Macherey-Nagel, Germany) for flavour analysis. Both samples were incubated at 30°C. Samples were prepared in triplicate for each time point. Conical tubes were collected immediately after inoculation and after different incubation times (24, 48, and 72 h) and used for cell counting by selective plating or live/dead counting. Vials were stored at −40°C until further analysis of flavour compounds. For each experiment, uninoculated blanks were taken and incubated under the same conditions. Data presented are the average of 3 biological replicates.

Statistics

Experiments were performed in triplicate unless otherwise stated. Data were analysed by two-sample t-test assuming equal variances using Analysis ToolPak in Excel. Data are expressed as average ± standard deviation (SD), and statistically significant differences of $P < .01$ and $P < .05$ are indicated by ∗ or ∗∗, respectively.

Results

Development of *L. lactis* DS84445 strain with GDH activity

The effect of GDH activity and pyruvate supplementation on the transamination of leucine into α-ketoisocaproic acid was studied in *Lactococcus*. For this work, we selected *L. lactis* strain DS84445, which has a relatively high decarboxylase activity that decarboxylates α-ketoisocaproic acid into 3-methylbutanal. By using this strain, we aim to minimize the accumulation of α-ketoisocaproic acid, which could otherwise reduce the transamination rate of leucine. In addition, the production of 3-metylbutanal is used as an additional and indirect measure of the transamination rate of leucine. The α-ketoisocaproic acid decarboxylase activity of strain DS84445 was 19 nmol min^{-1} mg^{-1} (SD = 7.9), which is in line with other *Lactococcus* strains grown under similar conditions with a relatively high decarboxylase activity (Smit et al. 2005a). Strain DS84445 does not have GDH activity, so in order to study the effect of GDH activity, a derivative of strain DS84445 with GDH activity was developed. The conjugational transfer technique was used to transfer the plasmid pGdh442, which contains the *gdh* gene encoding for the GDH protein from the donor strain TIL504, into cells of strain DS84445 (Tanous et al. 2007). Unfortunately, we were not able to select conjugants of strain DS84445 that received the pGdh442 plasmid. Tanous et al. (2006) suggested that the pGdh442 plasmid is incompatible with plasmids that are already present in the recipient strain. Indeed, strain DS84445 carries a plasmid containing genes required for lactose metabolism, which has replication genes comparable to those found on plasmid pGdh442. We performed a plasmid curation step and selected a lactose-negative variant of strain DS84445, called strain DS84445-lac^{neg}. The donor strain TIL504 and the recipient strain DS84445-lac^{neg} were mixed and grown on a GM17 agar plate for conjugational transfer of the plasmid pGdh442. After incubation, the cells were harvested and plated on agar containing xylose and cadmium chloride, which are selective conditions for the growth of the DS84445-lac^{neg} strain that has acquired the pGdh442 plasmid. DNA sequencing data confirmed that conjugant strain DS84445-lac^{neg}-pGdh442 contained the pGdh442 plasmid. The specific GDH activity of strains DS84445-lac^{neg}-pGdh442 and TIL504 grown in GM17 with $CdCl_2$ was comparable (Fig. 2). In addition, the stability of the pGdh442 plasmid during the pre-incubation and incubation step was tested by comparing the GDH activities of cells grown in the absence and presence of cadmium.

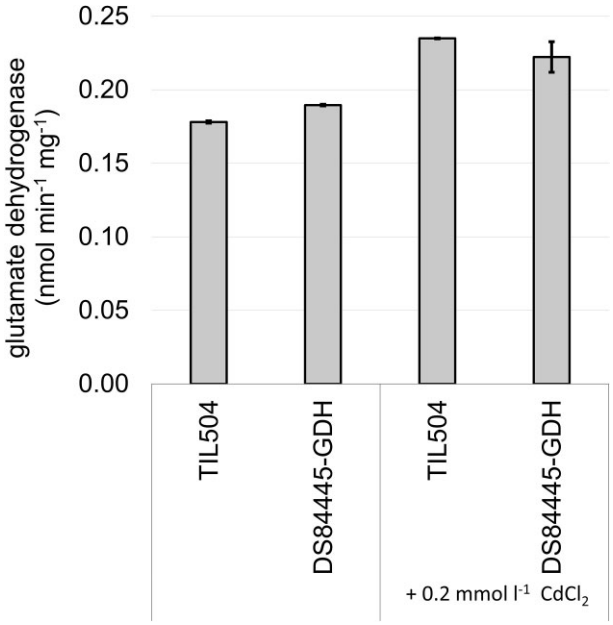

Figure 2. GDH activity of TIL504 and DS84445-lac^{neg}-pGdh442 (DS84445-GDH) strains grown in GM17 with and without 0.2 mmol l^{-1} $CdCl_2$ for 18 h at 30°C. Average of two biological replicates.

Cells incubated without cadmium lost ∼20% of the GDH activity, which is in agreement with the results of Tanous et al. (2006). In addition, DS84445-lac^{neg}-pGdh442 incubated without $CdCl_2$ was plated on GM17 agar, and 10 colonies were selected for PCR analysis. The *gdh* gene was found in 9 out of 10 colonies, supporting the observation that GDH activity was comparable to cells incubated with $CdCl_2$. Cadmium affects several genes involved in the stress response, including lactate dehydrogenase (Sheng et al. 2016). All subsequent experiments were performed in the absence of cadmium to ensure that the effect of pyruvate supplementation was not affected by the presence of cadmium.

Leucine transamination in sugar metabolizing *L. lactis* strains

Lactococcus starter cultures are specialized in converting lactose via the glycolytic pathway into lactic acid, resulting in rapid acidification of the cheese milk and depletion of sugars during the early cheese ripening period. During this phase of the cheese production, when residual sugars are still available, transamination of amino acids already takes place, resulting in the production of volatile flavours such as 3-methylbutanal (Ayad et al. 2001, Brandsma et al. 2021, Meng et al. 2021). We first investigated the impact of GDH activity on the transamination of leucine by cells incubated with an excess of glucose. The growth of strains DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 was found to be comparable in terms of live cell counts and pH development (Fig. 3). This permits a compari-

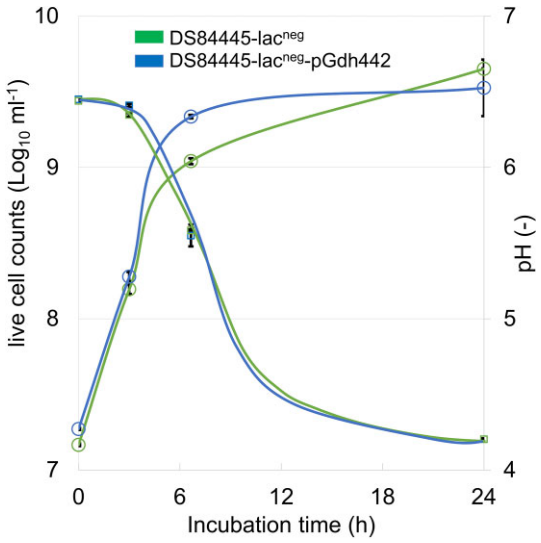

Figure 3. Live cell counts (circles) and pH development (squares) of strains DS84445-lac^{neg} (green) and DS84445-lac^{neg}-pGdh442 (blue) grown in CDM with 1% (wt/vol) glucose and incubated at 30°C. Average of three biological replicates.

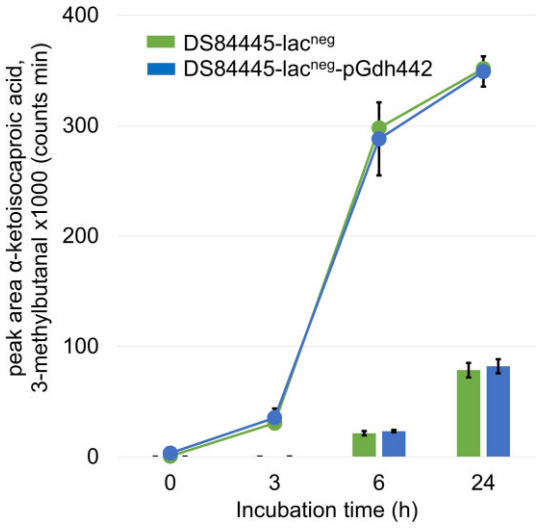

Figure 4. Levels of α-ketoisocaproic acid (bars) and 3-methylbutanal (lines) produced by strains DS84445-lac^{neg} (green) and DS84445-lac^{neg}-pGdh442 (blue) grown in CDM with 1% (wt/vol) glucose and incubated at 30°C. Average of three biological replicates.

son of the relative production of volatile compounds between the two strains.

The production of α-ketoisocaproic acid and 3-methylbutanal was measured by HS-GC-MS in biological triplicates. It was found that strain DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 produced comparable relative amounts of α-ketoisocaproic acid and 3-methylbutanal, indicating that GDH activity did not stimulate the transamination of leucine (Fig. 4).

Surprisingly, the accumulation of α-ketoisocaproic acid indicated that decarboxylation of α-ketoisocaproic acid was rate limiting for the production of 3-methylbutanal (Fig. 4). Leucine transamination was apparently not limited by the availability of an amino group acceptor in sugar metabolizing cells. Glycolysis produces relatively large amounts of pyruvate, which is predominantly converted into lactic acid (Thomas 1976, Thomas et al. 1980). However, a limited amount of pyruvate could be used for other cellular reactions, e.g. serving as an amino group acceptor for transamination reactions. This could explain why α-ketoglutarate was not limiting and GDH activity did not stimulate the production of α-ketoisocaproic acid and 3-methylbutanal. To test the effect of pyruvate and GDH activity on the transamination, cells were incubated under the same conditions but without sugar.

Leucine transamination in sugar-deprived cells

For most of the cheese ripening period, sugars are absent, and the pH stabilizes around 5.2. These conditions reduce cell viability and presumably reduce the availability of amino group acceptors for transamination reactions. In this context, cells with GDH activity are expected to be able to replenish α-ketoglutarate, which stimulates the transamination of leucine. For this reason, the DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 strains were incubated in CDM without sugar and at a controlled pH of 5.2. In contrast to sugar metabolizing cells, sugar-deprived cells of strain DS84445-lac^{neg} did not excrete α-ketoisocaproic acid, indicating that the transamination of leucine is the rate-limiting step in the production of 3-methylbutanal. Surprisingly, cells from strain DS84445-lac^{neg}-pGdh442 did not excrete α-ketoisocaproic acid or produced more 3-methylbutanal than strain DS84445-lac^{neg} (Fig. 5). Apparently, strain DS84445-lac^{neg}-pGdh442 is not able to produce enough α-ketoglutarate to support transamination. It is important to note that the deamination of glutamate requires NAD$^+$ to accept the proton released from glutamate. In fact, these sugar-deprived cells might have run out of NAD$^+$, which is unlikely to be the case in sugar metabolizing cells.

The strains were supplemented with pyruvate to test whether this would induce an effect in cells with GDH activity. Strains DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 produced equal amounts of 3-methylbutanal, showing that leucine transamination in cells with GDH activity was not stimulated by pyruvate supplementation. In fact, pyruvate stimulated the 3-methylbutanal production in both strains (Fig. 5). These observations could not be explained by differences in cell counts because live cell counts of both strains supplemented with and without pyruvate remained stable at 8.57 Log$_{10}$ (SD 0.04) counts per ml over the course of the 72-h incubation. We therefore argue that pyruvate stimulates the 3-methylbutanal production by acting as an amino group acceptor. Pyruvate can stimulate transamination directly by accepting amino groups from transaminated amino acids, or indirectly by first generating another amino group acceptor, e.g. α-ketoglutarate. In either cases, pyruvate accepts the amino group and converts into alanine. To test this, strain DS84445-lac^{neg} was incubated under the same sugar-deprived and pyruvate-supplemented conditions as before, but alanine was omitted from the CDM medium. Indeed, strain DS84445-lac^{neg} produced alanine, demonstrating that pyruvate acts as an amino group acceptor and stimulates transamination reactions (Figs 5 and 6).

Alanine production increased steadily throughout the 72 h of incubation, whereas 3-methylbutanal production stabilized after 24 h of incubation (Fig. 5). Most likely, pyruvate stimulates not only the transamination of leucine into α-ketoisocaproic acid but also the transamination of other amino acids. In fact, the production of 2-methylbutanal, which is derived from isoleucine, was also 2–3 times higher when pyruvate was added.

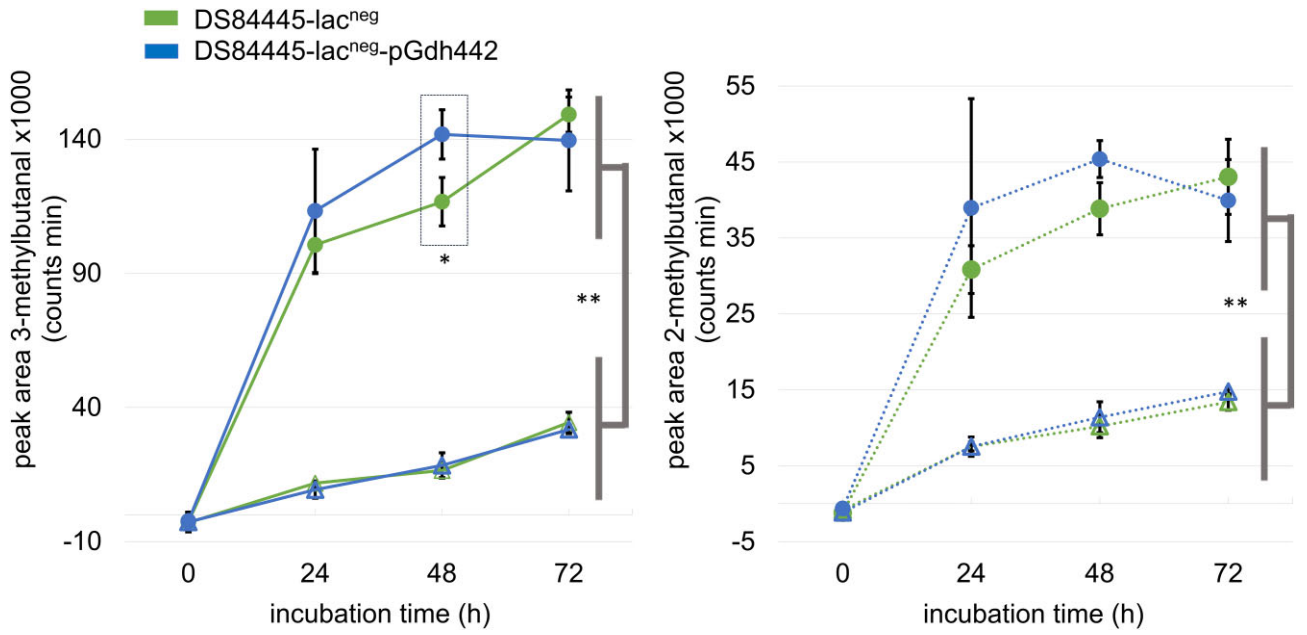

Figure 5. Levels of 3-metylbutanal (left panel) and 2-methylbutanal (right panel) produced by DS84445-lac^{neg} (green) and DS84445-lac^{neg}-pGdh442 (blue) in CDM supplemented with (closed circles) and without (open triangles) 10 mmol l^{-1} pyruvate at pH of 5.2 and incubated at 30°C. α-Ketoisocaproic acid was not detected in the samples. Average of 3 biological replicates and values indicated with $*$ and $**$ are significantly different at $P < .05$ and $P < .01$, respectively.

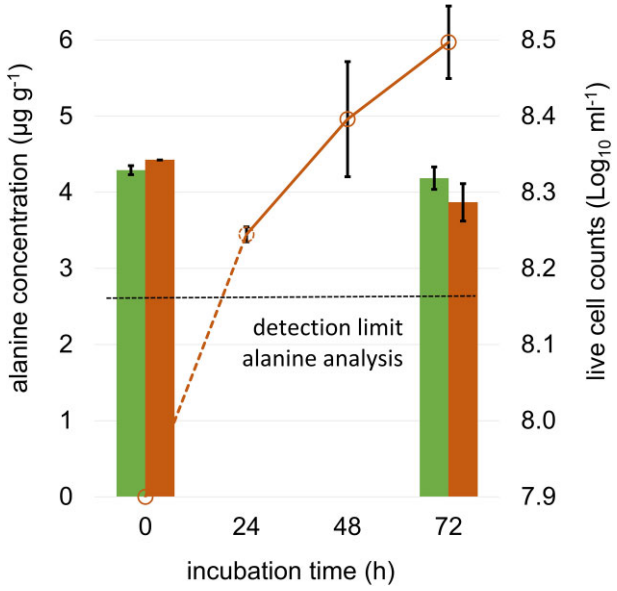

Figure 6. Alanine production by strain DS84445-lac^{neg} grown at 30°C in alanine-free CDM supplemented with and without pyruvate. Alanine concentrations of cells incubated without pyruvate were below the detection limit (2.7 µg alanine per gram) and samples incubated with pyruvate (brown). Average live cell counts after 0 and 72 h of incubation (bars) supplemented with (brown) and without pyruvate (green). Average of three biological replicates.

The decrease in 3-methylbutanal production after the first 24 h of incubation could also reflect a decrease in viable and/or live cell counts. Live cell counts were determined by the live/dead staining method, and viable cell counts were determined by using a plate counting method. As mentioned above, the average live cell counts of all variants remained stable at 8.57 Log_{10} (SD 0.04) in the sugar-deprived incubation experiments, with the number of dead cells being <5%. However, the viable cell counts decreased by more than 2 orders of magnitude to a level below 6.39 Log_{10} (SD 0.15) CFU ml^{-1}. Thus, the cells predominantly maintained the cell membrane integrity, but the ability to form colonies on agar plates was greatly reduced during the 72 h of incubation under sugar-deprived conditions. This raises the question of whether the intracellular enzymes involved in the transamination of leucine remain active under these typical cheese ripening conditions.

Enzyme stability in sugar deprived cells

Sugar deprivation reduces the cellular metabolism, leading, e.g. to the degradation of intracellular proteins (Douwenga et al. 2021). We investigated the effect of sugar deprivation on the stability of enzymes involved in leucine transamination. Cells were incubated in CDM with 0.5% (wt/vol) glucose, which allows acidification until sugar is depleted to a pH of 5.2 (time point 0 h), which then remained stable over the 72 h of incubation at an average value of 5.29 (SD 0.03). Viable cell counts at incubation time point 0 h were similar for both DS84445-lac^{neg} and DS84445-lac^{neg}-pGdh442 strains at approximately Log_{10} 9.2 CFU ml^{-1}. During incubation, the viable cell counts decreased (Fig. 7), which is consistent with the reduction of the viable cell counts in cheese during ripening (Brandsma et al. 2012).

At each time point, samples were taken to measure the specific leucine transaminase and GDH activities. The highest leucine transaminase activities of both strains were reached after 24 h of incubation and remained stable for the next 48 h (Fig. 8). Cells of strain DS84445-lac^{neg} were supplemented with pyruvate (Fig. 8, dashed green bars) and had comparable leucine transaminase activities as to the reference cells (Fig. 8, solid green bars). This shows that pyruvate does not affect the stability of intracellular leucine transaminase activities of cells,

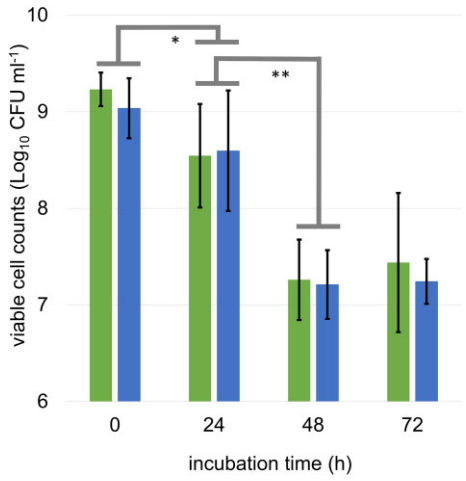

Figure 7. Viable cell counts (Log_{10} CFU ml^{-1}) of strains DS84445-lac^{neg} (green) and DS84445-lac^{neg}-pGdh442 (blue) in CDM with 0.5% (wt/vol) glucose incubated at 30°C. Incubation time $t = 0$ h represents the time when the acidification of the medium stabilizes at pH 5.2. Average of three biological replicates and values indicated with ∗ and ∗∗ are significantly different at $P < .05$ and $P < .01$, respectively.

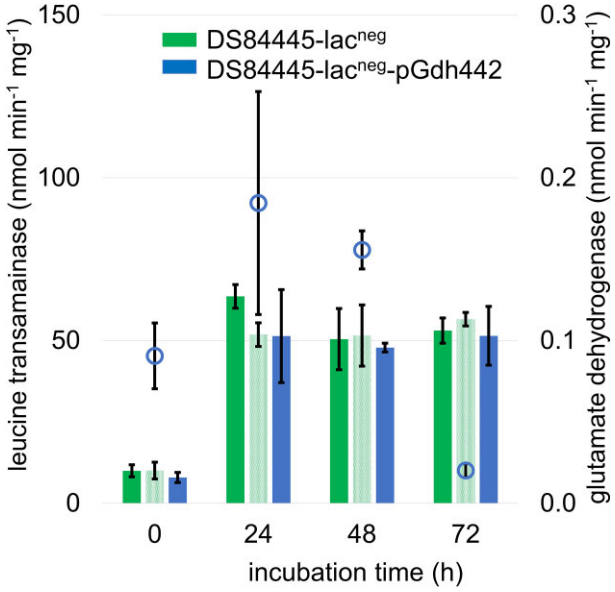

Figure 8. Leucine transaminase activity of strain DS84445-lac^{neg} grown in CDM with 0.5% (wt/vol) glucose in the absence (solid green bar) or presence (dashed green bar) of 10 mmol l^{-1} pyruvate and incubated at 30°C. Leucine transaminase activity (solid blue bar) and GDH activity (blue circles) of DS84445-lac^{neg}-pGdh442 were determined with cells grown in CDM with 0.5% (wt/vol) glucose and incubated at 30°C. Incubation time $t = 0$ h represents the time when glucose is depleted, and pH reaches 5.2. Average of three biological replicates.

but that pyruvate supports transaminase reactions by acting as an amino group acceptor.

The GDH activity of strain DS84445-lac^{neg}-pGdh442 followed a different pattern, increasing to 0.18 (SD 0.07) during the first 24 h but then decreasing to 0.02 (SD 0.00) nmol min^{-1} mg^{-1} during the following 48 h. Compared to leucine transaminase activity, the stability of GDH activity from sugar-deprived cells is low, which is consistent with the limited effect that GDH activity might have on amino acid transamination. As expected, GDH activity is not detected in strain DS84445-lac^{neg}.

Discussion

The transamination of amino acids is an important first step in the production of volatile flavours, and the rate of this reaction is thought to be limited by the availability of α-ketoglutarate (Yvon et al. 1998, Banks et al. 2001, Williams et al. 2004). In general, sugar-deprived bacteria have limited options left to produce α-ketoglutarate (Legendre et al. 2020). For lactic acid bacteria, such as lactobacilli, streptococci, and lactococci, the enzymatic deamination of glutamate by GDH is one of the most studied pathways to produce α-ketoglutarate (Williams et al. 2006, Peralta et al. 2016, Gómez de Cadiñanos et al. 2018). Derivative strains of *L. lactis* NCDO 712 with GDH activity were developed to study the effect on amino acid transamination and flavour production. It was found that GDH activity enhanced the catabolism of leucine and/or phenylalanine and increased the production of carboxylic acids (Rijnen et al. 2000, Tanous et al. 2006). However, effects of GDH activity on the production of key flavours such as 3-methylbutanal were not reported in these studies. Smit et al. (2005a) showed that *L. lactis* requires a complete gene encoding for an α-ketoisocaproic acid decarboxylase to produce 3-methylbutanal, which is not present in strain NCDO 712. For this reason, we used strain DS84445 in our study, which carries the complete gene encoding the α-ketoisocaproic acid decarboxylase. In contrast to these previous studies, we found no evidence for a stimulatory effect of GDH activity on amino acid transamination. There are several possible explanations for our observations. Rijnen et al. (2000) introduced the heterologous *gdh* gene from a *Peptoniphilus asaccharolyticus* strain into the GDH negative *L. lactis* NCDO 712 by using the high copy number plasmid pGEMT-Easy, resulting in a transformed *L. lactis* NCDO 712 strain with a GDH activity of ∼900 nmol mg^{-1} min^{-1}. On the contrary, the GDH activity of NCDO 712 and DS84445-lac^{neg} strains carrying pGdh442, derived from *L. lactis* NCDO 1867 is ∼1000 times lower. This could be due to a difference in enzyme activity but could also be influenced by plasmid characteristics such as copy number and stability. Tanous et al. (2006) found a higher phenylalanine degradation by strain NCDO 712 with pGdh442 (TIL504), but this experiment was performed in a buffered cell suspension with addition of the cofactor pyridoxal-5-phosphate and with an extremely high biomass density, expressed by optical density (OD_{600nm}), of 20. On the contrary, we performed incubations without pyridoxal-5-phosphate supplementation and with cell counts (10^8–10^9 counts per ml) comparable to those found during the first week of cheese ripening (Brandsma et al. 2012). The relatively high biomass concentration in combination with an incubation temperature of 37°C used by Tanous et al. (2006) might have resulted in partial lysis of the population, which could have stimulated the metabolic activity of cells that maintained integrity of the cell membrane (Corchero et al. 2001). However, apart from different incubation conditions, it is not clear whether the GDH activity of strains enriched with the pGdh442 plasmid is high enough to support the leucine transamination. The leucine transaminase activity of strain DS84445-lac^{neg}-pGdh442 is ∼150 nmol NADH min^{-1} mg^{-1}, which is in line with activities found for other *L. lactis* strains (Smit et al. 2004, Brandsma et al. 2012). On the other hand, the specific GDH activity found for the DS84445-lac^{neg}-pGdh442 strain and other *Lactococcus* strains is in the range of 0.15–3 nmol NADH min^{-1} mg^{-1} (Comez de Cadi-

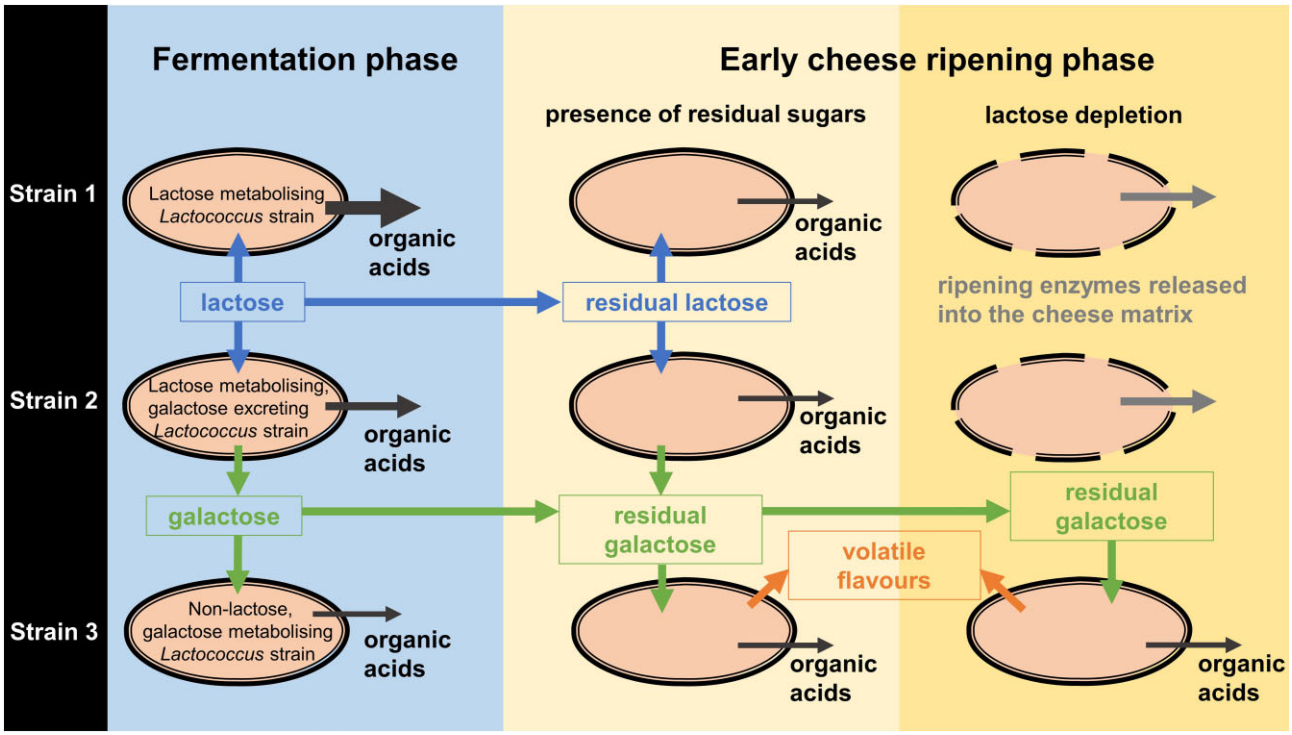

Figure 9. Schematic overview of a starter culture containing three *Lactococcus* strains with different sugar metabolizing properties during the fermentation phase and at the beginning of cheese ripening phase. The size of the black arrows indicates the amount of organic acid production.

nanos et al. 2018). It is important to note that these activities are determined by *in vitro* enzyme assays at a pH of 7.5, which might not be representative of the intracellular pH of cells during cheese ripening. The leucine transaminase activity might yet remain 50–100 times higher than the GDH activity in wild-type *Lactococcus* strains, and thus still not support amino acid transamination reactions. An alternative way for *Lactococcus* strains to produce α-ketoglutarate is through the uptake of citrate which can be converted into α-ketoglutarate via iso-citrate or via oxaloacetate (Tanous et al. 2005, Pudlik et al. 2012). A disadvantage of using citrate-converting strains is the production of CO_2, which can lead to crack and slit formation in cheese during ripening. Cheese types with a relatively firm texture, such as Cheddar, are particularly sensitive to CO_2 production.

The transamination of amino acids is an important first step in the production of volatile cheese flavours. Our results showed that the leucine transaminase activity is relatively stable compared to the GDH activity in sugar-deprived cells. Furthermore, we have shown that pyruvate acts as an amino group acceptor and thereby stimulating the transamination of amino acids, which supports the production of volatile flavours. For these reasons, we propose to increase the sugar content in cheese at the beginning of the ripening phase to increase the availability of pyruvate for the starter culture to produce flavours, rather than developing *Lactococcus* strains with GDH activity. However, increasing the sugar content will stimulate the production of organic acids, which will lower the pH and affect cheese characteristics such as flavour, texture, and appearance (Upreti and Metzger 2007). Maintaining the pH of cheese at a normal level requires a different interplay between the cheese manufacturing conditions and the starter culture. There are several ways to adjust the composition of the cheese matrix with the goal to buffer the additional organic acids produced. For example, flushing the cheese milk with CO_2 increases the buffering capacity. By modifying the milk coagulation and curd draining processes, the protein and mineral content of cheese can be altered to increase the buffering capacity (Salaün et al. 2005). The manufacturing conditions of cheese largely depend on the type of cheese produced. For this reason, we only discuss the design criteria for a starter culture to metabolize the additional sugar. In our opinion, such a starter culture consists of 3 *L. lactis* or *L. cremoris* strains with different sugar metabolism capabilities. Firstly, this starter culture contains a lactose-metabolizing *L. lactis* or *L. cremoris* strain, which is responsible for a rapid acidification of the cheese milk. Secondly, we propose a *Lactococcus* strain that only metabolizes the glucose moiety of lactose and excretes galactose, which increases the sugar content of the cheese. By combining a galactose-metabolizing strain and a non-galactose-metabolizing strain, the galactose content in cheese can be controlled. The additional galactose content is metabolized by a third *L. lactis* or *L. cremoris* strain, which is only able to metabolize galactose and is mainly responsible for the increased production of volatile flavours (Fig. 9). The advantage of specifically increasing the galactose content is that galactose is metabolized relatively slowly by *Lactococcus* (Thomas 1976, Thomas et al. 1980). This makes it easier to balance the increasing buffering capacity, as a result of, e.g. proteolysis (Salaün et al. 2005), with the additional organic acids produced during cheese ripening. In addition, by selecting a *L. lactis* or *L. cremoris* strain with a specific galactose transporter, the galactose uptake can be further optimized in relation to the buffer capacity of the cheese (Neves et al. 2010). These three strains should be combined in such a way that relatively more galactose is present at the beginning of the

cheese ripening phase and that adjusted cheese manufacturing conditions ensure that the pH of the cheese is maintained at the required level.

The contribution of a *Lactococcus* starter culture to the cheese flavour is a combined result of cells producing volatile flavour compounds and permeabized cells releasing intracellular enzymes into the cheese matrix (Lortal et al. 2005, Smid et al. 2014, Wilkinson and Lapointe 2020, Nugroho et al. 2021). In our starter culture design, we propose to use a fast lactose-metabolizing strain, which mainly drives the milk acidification, and also permeabilizes relatively fast and releases intracellular enzymes into the cheese matrix to stimulate casein degradation. On the other hand, the galactose-metabolizing strain remains metabolically active during the cheese ripening, producing additional pyruvate, which acts as an amino group acceptor to support amino acid transamination. Of particular interest is the degradation of methionine, branched-chain amino acids, and aromatic amino acids, which are associated with sulphur notes, mature cheese flavour, and floral flavour, respectively (Engels et al. 2000, Yvon and Rijnen 2001). We have shown that pyruvate stimulates the transamination of the branched-chain amino acid leucine and isoleucine in strain *L. lactis* DS84445. Strain diversity studies have shown that the aforementioned aminotransferases are active in *L. lactis* strains (Engels et al. 2000, Brandsma et al. 2008), suggesting that pyruvate can stimulate the transamination of a relatively large number of different amino acids. Further studies are required to demonstrate whether pyruvate acts as an amino group acceptor in the transamination of other amino acids. Interestingly, the presence of α-ketoacid decarboxylase activity seems to be required to decarboxylate the produced α-ketoacid into these volatile cheese flavours (Smit et al. 2005a). This suggests that the flavour profile of cheese can be steered by developing a starter culture comprising a galactose-metabolizing *L. lactis* strain that possesses α-ketoacid decarboxylase activity, which specifically converts α-ketoacids derived from methionine, branched-chain, and/or aromatic amino acids.

In conclusion, our work has shown that active sugar metabolism and pyruvate supplementation support leucine transamination, which in turn stimulates 3-methylbutanal production. In light of these findings, we propose to develop a novel *Lactococcus* starter culture that increases the residual galactose concentration in the early stage of cheese ripening in order to remain metabolically active and produce more volatile flavour at a later stage. Such a starter culture would enable cheese producers to accelerate the cheese ripening phase, thus contributes to the cost efficiency of cheese production.

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Author contributions

Johannes B. Brandsma (Conceptualization, Data curation, Investigation, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing), Judith Brinkman (Data curation, Formal analysis, Investigation, Validation), Judith C.M. Wolkers-Rooijackers (Data curation, Formal analysis, Investigation, Methodology, Validation), Iris van Swam (Data curation, Formal analysis, Investigation, Methodology, Validation), Kim van Uitert (Data curation, Formal analysis, Investigation, Methodology, Validation), Marcel H. Zwietering (Writing – review & editing), and Eddy J. Smid (Conceptualization, Supervision, Writing – review & editing)

Supplementary data

Supplementary data is available at *JAMBIO Journal* online.

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Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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