Food Microbiology 73 (2018) 216–226

Contents lists available at ScienceDirect

Food Microbiology

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

Quantitative physiology and aroma formation of a dairy *Lactococcus lactis* at near-zero growth rates

Oscar van Mastrigt [a], Tjakko Abee [a], Søren K. Lillevang [b], Eddy J. Smid [a, *]

[a] Laboratory of Food Microbiology, Wageningen University & Research, The Netherlands
[b] Arla Innovation Centre, Arla Foods Amba, Agro Food Park 19, 8200 Aarhus N, Denmark

ARTICLE INFO

Article history:
Received 17 November 2017
Received in revised form
29 January 2018
Accepted 30 January 2018
Available online 3 February 2018

Keywords:
Maintenance
Viable but non-culturable
Cheese
Hydrophobicity
Volatile organic compounds

ABSTRACT

During food fermentation processes like cheese ripening, lactic acid bacteria (LAB) encounter long periods of nutrient limitation leading to slow growth. Particular LAB survive these periods while still contributing to flavour formation in the fermented product. In this study the dairy *Lactococcus lactis* biovar diacetylactis FM03-V1 is grown in retentostat cultures to study its physiology and aroma formation capacity at near-zero growth rates. During the cultivations, the growth rate decreased from 0.025 h^{-1} to less than 0.001 h^{-1} in 37 days, while the viability remained above 80%. The maintenance coefficient of this dairy strain decreased by a factor 7 at near-zero growth rates compared to high growth rates (from 2.43 ± 0.35 to 0.36 ± 0.03 mmol ATP.$gDW^{-1}.h^{-1}$). In the retentostat cultures, 62 different volatile organic compounds were identified by HS SPME GC-MS. Changes in aroma profile resembled some of the biochemical changes occurring during cheese ripening and reflected amino acid catabolism, metabolism of fatty acids and conversion of acetoin into 2-butanone. Analysis of complete and cell-free samples of the retentostat cultures showed that particular lipophilic compounds, mainly long-chain alcohols, aldehydes and esters, accumulated in the cells, most likely in the cell membranes. In conclusion, retentostat cultivation offers a unique tool to study aroma formation by lactic acid bacteria under industrially relevant growth conditions.

1. Introduction

In natural environments, microorganisms generally live in a feast or famine existence due to variable availabilities of nutrient and energy sources (Koch, 1971; Morita, 1993) and it has been suggested that energy limitation is the prevailing physiological state among microorganisms on Earth (Brock, 1971; Morita, 1997). The physiological state of these organisms is poorly represented in laboratory experiments with nutrient-rich batch cultures, in which metabolic rates are several orders of magnitude higher than in oligotrophic environments (Hoehler and Jorgensen, 2013). Also during particular industrial fermentations bacteria may experience strongly restricted access to nutrients for long periods of time. For instance, during cheese ripening lactic acid bacteria (LAB) encounter such conditions (Martley and Crow, 1993; McSweeney and Fox, 2004; Thomas and Pearce, 1981). This leads to severe reduction of the growth rate. Particular LAB survive these periods of extremely slow growth, while still contributing to aroma formation in the fermented product (Erkus et al., 2013).

Conditions of strong nutrient restriction and extremely slow growth could be mimicked in the laboratory using retentostat cultivation (Boender et al., 2009). This unique fermentation technique is a modification of chemostat cultivation in which culture effluent is removed, but biomass is retained by a filter in the effluent line. Because nutrients are continuously fed to the reactor while biomass is retained, biomass will accumulate leading to a gradual decrease in substrate availability per cell. Metabolic energy is distributed to either growth or non-growth (maintenance) processes (Pirt, 1965). When the maintenance energy coefficient is assumed to be growth rate independent as proposed by Herbert and Pirt (1965) the decreasing substrate availability leads to growth rates approaching zero. Finally, all metabolic energy that can be

* Corresponding author. Laboratory of Food Microbiology, Wageningen University & Research, P.O. Box 17, 6700AA Wageningen, The Netherlands.
E-mail address: eddy.smid@wur.nl (E.J. Smid).

https://doi.org/10.1016/j.fm.2018.01.027

derived from the substrate will be used for maintenance processes.

Retentostat cultivation has been used for studying the physiology of some industrially relevant microorganisms, like *Saccharomyces cerevisiae, Bacillus subtilis, Lactobacillus plantarum* and *Lactococcus lactis* (Boender et al., 2009; Ercan et al., 2013, 2015a; Goffin et al., 2010; Overkamp et al., 2015). Doubling times increased till one year and the viability remained high. Different adaptation strategies were observed, like alternative carbon-source utilisation, upregulation of stress response genes and increased robustness (Boender et al., 2011; Ercan et al., 2015a, 2015b; Vos et al., 2016). However, the effect of extremely slow growth on aroma formation has never been studied using retentostat cultivation. Moreover, the study with *L. lactis* was performed with the plant-associated *L. lactis* subsp. *lactis* KF147, while the main aroma producers of this species during cheese ripening are strains of *L. lactis* subsp. *lactis* biovar diacetylactis (Erkus et al., 2013). This biovariety is able to consume citrate which can be converted into the buttery aromas diacetyl and acetoin (Harvey and Collins, 1961).

Besides these buttery aromas, various other categories of compounds are responsible for aroma in fermented foods, such as alcohols, aldehydes, ketones, fatty acids, esters and sulphur compounds (Engels et al., 1997). Precursors for these compounds originate from all main components in food materials (i.e. proteins, lipid and carbohydrates) (Smit et al., 2005b). Conversion of most precursors into aroma compounds relies on functional metabolic pathways (Smid and Kleerebezem, 2014) and microorganisms might activate certain pathways in response to specific conditions.

In this study, a dairy strain of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis was grown in retentostat cultures to study the effect of near-zero growth rates on the physiology and aroma formation capacity of this bacterium.

2. Materials and methods

2.1. Strain and media

In this study *Lactococcus lactis* subsp. *lactis* biovar diacetylactis FM03-V1 has been used, which has been isolated from 10-weeks-old Samsø cheese (van Mastrigt et al., 2017). For chemostat and retentostat cultivations, the bacteria were streaked from a −80 °C stock on M17 agar plates supplemented with 0.5% (w/v) lactose (LM17). After incubation at 30 °C for 2 days, a single colony was inoculated in 10 mL lactose-limited chemically-defined medium (van Mastrigt et al., 2017) and grown overnight at 30 °C. This overnight culture was used to inoculate the bioreactor at 1% (v/v) inoculation level. The chemically-defined medium that was used for all retentostat cultivations contained as main carbon and nitrogen sources 0.5% (w/w) lactose, 10 mM $(NH_4)_3$citrate and 1% (w/w) Bacto-Tryptone and was prepared in 20 L batches. For chemostat cultivations, the media contained either 0 or 10 mM $(NH_4)_3$citrate.

2.2. Chemostat cultivation

Bacteria were grown at dilution rates between 0.05 and 0.4 h^{-1} in bioreactors with a working volume of 0.5 L (Multifors, Infors HT, Switzerland). The stirring speed was set at 300 rpm, the temperature was kept constant at 30 °C and the pH was controlled at 5.5 by automatic addition of 5M NaOH. The headspace was flushed with nitrogen gas at a rate of 0.1 L/min to maintain anaerobic conditions. The optical density at 600 nm was continuously measured using an internal probe (Trucell2, Finesse, USA). Samples were taken after reaching steady-state conditions, which were considered to be achieved after a minimum of five volume changes at which the optical density remained constant. The dilution rate was changed by controlling the feed rate, which was monitored by continuously weighing the medium vessel.

2.3. Retentostat cultivation

Independent retentostat cultivations were performed in bioreactors with a working volume of 1 L (Multifors, Infors HT, Switzerland). All conditions were the same as in the chemostat cultivation except that a stirring speed of 400 rpm was used. After a steady-state had been achieved in chemostat cultivation at a dilution rate of 0.025 h^{-1}, retentostat cultivation was initiated by removing the effluent via a sterilisable polyethersulfone cross-flow filter (Spectrum laboratories, USA). This filter was connected via an outer loop. Residence times of biomass in the outer loop were minimised. As removal of samples could interfere with biomass accumulation, sample volumes and frequency were minimised to maximum 10 ml every 3–4 days.

2.4. Cell dry weight determination

The cell dry weight was determined as described by Van Mastrigt and co-workers (van Mastrigt et al., 2017). Briefly, 2 and 5 mL sample was passed through a pre-weighted 0.2 μm membrane filter (Pall Corporation, USA) by a vacuum filtration unit. Subsequently, the filter was dried at 80 °C for 48 h and weighted again on an analytical balance.

2.5. Analysis of extracellular metabolites

Lactose, citrate, lactate, acetate, ethanol, formate, pyruvate and acetoin were quantified by High Performance Liquid Chromatography (HPLC) as described by Van Mastrigt and co-workers (van Mastrigt et al., 2017).

2.6. Volatile organic compounds analysis

2.6.1. VOC detection by HS SPME GC-MS

To determine the volatile organic compounds (VOCs), 100 μl sample was transferred to a 5 ml GC vial. Samples were kept frozen (−20 °C) until analysis by headspace solid phase microextraction gas chromatography mass spectrometry (HS SPME GC-MS) (van Rijswijck et al., 2017). Samples were defrosted and incubated for 5 min at 60 °C with agitation. Subsequently, volatile organic compounds were extracted from the samples for 20 min at 60 °C using a Solid Phase Microextraction fiber (85 mm CAR/PDMS, Supelco, USA). The compounds were desorbed from the fiber for 10 min on a Stabilwax®-DA-Crossband®-Carbowax®-polyethylene-glycol column (30 m length, 0.25 mmID, 0.5 μm df). The settings on the gas chromatograph were PTV Split-less mode 5 min at 250 °C. Helium was used as carrier gas at a constant flow of 1.5 mL/min. The temperature of the GC oven was initially 40 °C. After 2 min, the temperature was raised to 240 °C at a rate of 10 °C/min and kept at 240 °C for 5 min. Mass spectral data was collected over a range of m/z 33–250 in full scan mode with 3.0030 scans/second.

2.6.2. VOC identification and quantification

VOC profiles were analysed with Chromeleon 7.2 software. The ICIS algorithm was used for peak integration and the NIST main library was used for identification by matching mass spectral profiles with the profiles in NIST. One quantifying peak (in general the

highest m/z peak per compound) was used per compound for quantification, while 1 or 2 confirming peaks were used for confirmation.

2.6.3. VOC statistics

Every sample was analysed in duplicate and the average was taken of these technical replicates. To identify if compounds were produced by the bacteria, we used the criteria that the quantifying peak area in the retentostat samples had to be higher than the quantifying peak area in the medium. To determine if production of the volatile organic compounds changed in time, linear mixed effect modelling was used to correct for the nested structure of the data: samples were taken at different times from the same cultivation. Therefore, time nested within cultivation was used as random factor. A linear mixed effect model (LME) with time as explanatory variable was compared with a generalised least square model (GLS) by restricted maximum likelihood estimation (REML). A compound was considered to change significantly in time if the p-value for the fixed factor time was lower than 0.00094 (Bonferroni correction; $p < 0.05/53$).

To analyse if compounds were located in the cells, the volatile organic compounds of complete samples and cell-free samples were quantified. Subsequently, paired t-tests were performed for each compound to determine if compounds were significantly more abundant in complete samples. To correct for the large number of compounds, Bonferroni correction was used (p-value $< 0.05/61$).

2.6.4. LogP

The octanol-water partitioning coefficient ($\log P_{oct/wat}$) was either taken from literature (Burkhard et al., 1985; Hansch et al., 1995; Sangster, 1989; Tewari et al., 1982; Valvani et al., 1981; Yamagami et al., 1991) or if not found in literature it was predicted by EPIWEB 4.1 software.

2.7. Cell viability

The viability of cells in the culture was determined by LIVE/DEAD *Bac*light Bacterial Viability kit (Molecular Probes Europe, The Netherlands). To stain the bacteria, 100 μl culture was incubated with 3.34 μM green fluorescent SYTO9 and 20 μM red fluorescent propidium iodide for 10 min at room temperature in the dark. A fluorescent microscope was used for visualisation and estimation of the number of green and red cells. In addition, appropriate dilutions of the culture in peptone physiological salt solution were plated in duplicate on LM17 plates for quantification of colony forming units (CFUs).

2.8. Scanning electron microscopy

The morphology of the cells was analysed by scanning electron microscopy (SEM). Samples were centrifuged (17000×*g* for 1 min), washed with peptone physiological salt solution (PPS) and resuspended in PPS. A droplet of the suspension was placed onto poly-L-lysine coated coverslips (Corning BioCoat, USA) and allowed to stand for 1 h at room temperature. After rinsing in phosphate buffered saline (PBS), the samples were fixed in 3% glutaraldehyde in buffer for 1 h. This was followed by further rinsing and post-fixing in 1% osmium tetroxide in buffer for 1 h. Subsequently, the samples were dehydrated in a graded series of ethanol followed by critical point drying with CO_2 (Leica EM CPD300, Leica Microsystems, Germany). The coverslips were fitted onto sample stubs using carbon adhesive tabs and sputter coated with 10 nm tungsten (Leica SCD500). Samples were imaged at 2 KV, 6 pA, at room temperature in a field emission scanning electron microscope (Magellan 400, FEI Company, USA).

2.9. Protein content

The protein content has been determined by cell lysis using bead-beating followed by protein determination using the Pierce™ Coomassie Plus (Bradford) assay kit (Thermo Scientific, USA). Cells were either harvested at the end of retentostat cultures or from batch cultures in the exponential phase. For the batch cultures, *L. lactis* FM03-V1 was streaked onto a LM17 plate and incubated for 3 days at 30 °C. A single colony was inoculated in chemically-defined medium with lactose and citrate as main carbon sources and grown overnight at 30 °C. One mL was transferred to 14 mL fresh medium and incubated for 3 h at 30 °C. When the optical density at 600 nm reached 0.5–0.6, 5 mL was used to determine the cell dry weight according to the previous described protocol. The remaining sample was centrifuged (6000×*g*, 5 min) and washed three times with 1 mL 0.85% NaCl. The sample was resuspended in 1 mL demineralised water and transferred to a lysis matrix tube (Matrix B, 0.1 mm silica spheres, Bio-Rad). The suspension was bead-beated in six cycles of 30 s beating (6.0 m/s; Bio-Rad Fastprep-24) and 5 min cooling on ice. Subsequently, tubes were centrifuged (17000×*g* for 20 s) and the supernatant was transferred to a new tube and used for the protein determination.

For the retentostat cultivations, cells were harvested at the end of the cultivation (37 days). The biomass concentration was determined as described before. Due to the high biomass concentration only 0.5 mL sample was used. The cells were washed three times with 1 ml 0.85% NaCl and resuspended in 1 mL demineralised water. The rest of the protocol is the same as for the batch cultures described before.

The protein concentration in the suspensions was determined with the Pierce™ Coomassie Plus (Bradford) assay kit (Thermo Scientific, USA) according to the manufacturer's procedure. Bovine serum albumin (BSA) was used as standard. Both standards and samples were analysed in triplicate.

The protein content was determined for three independent batch cultures and two independent retentostat cultures were analysed in triplicate.

2.10. Modelling biomass accumulation for estimation maintenance

The accumulation of biomass was modelled using a modified Verseveld equation (Van Verseveld et al., 1986), in which the metabolic changes resulting in a different ATP yield on substrate were taken into account:

$$C_X(t) = \left(C_{X,0} - \frac{D.(C_{S,in} - C_S)\cdot Y_{ATP/S}}{m_{ATP}}\right).e^{-m_{ATP}\cdot Y^{max}_{x/ATP}\cdot t} + \frac{D.(C_{S,in} - C_S)\cdot Y_{ATP/S}}{m_{ATP}} \quad (1)$$

in which C_X is the biomass concentration (gDW/kg), $C_{X,0}$ is the initial biomass concentration (gDW/kg), D is the dilution rate (h^{-1}), $C_{S,in}$ is the substrate concentration in the medium (14.6 mmol/kg lactose and 10 mmol/kg citrate), C_S is the substrate concentration in the effluent, $Y_{ATP/S}$ is the ATP yield on substrate (mol ATP/CmolS), m_{ATP} is the maintenance coefficient (mol ATP.gDW^{-1}.h^{-1}), $Y^{max}_{X/ATP}$ is the maximum biomass yield on ATP (gDW/mol ATP) and t is the time (h).

The ATP yield on substrate was calculated based on the measured metabolite production with equation (2):

$$Y_{ATP/S} = \frac{R_{lactate} + R_{ethanol} + R_{pyruvate} + 2 \cdot R_{acetoin} + 2 \cdot R_{acetate} + 2 \cdot R_{citrate}}{-(12 \cdot R_{lactose} + 6 \cdot R_{citrate})} \quad (2)$$

in which R_i is the production rate (mol/h) of compound i. Based on chemostat cultivations with and without citrate, it was assumed that uptake of 1 mol citrate and its conversion to pyruvate generated 1 mol ATP.

Input data for the modelling were the online optical density measurements, which were first converted into biomass dry weight concentrations using a second-order polynomial relation (Suppl. Fig. S1).

For fitting the data to equation (1), two different models were used: i) both $Y^{max}_{X/ATP}$ and m_{ATP} were constant and fitted and ii) $Y^{max}_{X/ATP}$ was estimated from chemostat cultivations between 0.05 and 0.4 h^{-1} and the m_{ATP} was linearly-dependent on the biomass specific ATP production rate (q_{ATP}) with a maximum m^{max}_{ATP} (equation (3)).

$$m_{ATP} = a + b \cdot q_{ATP} \; with \; m_{atp} \leq m^{max}_{ATP} \quad (3)$$

For this model, a, b and m^{max}_{ATP} were fitted. Because q_{ATP} and the growth rate are related to each other, both models are equivalent when $m_{ATP} \leq m^{max}_{ATP}$ (Supplement A), although the interpretation is different. The second model represents the situation that the maintenance coefficient changes at very low metabolic activities and thus at very low growth rates, while the first model would assume a different maximum biomass yield in chemostat and retentostat cultures. The variable parameters were optimised by minimising the sum of squared errors between the model and the data. This was done using the solver add-in of Excel.

3. Results

3.1. Biomass accumulation, viability and cell morphology

Lactococcus lactis subsp. *lactis* biovar diacetylactis FM03-V1 was grown anaerobically in retentostat cultivations on a chemically-defined medium containing both lactose and citrate. Two independent cultivations of 37 days were performed to investigate physiological adaptations of this dairy strain towards near-zero growth rates (retentostat 1 and 2). During the cultivations the biomass concentration and the corresponding growth rates were determined every 3–4 days (Fig. 1).

In both cultures the biomass concentration continuously increased and the growth rate gradually decreased till less than 0.001 h^{-1} at the end of the cultivation. This corresponds to a doubling time of more than one month. The culture viability was determined using SYTO9 and propidium iodide (PI) as fluorescent markers combined with fluorescent microscopy. The population was divided into two subpopulations based on the staining characteristics: i) strong green and weak red fluorescence was considered to represent live cells with intact cell membranes, ii) strong red fluorescence was considered to represent damaged or dead cells, of which the cell membrane is permeable to PI. During cultivation 1, the viability, which was calculated by dividing the number of living cells by the total number of cells, gradually decreased from 99 ± 1% to 79 ± 4% (Fig. 2). To determine the number of cells that remain culturable during the cultivations, serial dilutions were plated on LM17 plates. The plate counts hardly increased during the cultivations (Fig. 2), while the biomass concentrations increased 8-fold (Fig. 1). This difference could not be explained by increased chain lengths of the bacteria because the average chain length even decreased approximately 2-fold during the cultivation from 13 to 7 cells per chain (Suppl. Fig. S2). The above observations indicate that a large fraction of the bacteria remained viable but lost culturability on LM17 plates. The morphology of the retentostat-grown cells was examined using scanning electron microscopy (Fig. 3). This showed that the population became more heterogeneous during the cultivations and the fraction of cells with an altered appearance gradually increased. These cells were bigger or longer (more rod-shaped) (Suppl. Fig. S3). Moreover, the surface of these altered cells was rougher than that of fast growing cells and was comparable to the cell surface near the division plane of growing cells (compare cells at t = 0 and t = 37 in Fig. 3).

3.2. Metabolism

Concentrations of the main carbon sources (lactose and citrate) and the main fermentation products (lactate, acetate, ethanol, formate, acetoin and pyruvate) were determined with HPLC. In all cultivations no residual lactose and citrate was detected. Lactose and citrate were mainly converted into a mixture of lactate, formate, acetate and ethanol, but also small amounts of acetoin and pyruvate were detected. The product concentrations were converted into production rates using the effluent rate and compared with the consumption rates of lactose and citrate to determine how

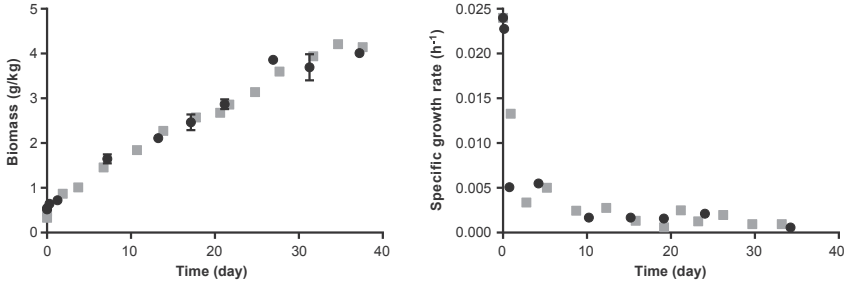

Fig. 1. Growth of *L. lactis* FM03-V1 during retentostat cultivation. Circles and squares represent data points of retentostat cultivation 1 and 2, respectively. At time zero a chemostat culture in steady-state was switched to retentostat mode by insertion of a filter in the effluent. Left: measured biomass concentration; data points represent average ± standard deviation of duplicate samples. Right: Calculated specific growth rates based on cell dry weight measurements.

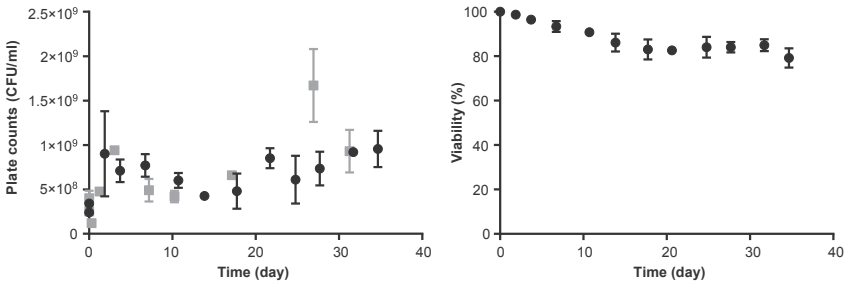

Fig. 2. Culturable cells and viability of *L. lactis* FM03-V1 during retentostat cultivation. At time zero a chemostat culture in steady-state was switched to retentostat mode by insertion of a filter in the effluent. Left: Culturable cells from retentostat 1 (circle) and 2 (square) determined by plating on M17 supplemented with 0.5% lactose. Data points represent average ± standard deviation of duplicate samples. Right: Viability of cells in retentostat cultivation 1 determined using SYTO9 and propidium iodide as fluorescent markers followed by fluorescent microscopy and manual counting. Cells with strong green and weak red fluorescence were considered live and cells with weak green and strong red fluorescence were considered dead. Data points represent the average ± standard deviation of at least five pictures taken from the same sample.

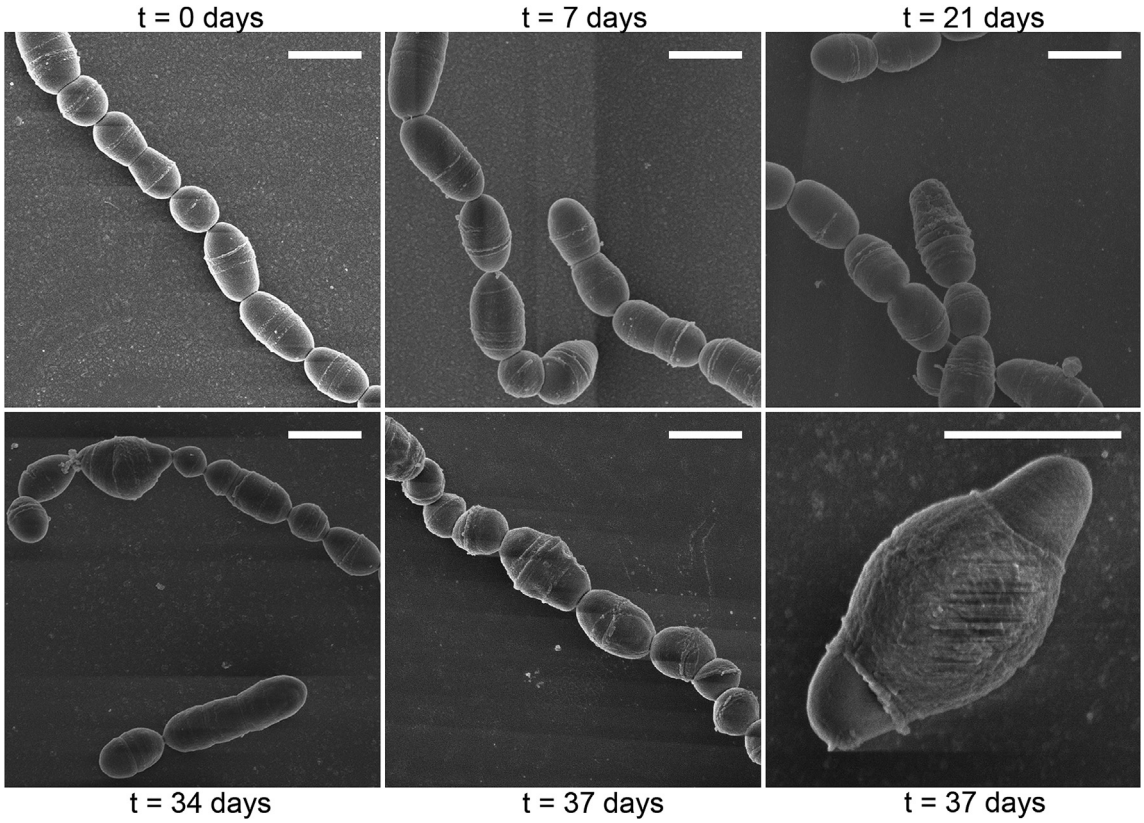

Fig. 3. Morphology of *L. lactis* FM03-V1 during cultivation in retentostat 1 analysed using scanning electron microscopy. Similar morphology was found for cells in retentostat 2. Samples were taken at different time points and stored at −20 °C until used for analysis by scanning electron microscopy. Morphology of cells was not affected by the freezing (data not shown). White bars correspond to 1 μm.

substrate was distributed over the various metabolic pathways of *L. lactis* (Fig. 4). During the first 10 days of retentostat cultivation, lactate production via lactate dehydrogenase relatively increased from approximately 50% to 60–70%, while acetate and ethanol production via acetyl-CoA forming reactions decreased from 40-50%–30%. This distribution remained constant during the rest of the cultivations. During retentostat cultivation 2, acetoin production decreased from 11% to approximately 2%. In contrast, in retentostat cultivation 1 only 2% of the substrate was converted into acetoin throughout the cultivation. In both cultivations formate production rates were low compared to production rates of ethanol and acetate (even after correction for acetate production by citrate lyase). This indicates that pyruvate formate lyase (PFL) was not the only enzyme producing acetyl-CoA and most likely pyruvate dehydrogenase (PDH) was active despite the anaerobic conditions.

3.3. Maintenance requirements

The maximum biomass yield on ATP ($Y^{max}_{X/ATP}$) and the maintenance coefficient (m_{ATP}) were estimated by fitting the biomass accumulation data to a modified Verseveld equation in which changes in metabolism were taken into account (Eq. (1)). Two models were used: i) both $Y^{max}_{X/ATP}$ and m_{ATP} were constant and fitted and ii) $Y^{max}_{X/ATP}$ was constant and estimated from chemostat cultivations, while m_{ATP} was fitted and linearly-dependent on the biomass specific ATP production rate (q_{ATP}) with a maximum m^{max}_{ATP}.

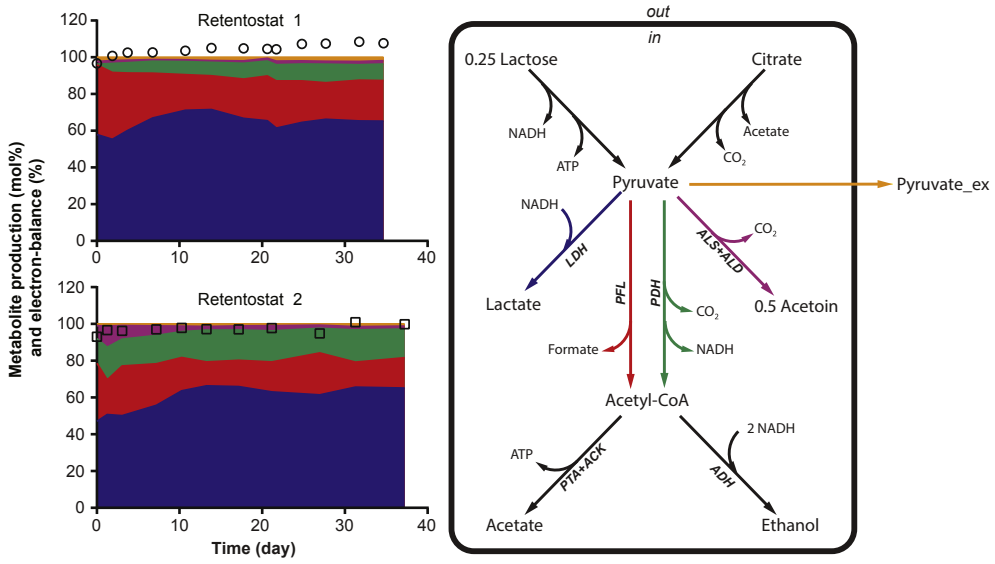

Fig. 4. Metabolite production by *L. lactis* FM03-V1 during retentostat cultivation. Left: Distribution of pyruvate over five different metabolic pathways during retentostat cultivation 1 and 2. The distribution is normalised to 100%. Every colour corresponds to a particular pathway highlighted in the right picture: LDH (purple), PFL (red), PDH (green), ALS (pink), pyruvate efflux (orange). Circles and squares represent the electron-balances of retentostat 1 and 2, respectively, which were calculated based on the degree of reduction of the main substrate and products. At time zero a chemostat culture in steady-state was switched to retentostat mode by insertion of a filter in the effluent. Right: Overview of the central metabolism of *L. lactis* FM03-V1 during growth on lactose and citrate according to stoichiometry. Reactions are normalised based on the production or consumption of 1 pyruvate or 1 acetyl-CoA. LDH: lactate dehydrogenase; PFL: pyruvate formate lyase; PDH: pyruvate dehydrogenase; ALS: acetolactate synthase; ALD: acetolactate decarboxylase; PTA: phosphotransacetylase; ACK: acetate kinase; ADH: alcohol dehydrogenase.

Table 1
Obtained values for maximum specific biomass yield on ATP ($Y^{max}_{X/ATP}$) and maintenance coefficient (m_{ATP}) by fitting of chemostat and retentostat cultivations. The $Y^{max}_{X/ATP}$ and m_{ATP} for the chemostat cultivations were determined in four independent chemostat cultivations (two with 0 and two with 10 mM citrate as co-substrate). In both retentostat cultivations citrate was used as co-substrate. For model 2 the same $Y^{max}_{X/ATP}$ as found in the chemostat cultivation was used. For the maintenance coefficient in model 2, the start and end value during the fermentations are given. The square root of the mean squared error was used to compare how good the models fitted the data. The estimated biomass concentration in the chemostat phase was calculated using equation (4).

		Cultivation	$Y^{max}_{X/ATP}$ (gDW/mol ATP)	m_{ATP} (mmolATP.gDW^{-1}.h^{-1})	$\sqrt{MSE}$ (gDW/kg)	Estimated biomass concentration in chemostat (D = 0.025 h^{-1}) (gDW/kg)
Chemostat[a]		0 mM citrate	14.89 ± 0.61	1.15 ± 0.63	–	–
		10 mM citrate	15.94 ± 0.42	2.43 ± 0.35	–	–
Retentostat	Model 1: $Y^{max}_{X/ATP}$ and m_{ATP} constant fitted parameter	1	2.9	0.19	0.030	0.23
		2	3.9	0.22	0.111	0.27
	Model 2: $Y^{max}_{X/ATP}$ as in chemostat, m_{ATP} q_{ATP}-dependent fitted parameter	1	15.9	2.11 → 0.39	0.028	0.53
		2	15.9	1.30 → 0.33	0.098	0.62

[a] $Y^{max}_{X/ATP}$ and m_{ATP} are −1/slope ± standard error and the intercept ± standard error in a plot of the biomass specific ATP production rate versus the growth rate, respectively (Fig. 5).

The models were compared based on i) how well they fitted the biomass accumulation, ii) the estimated biomass concentrations in the chemostat phase and iii) biological interpretation of changes in $Y^{max}_{X/ATP}$ and m_{ATP}. The biomass concentration in the chemostat was estimated using equation (4) (derivation in Supplement B).

$$C_{x,chemostat} = \frac{D^*\left(C_{s,in} - C_{s,out}\right)\cdot Y_{ATP/S}}{\frac{D}{Y^{max}_{x/ATP}} + m_{ATP}} \quad (4)$$

Both models fitted the biomass accumulation very well, but only for model 2 the obtained $Y^{max}_{X/ATP}$ and m_{ATP} could also predict the biomass concentration in the chemostat phase (~0.6 gDW/kg) (Table 1 and Suppl. Fig. S4). Moreover, model 1 predicted a 4 times lower $Y^{max}_{X/ATP}$ during retentostat cultivation compared to chemostat cultivation, which is unlikely because the $Y^{max}_{X/ATP}$ is assumed to be a constant growth parameter when using the same medium and environmental conditions. In contrast, model 2 assumed the same $Y^{max}_{X/ATP}$ as in chemostat cultivations and predicted that the maintenance requirement gradually decreased and approached a minimum of 0.36 ± 0.03 mmol ATP.gDW^{-1}.h^{-1}. It has been suggested that microorganisms can adapt to situations of extreme calorie restriction and lower their maintenance requirements (Kempes et al., 2017). We conclude that model 2 better describes the physiological changes during retentostat cultivation, which suggests that the maintenance coefficient of our *L. lactis* strain decreases at near-zero growth rates.

Using model 2, the growth rate was predicted and plotted against the biomass specific ATP production rate (q_{ATP}) and compared with data from chemostat cultivations used to estimate the $Y^{max}_{X/ATP}$ and m_{ATP} (Fig. 5). This shows that these estimates are

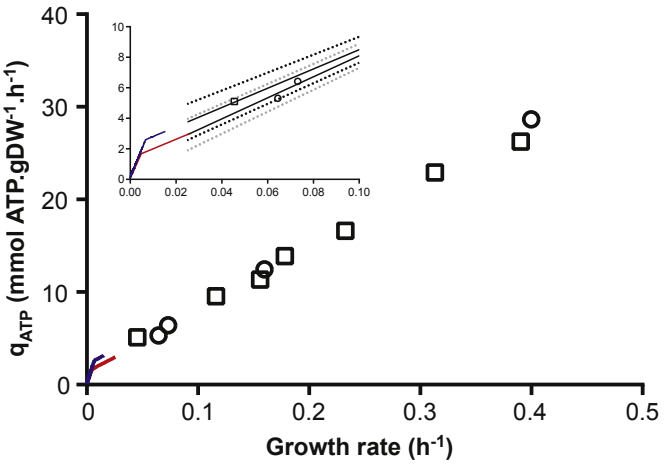

Fig. 5. Relation between the specific growth rate and the biomass specific ATP production rate (q_{ATP}) in chemostat and retentostat cultivations. Data from chemostat cultivations with and without citrate as co-substrate are represented by squares and circles, respectively. The black lines in the inset represent linear regression lines of the chemostat data points used to estimate the maximum biomass yield on ATP and the maintenance coefficient. Dotted lines in the inset represent the 95% confidence intervals of the linear regression lines. The biomass specific ATP production rate and growth rate in retentostat cultivations 1 and 2 were calculated based on biomass predictions of model 2 and shown by blue and red lines, respectively. Axis titles in the inset are the same as the main figure.

only valid at growth rates above approximately 0.01 h^{-1}. At near-zero growth rates, the ATP required for growth is significantly lower than predicted from the chemostat cultivations due to the decreasing maintenance requirements.

3.4. Maintenance components

Kempes et al. (2017) determined for a wide range of cell sizes what the major components of the maintenance requirements are. For *L. lactis* with a volume of about 5.10^{-19} m^3 (sphere with a diameter of 1 μm) it is expected that protein repair and maintaining trans-membrane proton gradients are the major constituents of maintenance and other components, like RNA turnover, play a minor role. Due to the smaller surface to volume ratio in bigger cells, proton leakage is generally smaller in bigger cells. Both in this study and in retentostat studies with the plant-associated *L. lactis* KF147 the cell size increased (Ercan et al., 2013). However, cell size decreased for microorganisms upon starvation (Lever et al., 2015). This suggests a different response towards starvation and near-zero growth conditions.

One way for a cell to minimise its energy need for protein repair is to decrease its protein content. The protein content was determined at the end of the retentostat cultivations and compared with the protein content of exponential growing cells. The protein content was not significantly different between fast and slow growing cell cultures suggesting that the bacteria adapted in a different way to extreme calorie restriction (Fig. 6).

The *in vivo* utilisation capacity of lactose and citrate of retentostat-grown cells was compared with exponentially-grown cells to see adaptation at the level of enzyme capacities. The lactose and citrate utilisation capacity were 11 and 18-fold lower in the retentostat-grown cells, respectively (Fig. 6). This suggests that the bacteria might have adapted to the extreme calorie restriction by reducing the repair and/or replacement of damaged proteins resulting in a similar protein content but less functional proteins.

3.5. Aroma formation during retentostat cultivation

During the retentostat cultivations, samples were taken to monitor the formation of volatile organic compounds (VOCs) using headspace solid phase microextraction gas chromatography (HS SPME GC-MS). Samples were analysed without removal of cells because particular aromas might be located in the cells due to their hydrophobic nature. In total 62 compounds were identified. Comparison of peak areas of each compound in retentostat samples and medium samples, revealed that 56 compounds were higher in concentration in the retentostat samples and therefore considered to be produced during the cultivation. The produced compounds were assigned to different groups of chemicals: acids, alcohols, aldehydes, ketones, esters, sulphur-containing compounds, pyrazines, and other compounds (Suppl. Table S1, Fig. 7). The compounds mainly originated from primary metabolism and the degradation of amino acids (methionine, isoleucine, leucine, phenylalanine, threonine and tyrosine) and fatty acids.

The abundance of 14 compounds significantly changed in time and thus were affected by the low growth rates. These compounds include products of amino acid degradation (benzaldehyde, benzeneacetyladehyde, 3-(methylthio)-propanal and acetophenone), products of degradation of medium and long chain fatty acids (octanoic acid, 2-heptanone, tridecanal, 1-tetradecanol, 1-hexadecanol and decanoic acid, ethyl ester), and some other compounds (3-methyl-2-butenal, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 3-methyl-1-hexanol). All of these compounds increased in time (Fig. 8), which suggests that production of these compounds increases in cells that are growing very slowly.

Fat-derived compounds play a crucial role to achieve a balanced cheese flavour (Urbach, 1993). Although milk fat and fatty acids were not present in the chemically-defined medium, some fat-

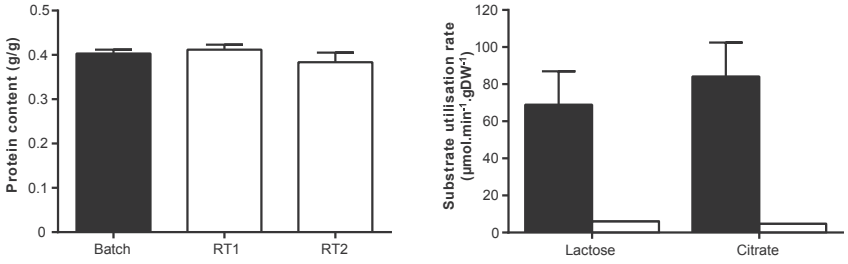

Fig. 6. Protein content and substrate utilisation rate of *L. lactis* FM03-V1 grown on chemically-defined medium with citrate in either batch (filled bars) or retentostat cultures (open bars). Cells were grown in three independent batch cultures and were harvested in exponential phase. For the retentostat cultivations, cells were harvested at the end of the cultivation (37 days). Left: Protein content. RT1 and RT2 represent retentostat cultivation 1 and 2, respectively. Error bars represent the standard deviation of triplicate measurements. Right: Substrate utilisation rates of lactose and citrate. Only retentostat culture 1 has been analysed. The error bars represent the standard deviation of biological triplicates.

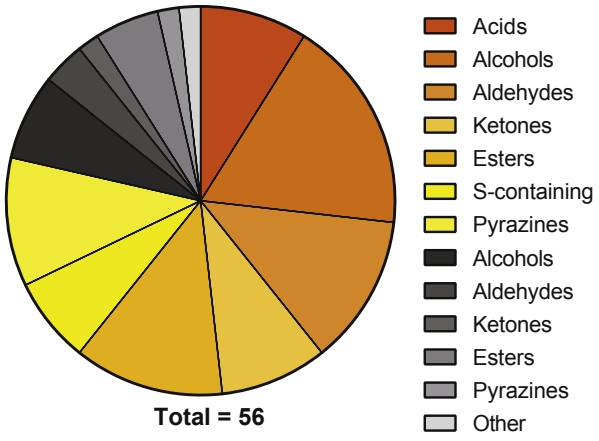

Fig. 7. Composition of the volatile organic compounds produced by *L. lactis* during retentostat cultivations. Every pie sector corresponds to a group of compounds (Suppl. Table 1) and the colour represents if the group is found in cheese (orange to yellow) or not (grey). Five studies describing aromas in various cheeses, including Gouda, Cheddar, Parmigiano-Reggiano and Parmasan, were selected for comparison (Barbieri et al., 1994; Curioni and Bosset, 2002; Qian and Reineccius, 2002; Singh et al., 2003; Van Leuven et al., 2008).

related compounds were produced (Suppl. Table S1) and some even increased during the cultivations (tridecanal, 1-tetradecanol, 1-hexadecanol and decanoic acid, ethyl ester) (Fig. 8). These compounds might originate from the turnover or degradation of phospholipids of the cell membrane. Interestingly, these compounds were hardly detected in cell-free samples in which cells were removed by centrifugation (data not shown). This shows that these compounds were associated with cells.

3.6. Partitioning of VOCs

To get further insight in the partitioning of compounds, we compared the VOC profiles of complete samples (cells and extracellular liquid) and cell-free samples (only extracellular liquid; cells removed by centrifugation). Sixteen compounds were significantly more abundant in the complete samples (Suppl. Fig. S5) indicating that significant fractions of these compounds were located in the cells. More hydrophobic compounds were relatively more abundant in the cells (Fig. 9) suggesting that the hydrophobic compounds predominantly accumulated in the cell membrane. Because cells were retained in the retentostat cultivation, also these aromas were retained, which explains why some fat-related compounds increased in time (Fig. 8).

4. Discussion

4.1. Physiology of L. lactis at near-zero growth rates

The physiology of a dairy *Lactococcus lactis* subsp. *lactis* biovar diacetylactis growing at near-zero growth rates has been investigated by inducing extreme carbon and energy limitation using retentostat cultivation. Biomass accumulation, culturability, viability, morphology and metabolite production were analysed revealing physiological adaptations to near-zero growth rates. Moreover this is, to the best of our knowledge, the first study of

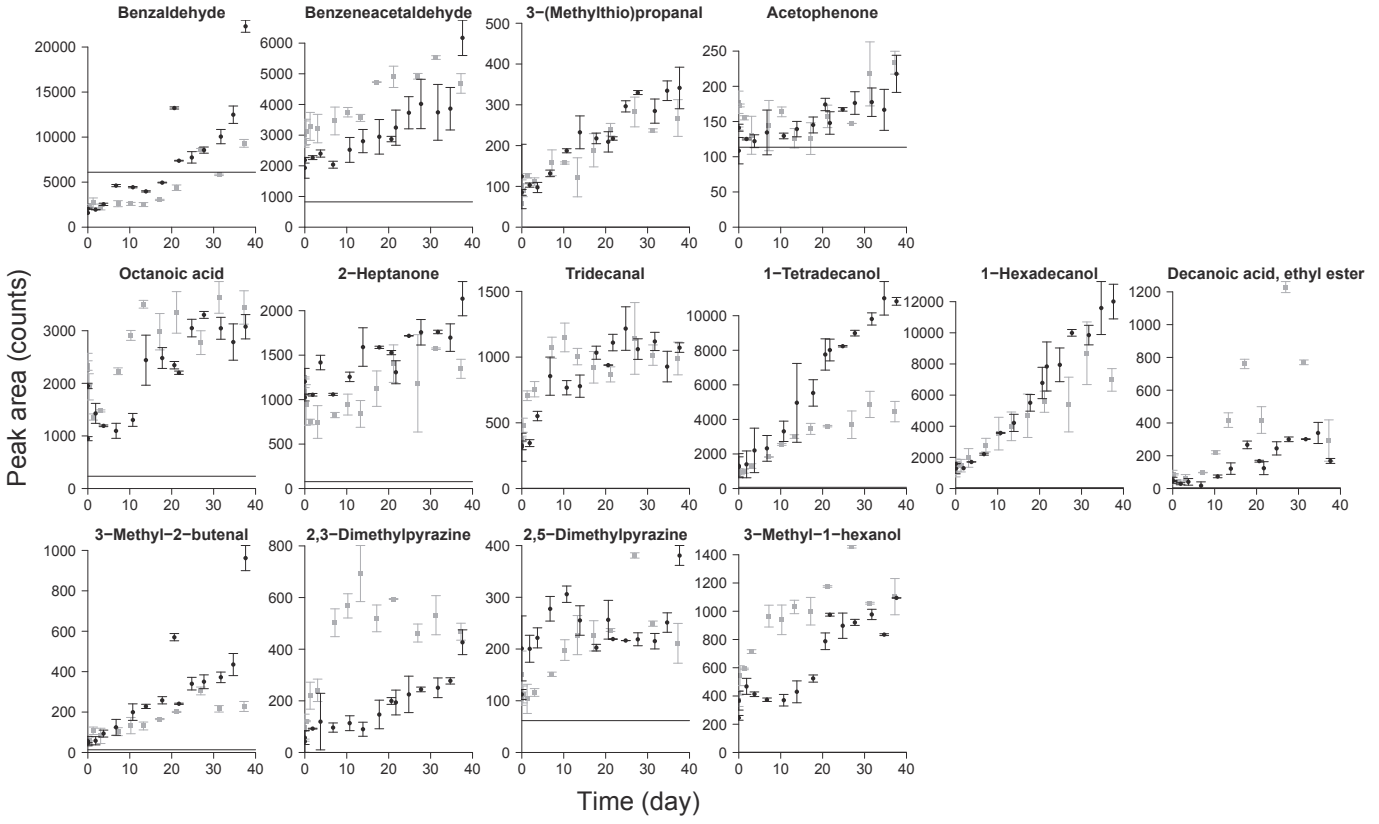

Fig. 8. Area of the quantitative peak of volatile organic compounds during retentostat cultivations of *L. lactis* FM03-V1. Only compounds that changed significantly in time are shown. Circles and squares represent retentostat cultivations 1 and 2, respectively. The black lines represent the abundance of the compounds in the chemically-defined medium. Error bars represent the standard deviation of technical duplicates.

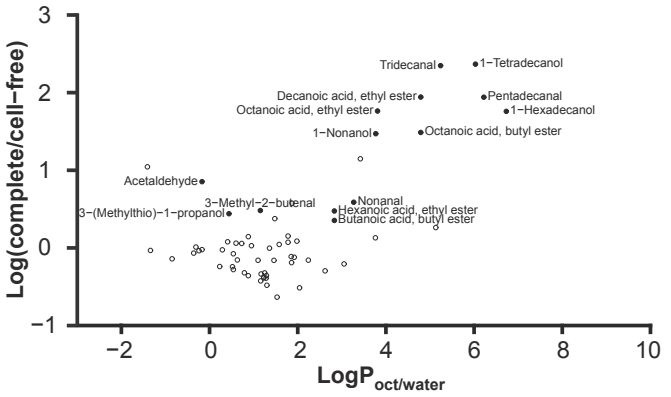

Fig. 9. Abundance of compounds in complete samples and cell-free samples as function of their hydrophobicity ($logP_{oct/water}$). Filled circles represent compounds that were significantly more abundant in complete samples and their names are given. Open circles represent compounds that were not significantly more abundant in complete samples. Data of both cultivations was taken into account by plotting the abundances in complete and cell-free samples (Suppl. Fig. S5), calculating the slopes and taking the logarithm of these slopes.

aroma formation during the retentostat cultivations revealing changes in the secondary metabolism of this bacteria upon extreme calorie restriction.

During 37 days of retentostat cultivation, the biomass concentration increased 8-fold while the growth rate decreased till less than 0.001 h^{-1}. Compared to the plant-associated *L. lactis* KF147, biomass accumulated much slower and no plateau was reached within 37 days. This was most likely caused by adaptation of the strain to the near-zero growth rates resulting in decreased maintenance costs. Decreasing maintenance costs during retentostat cultivation has also been described for *Pichia pastoris* and *Pseudomonas putida* (Panikov et al., 2015; Rebnegger et al., 2016). After 37 days of retentostat cultivation, the estimated maintenance coefficient of *L. lactis* FM03-V1 was approximately 7-fold lower than the value observed during chemostat cultivation and was similar to the maintenance coefficient of *L. lactis* KF147 (1.04 ± 0.07 and 1.11 ± 0.04 mCmolS.$gDW^{-1}.h^{-1}$, respectively) (Ercan et al., 2013). Kempes et al. (2017). combined data about maintenance costs from a very broad range of organisms and concluded that protein repair and proton leakage are the main components of the maintenance costs for organisms with a diameter of approximately 1 μm. Lahtvee et al. (2014). measured protein turnover rates in *L. lactis* and showed that protein turnover accounts for the highest maintenance costs in *L. lactis*. Moreover, they showed that the protein turnover rate decreases at lower growth rates. In our study the capacity to utilise lactose and citrate significantly decreased in retentostat-grown cells compared to exponential growing cells, while the protein content remained constant. In a retentostat study with *Lactobacillus plantarum* the protein content also remained constant (Goffin et al., 2010). We speculate that the bacteria might have adapted to the nutrient limitation by decreasing the protein turnover (i.e. repair and replacement of damaged proteins). This would result in less functional proteins (lower capacity), while the amount of proteins will remain the same (same protein content) and could greatly reduce the energy required for protein repair. Such response has also been observed for *Bacillus subtilis* and *Saccharomyces cerevisiae*, which showed a reduced expression of the translational machinery during retentostat cultivation (Boender et al., 2011; Overkamp et al., 2015).

Comparing the yield and maintenance coefficient of *L. lactis* FM03-V1 to other strains revealed that this strain, when grown in chemostat cultures, has very similar growth parameters as other dairy *L. lactis* strains (Adamberg et al., 2003; Meghrous et al., 1992; Otto et al., 1980; Ten Brink et al., 1985; Thomas et al., 1979). The low maximum biomass yield in combination with the high maintenance costs of dairy strains suggests adaptation towards the nutrient-rich dairy environment. In contrast, the plant-associated environment is commonly classified as a poor condition for microbial growth due to low pH, high osmolarity and/or low nutrient conditions and therefore the plant-isolated *L. lactis* KF147 may have evolved to these harsh environments by minimising its maintenance costs (Ercan et al., 2013).

During the cultivations, a significant fraction of the population lost the ability to grow on LM17 plates, while maintaining a high viability as determined by staining with SYTO9 and propidium iodide. The increase in viable but non-culturable cells (VBNC) has also been observed for *Pseudomonas putida* during retentostat cultivation and was linked to a decreased ribosome content in VBNC cells (Panikov et al., 2015). Also during cheese ripening *L. lactis* loses the capability to grow on M17 plates, while remaining its transcriptional activity (Desfossés-Foucault et al., 2013; Ruggirello et al., 2014, 2016). The relative increase in VBNC cells during cheese ripening was also found by quantification of culturable cells (by plate counting) and viable cells (using qPCR with a propidium monoazide treatment) (Erkus et al., 2013, 2016).

Based on the metabolite production profile, we concluded that despite the anaerobic conditions pyruvate dehydrogenase was active and responsible for part of the conversion of pyruvate into acetyl-CoA. In *L. lactis*, pyruvate dehydrogenase (PDH) is highly sensitive for the NADH/NAD ratio, which prevents *in vivo* activity under aerobic conditions (Condon, 1987; Snoep et al., 1992, 1993), but apparently a small activity remained under anaerobic conditions. The highest flux through PDH was 0.26 mmol.$gDW^{-1}.h^{-1}$ which is lower than found as *in vitro* activity for PDH for *L. lactis* MG1363 grown under anaerobic conditions at a dilution rate of 0.1 h^{-1} (Jensen et al., 2001). This indicates that PDH activity was in absolute terms not high in this experiment, but relative abundant because all other metabolic fluxes are also very low in retentostat cultivation. This allows relatively low metabolic fluxes to contribute significantly to the overall metabolism during retentostat cultivation.

4.2. Aroma formation in retentostat cultures resembles aroma formation during cheese ripening

Volatile organic compounds (VOCs) were analysed to detect changes in the secondary metabolism of the bacterium and to compare aroma formation at near-zero growth rates and during cheese ripening. Of the VOCs that were produced during the retentostat cultivations, 79% can be found in ripened cheese (Suppl. Table S1). Production of particular aroma compounds increased at near-zero growth rates including some specific cheese flavours thereby resembling reactions occurring during cheese ripening.

Degradation of amino acids is a major source of flavour compounds in cheese (Yvon and Rijnen, 2001). In the retentostat cultivations in total 12 amino acid degradation products were identified, which could originate from degradation of phenylalanine, tyrosine, threonine, methionine, leucine and isoleucine. The abundance of some of these products increased significantly in time (benzaldehyde, benzeneacetaldehyde, 3-(methylthio)propanal and acetophenone) indicating that production of these compounds was higher in a culture growing very slowly. Although products of branched chain amino acids degradation are important flavours produced by *L. lactis* during cheese ripening, production of these compounds was limited. This can be explained by the large deletion in the gene encoding the branched chain α-keto acid

decarboxylase (*kdcA*) in *L. lactis* FM03-V1 as also described for *L. lactis* IL1403 (Smit et al., 2005a).

Ketones are common constituents of cheese and especially methyl ketones are known for their contribution to the aroma of blue-veined and surface mould-ripened and cheese (Curioni and Bosset, 2002). 2-Heptanone has a blue cheese note and its formation in surface-ripened cheese has been related to the enzymatic activity of moulds, which convert octanoic acid into 2-heptanone (Gehrig and Knight, 1961). In the retentostat cultures both octanoic acid and 2-heptanone increased in time suggesting that also *L. lactis* is able to produce 2-heptanone and that production of 2-heptanone is increased in cells growing at near-zero growth rates. During retentostat cultivation 1, 2-butanone increased 9-fold, while the acetoin concentration remained low (Suppl. Fig. S6). Acetoin might have been continuously produced, but further converted into 2-butanone resembling reactions during cheese ripening (Keen et al., 1974). This would also explain the higher abundance of 2-butanone in retentostat 2 in which slightly more acetoin was produced at the onset of the cultivation (Suppl. Fig. S6).

During the retentostat cultivations several ethyl and butyl esters increased in time. Butyl esters can be formed by condensation of a carboxylic acid with 1-butanol, which was present in high concentrations in the medium as part of the bacto-tryptone, and are generally not found in cheese. Most common in cheese are ethyl esters of straight-chain fatty acids of C2-C10, which could be formed enzymatically by the bacteria by condensation of ethanol and a carboxylic acid (Curioni and Bosset, 2002; Liu et al., 2004b). These ethyl esters are responsible for the fruitiness of Italian-type cheeses, but at high concentrations they result in a fruity off-flavour. In general, ethanol is the limiting factor in ester synthesis in cheese (Liu et al., 2004b; Thierry et al., 2006). In contrast, the ethanol concentration in the retentostat cultures was high due to the mixed acid fermentation behaviour of the culture, which resulted in formation of various esters. In *L. lactis* FM03-V1 the *estA* gene encodes an alcohol acyltransferase, which might be responsible for the ester formation (Liu et al., 2004a; Nardi et al., 2002).

In all retentostat cultivations 3-methyl-2-butenal increased in time. The odour of this compound has been described as almond (Burdock, 2010) and as cheese, fruity and green (Curioni and Bosset, 2002) and this compound is sometimes found in cheese (Gogus et al., 2006; Majcher et al., 2010, 2011) and yoghurt (Cheng, 2010). Based on metabolism of *L. lactis*, we speculate that this compound is chemically produced from dimethylallyl pyrophosphate (DMAPP), which is the end-product of the mevalonate pathway required for isoprenoid synthesis. Therefore, the increase in 3-methyl-2-butenal suggests accumulation of DMAPP caused by the near-zero growth rates.

Several pyrazines were produced during the retentostat cultivations. Pyrazines have an earthy, roasty odour and are important contributors to cheese flavour, especially in Cheddar and Gruyère (Curioni and Bosset, 2002). The pyrazines found in this study can be linked to enzymatic reactions involving diketones, such as 2,3-butanedione (diacetyl) and 2,3-pentanedione (Dickschat et al., 2010).

Although fat and fatty acids were not present in the chemically-defined medium, some fat-related compounds were produced and even increased during the retentostat cultivations. Most likely these compounds originated from the turnover of phospholipids of the cell membrane or from degradation of phospholipids of dead cells. Most of the fat-related compounds are hydrophobic and therefore accumulate in the lipid bilayer of the cytoplasmic membrane. Because cells were retained in the bioreactor, also these hydrophobic compounds were largely retained and also accumulated in time.In conclusion, this study shows that retentostat cultivation is a unique tool to study microorganisms under industrially relevant conditions of slow growth. Physiological responses of *L. lactis* subsp. *lactis* biovar diacetylactis in the retentostat cultivations show similarities with the *in situ* behaviour of this lactic acid bacterium during cheese ripening e.g. viable but non-culturable cells and characteristic aroma formation. The similarities in aroma formation between lactococcal cells captured in the cheese matrix and cells in a retentostat bioreactor, indicates that retentostat cultivation might not only be used as study platform, but also as production platform for specific cheese aromas.

Acknowledgement

This work was supported by Arla Foods (Aarhus, Denmark). We thank Judith Wolkers-Rooijackers and Irma van Rijswijck for their assistance in the GC-MS analysis. We thank Marcel Giesbers (Wageningen Electron Microscopy Centre, Wageningen University & Research) for his assistance in SEM.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.027.

References

Adamberg, K., Kask, S., Laht, T.-M., Paalme, T., 2003. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. Int. J. Food Microbiol. 85, 171–183. https://doi.org/10.1016/S0168-1605(02)00537-8.

Barbieri, G., Bolzoni, L., Careri, M., Mangia, A., Parolari, G., Spagnoli, S., Virgili, R., 1994. Study of the volatile fraction of Parmesan cheese. J. Agric. Food Chem. 42, 1170–1176. https://doi.org/10.1021/jf00041a023.

Boender, L.G.M., de Hulster, E.A.F., van Maris, A.J.A., Daran-Lapujade, P.A.S., Pronk, J.T., 2009. Quantitative physiology of *Saccharomyces cerevisiae* at near-zero specific growth rates. Appl. Environ. Microbiol. 75, 5607–5614. https://doi.org/10.1128/Aem.00429-09.

Boender, L.G.M., van Maris, A.J.A., de Hulster, E.A.F., Almering, M.J.H., van der Klei, I.J., Veenhuis, M., de Winde, J.H., Pronk, J.T., Daran-Lapujade, P., 2011. Cellular responses of *Saccharomyces cerevisiae* at near-zero growth rates: transcriptome analysis of anaerobic retentostat cultures. FEMS Yeast Res. 11, 603–620. https://doi.org/10.1111/j.1567-1364.2011.00750.x.

Brock, T.D., 1971. Microbial growth rates in nature. Bacteriol. Rev. 35, 39–58.

Burdock, G.A., 2010. Fenaroli's Handbook of Flavor Ingredients, sixth ed. CRC Press, Boca Raton, USA.

Burkhard, L.P., Kuehl, D.W., Veith, G.D., 1985. Evaluation of reverse phase liquid chromatography/mass spectrometry for estimation of n-octanol/water partition coefficients for organic chemicals. Chemosphere 14, 1551–1560. https://doi.org/10.1016/0045-6535(85)90010-4.

Cheng, H., 2010. Volatile flavor compounds in yogurt: a review. Crit. Rev. Food Sci. Nutr. 50, 938–950. https://doi.org/10.1080/10408390903044081.

Condon, S., 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol. Lett. 46, 269–280. https://doi.org/10.1016/0378-1097(87)90112-1.

Curioni, P.M.G., Bosset, J.O., 2002. Key odorants in various cheese types as determined by gas chromatography-olfactometry. Int. Dairy J. 12, 959–984. https://doi.org/10.1016/S0958-6946(02)00124-3.

Desfossés-Foucault, É., LaPointe, G., Roy, D., 2013. Dynamics and rRNA transcriptional activity of lactococci and lactobacilli during Cheddar cheese ripening. Int. J. Food Microbiol. 166, 117–124. https://doi.org/10.1016/j.ijfoodmicro.2013.06.022.

Dickschat, J.S., Wickel, S., Bolten, C.J., Nawrath, T., Schulz, S., Wittmann, C., 2010. Pyrazine biosynthesis in *Corynebacterium glutamicum*. Eur. J. Org Chem. 2010, 2687–2695. https://doi.org/10.1002/ejoc.201000155.

Engels, W.J.M., Dekker, R., de Jong, C., Neeter, R., Visser, S., 1997. A comparative study of volatile compounds in the water-soluble fraction of various types of ripened cheese. Int. Dairy J. 7, 255–263. https://doi.org/10.1016/S0958-6946(97)00003-4.

Ercan, O., Bisschops, M.M.M., Overkamp, W., Jorgensen, T.R., Ram, A.F., Smid, E.J., Pronk, J.T., Kuipers, O.P., Daran-Lapujade, P., Kleerebezem, M., 2015a. Physiological and transcriptional responses of different industrial microbes at near-zero specific growth rates. Appl. Environ. Microbiol. 81, 5662–5670. https://doi.org/10.1128/Aem.00944-15.

Ercan, O., Smid, E.J., Kleerebezem, M., 2013. Quantitative physiology of *Lactococcus lactis* at extreme low-growth rates. Environ. Microbiol. 15, 2319–2332. https://doi.org/10.1111/1462-2920.12104.

Ercan, O., Wels, M., Smid, E.J., Kleerebezem, M., 2015b. Molecular and metabolic adaptations of *Lactococcus lactis* at near-zero growth rates. Appl. Environ. Microbiol. 81, 320–331. https://doi.org/10.1128/Aem.02484-14.

Erkus, O., de Jager, V.C.L., Geene, R.T.C.M., van Alen-Boerrigter, I., Hazelwood, L., van Hijum, S.A.F.T., Kleerebezem, M., Smid, E.J., 2016. Use of propidium monoazide for selective profiling of viable microbial cells during Gouda cheese ripening. Int. J. Food Microbiol. 228, 1–9. https://doi.org/10.1016/j.ijfoodmicro.2016.03.027.

Erkus, O., de Jager, V.C.L., Spus, M., van Alen-Boerrigter, I.J., van Rijswijck, I.M.H., Hazelwood, L., Janssen, P.W.M., van Hijum, S.A.F.T., Kleerebezem, M., Smid, E.J., 2013. Multifactorial diversity sustains microbial community stability. ISME J. 7, 2126–2136. https://doi.org/10.1038/ismej.2013.108.

Gehrig, R.F., Knight, S.G., 1961. Formation of 2-heptanone from caprylic acid by spores of various filamentous fungi. Nature 192, 1185–1185. https://doi.org/10.1038/1921185a0.

Goffin, P., van de Bunt, B., Giovane, M., Leveau, J.H.J., Höppener-Ogawa, S., Teusink, B., Hugenholtz, J., 2010. Understanding the physiology of *Lactobacillus plantarum* at zero growth. Mol. Syst. Biol. 6. https://doi.org/10.1038/msb.2010.67.

Gogus, F., Ozel, M.Z., Lewis, A.C., 2006. Analysis of the volatile components of Cheddar cheese by direct thermal desorption-GC×GC-TOF/MS. J. Sep. Science 29, 1217–1222. https://doi.org/10.1002/jssc.200500400.

Hansch, C., Leo, A., Hoekman, D., 1995. Exploring QSAR: Hydrophobic, Electronic, and Steric Constants. American Chemical Society, Washington, DC.

Harvey, R.J., Collins, E.B., 1961. Role of citritase in acetoin formation by *Streptococcus diacetilactis* and *Leuconostoc citrovorum*. J. Bacteriol. 82, 954–959.

Hoehler, T.M., Jorgensen, B.B., 2013. Microbial life under extreme energy limitation. Nat. Rev. Microbiol. 11, 83–94. https://doi.org/10.1038/nrmicro2939.

Jensen, N.B.S., Melchiorsen, C.R., Jokumsen, K.V., Villadsen, J., 2001. Metabolic behavior of *Lactococcus lactis* MG1363 in microaerobic continuous cultivation at a low dilution rate. Appl. Environ. Microbiol. 67, 2677–2682. https://doi.org/10.1128/aem.67.6.2677-2682.2001.

Keen, A.R., Walker, N.J., Peberdy, M.F., 1974. The formation of 2-butanone and 2-butanol in Cheddar cheese. J. Dairy Res. 41, 249–257. https://doi.org/10.1017/s002202990001966x.

Kempes, C.P., van Bodegom, P.M., Wolpert, D., Libby, E., Amend, J., Hoehler, T., 2017. Drivers of bacterial maintenance and minimal energy requirements. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00031.

Koch, A.L., 1971. The adaptive responses of *Escherichia coli* to a feast and famine existence. Adv. Microb. Physiol. 6, 147–217. https://doi.org/10.1016/s0065-2911(08)60069-7.

Lahtvee, P.-J., Seiman, A., Arike, L., Adamberg, K., Vilu, R., 2014. Protein turnover forms one of the highest maintenance costs in *Lactococcus lactis*. Microbiology 160, 1501–1512. https://doi.org/10.1099/mic.0.078089-0.

Lever, M.A., Rogers, K.L., Lloyd, K.G., Overmann, J., Schink, B., Thauer, R.K., Hoehler, T.M., Jorgensen, B.B., 2015. Life under extreme energy limitation: a synthesis of laboratory- and field-based investigations. FEMS Microbiol. Rev. 39, 688–728. https://doi.org/10.1093/femsre/fuv020.

Liu, S.-Q., Baker, K., Bennett, M., Holland, R., Norris, G., Crow, V.L., 2004a. Characterisation of esterases of *Streptococcus thermophilus* ST1 and *Lactococcus lactis* subsp. *cremoris* B1079 as alcohol acyltransferases. Int. Dairy J. 14, 865–870. https://doi.org/10.1016/j.idairyj.2004.02.014.

Liu, S.-Q., Holland, R., Crow, V.L., 2004b. Esters and their biosynthesis in fermented dairy products: a review. Int. Dairy J. 14, 923–945. https://doi.org/10.1016/j.idairyj.2004.02.010.

Majcher, M., Ławrowski, P., Jeleń, H., 2010. Comparison of original and adulterated Oscypek cheese based on volatile and sensory profiles. Acta Sci. Pol. Technol. Aliment 9, 265–275.

Majcher, M.A., Goderska, K., Pikul, J., Jeleń, H.H., 2011. Changes in volatile, sensory and microbial profiles during preparation of smoked ewe cheese. J. Sci. Food Agric. 91, 1416–1423. https://doi.org/10.1002/jsfa.4326.

Martley, F.G., Crow, V.L., 1993. Interactions between non-starter microorganisms during cheese manufacture and repening. Int. Dairy J. 3, 461–483. https://doi.org/10.1016/0958-6946(93)90027-W.

McSweeney, P.L.H., Fox, P.F., 2004. Metabolism of Residual Lactose and of Lactate and Citrate, Cheese: Chemistry, Physics and Microbiology. Academic Press, pp. 361–371. https://doi.org/10.1016/S1874-558X(04)80074-5.

Meghrous, J., Huot, E., Quittelier, M., Petitdemange, H., 1992. Regulation of nisin biosynthesis by continuous cultures and by resting cell of *Lactococcus lactis* subsp. *lactis*. Res. Microbiol. 143, 879–890. https://doi.org/10.1016/0923-2508(92)90075-Y.

Morita, R.Y., 1993. Bioavailability of energy and the starvation state. In: Kjelleberg, S. (Ed.), Starvation in Bacteria. Springer US, Boston, MA, pp. 1–23. https://doi.org/10.1007/978-1-4899-2439-1_1.

Morita, R.Y., 1997. Bacteria in Oligotrophic Environments: Starvation-survival Lifestyle. Chapman & Hall, New York. https://doi.org/10.4319/lo.1998.43.5.1021.

Nardi, M., Fiez-Vandal, C., Tailliez, P., Monnet, V., 2002. The EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters in vitro. J. Appl. Microbiol. 93, 994–1002. https://doi.org/10.1046/j.1365-2672.2002.01793.x.

Otto, R., Sonnenberg, A.S., Veldkamp, H., Konings, W.N., 1980. Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. Proc. Natl. Acad. Sci. 77, 5502–5506. https://doi.org/10.1073/pnas.77.9.5502.

Overkamp, W., Ercan, O., Herber, M., van Maris, A.J.A., Kleerebezem, M., Kuipers, O.P., 2015. Physiological and cell morphology adaptation of *Bacillus subtilis* at near-zero specific growth rates: a transcriptome analysis. Environ. Microbiol. 17, 346–363. https://doi.org/10.1111/1462-2920.12676.

Panikov, N.S., Mandalakis, M., Dai, S., Mulcahy, L.R., Fowle, W., Garrett, W.S., Karger, B.L., 2015. Near-zero growth kinetics of *Pseudomonas putida* deduced from proteomic analysis. Environ. Microbiol. 17, 215–228. https://doi.org/10.1111/1462-2920.12584.

Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. Proc. R. Soc. Lond. B Biol. Sci. 163, 224–231. https://doi.org/10.1098/rspb.1965.0069.

Qian, M., Reineccius, G., 2002. Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. J. Dairy Sci. 85, 1362–1369. https://doi.org/10.3168/jds.S0022-0302(02)74202-1.

Rebnegger, C., Vos, T., Graf, A.B., Valli, M., Pronk, J.T., Daran-Lapujade, P., Mattanovich, D., 2016. *Pichia pastoris* exhibits high viability and a low maintenance energy requirement at near-zero specific growth rates. Appl. Environ. Microbiol. 82, 4570–4583. https://doi.org/10.1128/aem.00638-16.

Ruggirello, M., Cocolin, L., Dolci, P., 2016. Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models. Food Microbiol. 59, 112–118. https://doi.org/10.1016/j.fm.2016.05.001.

Ruggirello, M., Dolci, P., Cocolin, L., 2014. Detection and viability of *Lactococcus lactis* throughout cheese ripening. PLoS One 9, e114280. https://doi.org/10.1371/journal.pone.0114280.

Sangster, J., 1989. Octanol-water partition coefficients of simple organic compounds. J. Phys. Chem. Ref. Data 18, 1111–1229. https://doi.org/10.1063/1.555833.

Singh, T.K., Drake, M.A., Cadwallader, K.R., 2003. Flavor of Cheddar cheese: a chemical and sensory perspective. Compr. Rev. Food Sci. Food Saf. 2, 166–189. https://doi.org/10.1111/j.1541-4337.2003.tb00021.x.

Smid, E.J., Kleerebezem, M., 2014. Production of aroma compounds in lactic fermentations. Annu. Rev. Food Sci. Technol 5, 313–326. https://doi.org/10.1146/annurev-food-030713-092339.

Smit, B.A., Vlieg, J.E.T.V., Engels, W.J.M., Meijer, L., Wouters, J.T.M., Smit, G., 2005a. Identification, cloning, and characterization of a *Lactococcus lactis* branched-chain alpha-keto acid decarboxylase involved in flavor formation. Appl. Environ. Microbiol. 71, 303–311. https://doi.org/10.1128/aem.71.1.303-311.2005.

Smit, G., Smit, B.A., Engels, W.J.M., 2005b. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29, 591–610. https://doi.org/10.1016/j.femsre.2005.04.002.

Snoep, J.L., de Graef, M.R., Westphal, A.H., de Kok, A., Teixeira de Mattos, M.J., Neijssel, O.M., 1993. Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of *Enterococcus faecalis, Lactococcus lactis, Azotobacter vinelandii* and *Escherichia coli*: implications for their activity in vivo. FEMS Microbiol. Lett. 114, 279–283. https://doi.org/10.1016/0378-1097(93)90284-9.

Snoep, J.L., Teixeira de Mattos, M.J., Starrenburg, M.J., Hugenholtz, J., 1992. Isolation, characterization, and physiological role of the pyruvate dehydrogenase complex and alpha-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. J. Bacteriol. 174, 4838–4841. https://doi.org/10.1128/jb.174.14.4838-4841.1992.

Ten Brink, B., Otto, R., Hansen, U.P., Konings, W.N., 1985. Energy recycling by lactate efflux in growing and nongrowing cells of *Streptococcus cremoris*. J. Bacteriol. 162, 383–390.

Tewari, Y.B., Miller, M.M., Wasik, S.P., Martire, D.E., 1982. Aqueous solubility and octanol/water partition coefficient of organic compounds at 25.0 °C. J. Chem. Eng. Data 27, 451–454. https://doi.org/10.1021/je00030a025.

Thierry, A., Maillard, M.-B., Richoux, R., Lortal, S., 2006. Ethyl ester formation is enhanced by ethanol addition in mini Swiss cheese with and without added aropionibacteria. J. Agric. Food Chem. 54, 6819–6824. https://doi.org/10.1021/jf060673m.

Thomas, T.D., Ellwood, D.C., Longyear, V.M.C., 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J. Bacteriol. 138, 109–117.

Thomas, T.D., Pearce, K.N., 1981. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. New Zeal J Dairy Sci 16, 253–259.

Urbach, G., 1993. Relations between cheese flavour and chemical composition. Int. Dairy J. 3, 389–422. https://doi.org/10.1016/0958-6946(93)90025-U.

Valvani, S.C., Yalkowsky, S.H., Roseman, T.J., 1981. Solubility and partitioning IV: aqueous solubility and octanol-water partition coefficients of liquid non-electrolytes. J. Pharm. Sci. 70, 502–507. https://doi.org/10.1002/jps.2600700510.

Van Leuven, I., Van Caelenberg, T., Dirinck, P., 2008. Aroma characterisation of Gouda-type cheeses. Int. Dairy J. 18, 790–800. https://doi.org/10.1016/j.idairyj.2008.01.001.

van Mastrigt, O., Mager, E.E., Jamin, C., Abee, T., Smid, E.J., 2017. Citrate, low pH and amino acid limitation induce citrate utilisation in *Lactococcus lactis* biovar diacetylactis. Microb. Biotechnol. https://doi.org/10.1111/1751-7915.13031.

van Rijswijck, I.M.H., Wolkers – Rooijackers, J.C.M., Abee, T., Smid, E.J., 2017. Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production. Microb. Biotechnol 10, 1591–1602. https://doi.org/10.1111/1751-7915.12717.

Van Verseveld, H.W., De Hollander, J.A., Frankena, J., Braster, M., Leeuwerik, F.J., Stouthamer, A.H., 1986. Modeling of microbial substrate conversion, growth and product formation in a recycling fermentor. Antonie Leeuwenhoek 52, 325–342. https://doi.org/10.1007/bf00428644.

Vos, T., Hakkaart, X.D.V., de Hulster, E.A.F., van Maris, A.J.A., Pronk, J.T., Daran-Lapujade, P., 2016. Maintenance-energy requirements and robustness of *Saccharomyces cerevisiae* at aerobic near-zero specific growth rates. Microb. Cell Factories 15 (111). https://doi.org/10.1186/s12934-016-0501-z.

Yamagami, C., Takao, N., Fujita, T., 1991. Hydrophobicity parameter of diazines. II: analysis and prediction of partition coefficients of disubstituted pyrazines. J. Pharm. Sci. 80, 772–777. https://doi.org/10.1002/jps.2600800814.

Yvon, M., Rijnen, L., 2001. Cheese flavour formation by amino acid catabolism. Int. Dairy J. 11, 185–201. https://doi.org/10.1016/S0958-6946(01)00049-8.