Respiratory physiology of *Lactococcus lactis* in chemostat cultures
and its effect on cellular robustness in frozen and freeze-dried
starter cultures

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\textbf{Running Head:} Respiratory physiology of *L. lactis* in chemostats

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Abstract

In this study, we used chemostat cultures to analyze the quantitative effects of the specific growth rate and respiration on the metabolism in *L. lactis* CHCC2862, and on the downstream robustness of cells after freezing or freeze-drying. Under anaerobic conditions, metabolism remained homofermentative, although biomass yields varied with the dilution rate (*D*). In contrast, metabolism shifted with the dilution rate under respiration-permissive conditions. At *D* = 0.1 h⁻¹, no lactate was produced, while lactate formation increased with higher dilution rates. Thus, a clear metabolic shift was observed, from flavor-forming respiratory metabolism at low specific growth rate, to mixed-acid respiro-fermentative metabolism at higher specific growth rates. Quantitative analysis of the respiratory activity, lactose uptake rate and metabolite production rates showed that aerobic acetoin formation provided most of the NADH consumed in respiration. Moreover, the maintenance-associated lactose consumption under respiration-permissive conditions was only 10% of the anaerobic value, either due to higher respiratory yield of ATP on consumed lactose or due to lower maintenance-related ATP demand. The cultivation conditions also affected the quality of the starter cultures produced. Cells harvested under respiration-permissive conditions at *D* = 0.1 h⁻¹ were less robust after freeze-drying and had a lower acidification activity for subsequent milk acidification, whereas respiration-permissive conditions at the higher dilution rates led to robust cells that performed equally well or better than anaerobic cells.

Importance

*Lactococcus lactis* is used in large quantities by the food and biotechnological industries. *L. lactis* can use oxygen for respiration if heme is supplied in the growth medium. This has been extensively studied in batch cultures using various mutants, but quantitative studies of how the cell growth affects respiratory metabolism, energetics and cell quality are surprisingly scarce. Our results demonstrate that the respiratory metabolism of *L. lactis* is remarkably flexible and can be modulated by controlling the specific
growth rate. We also link the physiological state of cells during cultivation to the quality of frozen or freeze-dried cells, which is relevant to the industry that may lack understanding of such relationships.

This study extends our knowledge on respiratory metabolism in L. lactis and its impact on frozen and freeze-dried starter culture products, and it illustrates the influence of cultivation conditions and microbial physiology on the quality of starter cultures.

**Keywords**

Lactic acid bacteria, continuous cultures, respiration-permissive conditions, acidification activity, aerobic growth, microbial physiology
Introduction

Lactococcus lactis is extensively used within the dairy industry as a starter culture. Starter cultures are produced in a process involving cultivation, concentration, and preservation, for example, by freezing or freeze-drying (1). Lactic acid bacteria are commonly freeze-dried as this ensures not only long-term preservation, but also lower storage and transportation costs than for frozen cell suspensions. Bacterial tolerance to freeze-drying varies between strains, which can have considerable impact on the metabolic activity, e.g. acidification rate, in the final application. Moreover, the robustness of the starter culture, viz. cell survival and metabolic capacity after storage, is also affected by the conditions under which it is produced, e.g. cultivation conditions and exposure to cold and heat shock (2, 3). Industry strives to achieve starter culture production conditions that give high biomass yield without compromising cellular robustness.

L. lactis has long been described as an anaerobic homofermentative bacterium which converts at least 90% of consumed sugars to lactate. However, it is now known that L. lactis can undergo aerobic respiration in the presence of heme and oxygen, so-called respiration-permissive conditions (4-7). Heme is an essential cofactor for the cytochrome oxidase system. In the presence of exogenous heme, L. lactis can establish an electron transport chain (ETC) consisting of NADH dehydrogenase, menaquinone as an electron shuttle, and cytochrome oxidase, resulting in NADH oxidation and proton extrusion from the cell (6, 8-10).

L. lactis strains are generally considered to be homofermentative under anaerobic conditions, where pyruvate is reduced to lactate via lactate dehydrogenase (LDH) (Figure 1). Conditions such as carbon limitation and low specific growth rate can result in a decrease in the flux through LDH. In such conditions, pyruvate is instead metabolized via pyruvate formate lyase (PFL) or pyruvate dehydrogenase (PDH) leading to mixed-acid fermentation including production of formate, CO₂, acetate, and ethanol in
addition to lactate (11-14). The significant difference between PDH and PFL is that PDH produces NADH and CO₂, rather than formate, in addition to acetyl-CoA. Furthermore, PDH activity is induced by aeration, whereas PFL is inactivated. Irrespective of whether PDH or PFL is activated, the intermediate acetyl-CoA is further metabolized to either acetate or ethanol, via phosphotransacetylase (PTA) and acetate kinase (ACKA), or via acetaldehyde dehydrogenase (ADHE) and alcohol dehydrogenase (ADHA), respectively. Acetate formation generates additional ATP, while ethanol formation regenerates NAD⁺.

During anaerobic fermentation, when lactate is the main product, protons are extruded by the H⁺-ATPase to maintain the intracellular pH at the expense of ATP (8, 15). H⁺-ATPase has been recognized as being essential for the anaerobic growth of *L. lactis* (16). However, in the presence of heme, the ETC functions as a proton transport system causing respiration-driven efflux of protons in addition to, or instead of, H⁺-ATPase (17). *L. lactis* thus saves ATP through heme-activated respiration, as NADH dehydrogenase regenerates NAD⁺ while protons are extruded at no additional ATP cost. Some studies suggest that additional ATP generation may occur, however, this has not yet been fully verified (8, 15).

Furthermore, respiration-permissive conditions direct metabolism towards flavor-forming pathways. The flux from pyruvate to lactate decreases significantly and acetoin, diacetyl and CO₂ are formed instead. In the production of acetoin and diacetyl, pyruvate undergoes decarboxylation by acetolactate synthase (ALS) into acetolactate (Figure 1). Acetolactate is unstable and can either undergo spontaneous conversion to diacetyl by chemical reaction with O₂, or it can be enzymatically converted to acetoin by acetolactate decarboxylase (18).

The advantages of respiration-permissive conditions are well established in both the literature and in industrial practice: the intracellular oxygen level is decreased, acid production is reduced, and thus the stress on the cells is lower. This leads to an increase in biomass yield, resistance to oxygen, and long-term cellular survival in liquid-form cultures stored at 4°C (4, 5, 19). Nevertheless, respiration in *L. lactis*...
has exclusively been conducted in batch cultivations and at low sugar concentrations. Quantitative analyses of metabolic fluxes and energetics are scarce, and have considered neither the potential relationship between the specific growth rate and the beneficial effects of respiration, nor the potential effects on the robustness in terms of tolerance to downstream processing.

The aim of the present study was to understand the relationship between respiration-permissive conditions during starter culture production and the microbial robustness during downstream treatment of the culture. We mimicked conditions that prevail during different phases of industrial batch production by culturing *L. lactis* subsp. *lactis* to steady states at three dilution rates in respiration-permissive and anaerobic chemostat cultures. We performed quantitative metabolic analysis to investigate how cellular metabolism and energetics depend on respiration and the specific growth rate.

Furthermore, we explored how the specific growth rate and respiration influence the robustness of frozen and freeze-dried starter cultures by assessing the viability and acidification activity after industry-like downstream processing.

**Results**

**Metabolic fluxes and yields**

*Lactococcus lactis* CHCC2862 was grown in continuous cultures on rich medium with 40 g·l\(^{-1}\) lactose under respiration-permissive conditions, i.e. aerobically and with hemin supplementation, and under anaerobic conditions, in order to study the effects of respiration and specific growth rate on metabolite yields, energetics of growth, and robustness during and after downstream processing. The experimental procedures are schematically illustrated in Figure 2.
The residual lactose concentration increased with increasing dilution rate under both anaerobic and respiration-permissive conditions (Figure 3A, 3B). The biomass concentration was higher under respiration-permissive than in anaerobic conditions. Lactate was the main product under anaerobic conditions (Figure 3A, 3C), whereas acetoin was the main product under respiration-permissive conditions, together with CO$_2$ and lactate, depending on the dilution rate (Figure 3B, 3D, Table 1). At $D = 0.1$ h$^{-1}$, no detectable lactate was formed in the respiration-permissive chemostat.

Under anaerobic conditions, lactose was almost exclusively converted to lactate, and only minor amounts of formate, acetate, and ethanol were formed (Figure 3C, Table 1). The metabolism remained homofermentative, with mainly lactate being produced at all dilution rates. Under respiration-permissive conditions, the metabolite profile instead consisted of a mixture of acetoin, CO$_2$, acetate and lactate, with CO$_2$ exhibiting the highest specific flux (Figure 3D, Table 1). At $D = 0.1$ h$^{-1}$ the lactate flux was close to zero, but it increased linearly with increasing dilution rate. The specific O$_2$ uptake rate also increased with increasing dilution rate (Figure 3D, $p < 0.05$). However, the ratio between CO$_2$ production and O$_2$ uptake (the respiratory quotient, RQ) was higher at $D = 0.1$ h$^{-1}$, RQ = 1.97, than at $D = 0.5$ h$^{-1}$ and 0.8 h$^{-1}$ where RQ = 1.88 ($p < 0.05$). This was in line with the higher activity of CO$_2$-producing metabolic pathways at $D = 0.1$ h$^{-1}$, where acetoin was the main product (Table 1). Diacetyl was detected in all respiratory cultures, but at low concentrations.

Under anaerobic conditions, the biomass yield at $D = 0.1$ h$^{-1}$ was significantly lower (0.11 C-mol C-mol$^{-1}$) than at $D = 0.5$ h$^{-1}$ and $D = 0.8$ h$^{-1}$ (0.24 and 0.26 C-mol C-mol$^{-1}$, respectively; $p < 0.05$). Lactate was the main product and lactate yields were all similar at 0.96 – 0.99 C-mol C-mol$^{-1}$ (Table 1). Acetate yields were low and decreased significantly with increasing dilution rate ($p < 0.05$).

Under respiration-permissive conditions, the biomass yield at $D = 0.1$ h$^{-1}$ tended to be lower than that at $D = 0.5$ h$^{-1}$ and $D = 0.8$ h$^{-1}$, however, the differences were much smaller. At $D = 0.1$ h$^{-1}$ no lactate was
produced, and carbon was instead directed to the flavor-forming and mixed acid metabolites acetoin, acetate, and CO₂. The lactate yield on consumed lactose increased with increasing dilution rate, while acetoin and CO₂ yields decreased (Table 1, p < 0.05).

Energy and redox balances

The substrate-related maintenance coefficient (m_s) and maximum biomass yield on lactose (Y_{SX}^{max}) of L. lactis CHCC2862 were estimated from the specific lactose consumption by linear regression. The relationships between the specific lactose consumption rate and the dilution rate were linear and clearly significant under both anaerobic and respiration-permissive conditions (F_{obs} = 656 > F_{0.001,1,10} = 21 and F_{obs} = 1072 > F_{0.001,1,9} = 23, respectively), indicating that the maintenance coefficient was independent of the specific growth rate in both cases (Figure 4A). The maintenance coefficient was significantly lower under respiration-permissive conditions, 2.1 ± 4.7 mmoles of carbon per gram dry cell weight per hour (C-mmol·gDW⁻¹·h⁻¹, 95% confidence interval) than under anaerobic conditions, 26.0 ± 5.6 C-mmol·gDW⁻¹·h⁻¹.

The maximum biomass yields on lactose were very similar under respiration-permissive and anaerobic conditions, 1120 ± 8 and 1122 ± 10 gDW C-mmol⁻¹, respectively, corresponding to 0.33 ± 0.03 C-mol·C-mol⁻¹.

In consequence, the fraction of carbon (C-mol·C-mol⁻¹) used for maintenance under respiration-permissive conditions was 14 %, 3 %, and 2 % of the consumed lactose at D = 0.1, 0.5, and 0.8 h⁻¹, respectively (Figure 4B). The maintenance energy demand was higher under anaerobic conditions; 67 %, 29 %, and 22 % of the consumed lactose was used for maintenance at D = 0.1, 0.5, and 0.8 h⁻¹, respectively.

The NADH-NAD⁺ balance in the central carbon metabolism and the ATP produced via substrate-level phosphorylation, ATP_{SLP}, were estimated based on the lactose catabolism and yields of all by-products (Table 2). Under anaerobic conditions, the balance of NADH formed and NAD⁺ regenerated in catabolism
(Eq. 2) was almost complete, indicating small experimental errors. An excess of NADH was found in lactose catabolism under respiration-permissive conditions (Eq. 3). However, under respiration-permissive conditions NADH can donate electrons to the ETC with O$_2$ being the final acceptor. The estimated NADH re-oxidized in the ETC due to O$_2$ uptake (Eq. 4) showed that the overall NADH-NAD$^+$ balance was close to zero also under respiration-permissive conditions (Eq. 5, Table 2). Acetoin production was associated with 71 ± 13 % of the NADH consumed in respiration.

The estimated ATP formed by substrate-level phosphorylation ($ATP_{SLP}$, Eq. 6) was lower under respiration-permissive conditions at all dilution rates ($p < 0.05$), despite the fact that the biomass yields were significantly higher (Table 2).

Degree of cell damage at each process step

Cell robustness is essential throughout the production process to ensure starter cultures of high quality. To investigate the effect of fermentation conditions on cellular robustness, cells were harvested from each fermentation experiment after steady-state samples had been collected. The complete fermentation broth (FB) was divided into two equal parts that were used to produce frozen product (FP) and freeze-dried product (FDP), respectively (Figure 2). The robustness was assessed by measuring the number of cells and their membrane potential using flow cytometry, to estimate the percentage of damaged cells after each process step (Figure 5). The total number of cells per gram increased between the downstream process steps as cells were concentrated by centrifugation and freeze-drying (Figure 5A). According to cell counts measured in each of the process steps (FM, FP, FDP), cell recoveries after downstream treatments were complete within 3 ± 2 % and 7 ± 1 % for FP and FDP, respectively. In general, cells in the frozen and freeze-dried products had a higher degree of cell damage than those in the fermentation broth (Figure 5B). There was a tendency towards an increasing percentage of damaged cells with increasing dilution rate under anaerobic conditions, and the highest percentage of damaged
cells (14%) was determined in the frozen product harvested at $D = 0.8 \text{ h}^{-1}$. Cells harvested under respiration-permissive culture conditions showed the opposite trend; the percentage of damaged cells decreased with increasing dilution rate and was only about 3% in the frozen and freeze-dried products harvested at 0.5 h$^{-1}$ and 0.8 h$^{-1}$, respectively (Figure 5B).

Acidification performance

The effect of the cultivation conditions on the acidification performance after each process step was assessed by a standardized milk acidification assay (Figure 6). For anaerobically cultured cells, the downstream processing alone did not seem to influence the maximum acidification rate ($r_{\text{pH, max}}$) between cells in the fermentation broth (FB), frozen product (FP), and freeze-dried product (FDP). However, it was significantly affected by low dilution rate in all process steps. At $D = 0.1 \text{ h}^{-1}$ the maximum acidification rates of FB, FP and FDP were about 13% lower than at the higher dilution rates (Figure 6A, $p < 0.05$). No significant differences were found in the specific acidification time ($t_{\text{spe}}$) across the product type nor the dilution rate, although the freeze-dried products tended to perform slightly better at the higher dilution rates (Figure 6B).

In respiration-permissive conditions the maximum acidification rate ($r_{\text{pH, max}}$) appeared to be independent of the dilution rate and no significant differences were observed between the fermentation broth and the frozen and freeze-dried products prepared (Figure 6A). On the other hand, the specific acidification time ($t_{\text{spe}}$) depended on both the dilution rate and the downstream processing. After cultivation at the two higher dilution rates, the $t_{\text{spe}}$ for the FM, FB and FDP products were shorter (i.e. better) than at $D = 0.1 \text{ h}^{-1}$ (Figure 6B). At low dilution rate, the cells were highly sensitive to freeze-drying. The specific acidification time of the freeze-dried product was more than 60% longer than that of the frozen product at $D = 0.1 \text{ h}^{-1}$. The sensitivity to freeze-drying was less severe at the higher dilution rates, although a difference in $t_{\text{spe}}$ could still be observed between the freeze-dried and the frozen products at $D = 0.5 \text{ h}^{-1}$. 
Moreover, compared to the anaerobic conditions, the frozen products of \( D = 0.5 \text{ h}^{-1} \) and \( 0.8 \text{ h}^{-1} \) and the freeze-dried product of \( D = 0.8 \text{ h}^{-1} \) resulted in similar \( t_{\text{ph, max}} \) and shorter \( t_{\text{spe}} \) than their anaerobic counterparts, making the respiro-fermentative cells at \( D = 0.5 \text{ h}^{-1} \) and \( 0.8 \text{ h}^{-1} \) the best performing products among all conditions tested.

**Discussion**

Metabolic shifts and respiration in *Lactococcus lactis*

The metabolic shift between homofermentative and mixed-acid fermentation under anaerobic conditions can be strain dependent, although factors such as carbon excess, high glycolytic flux, and rapid growth are often associated with homofermentative metabolism. Mixed-acid fermentation, on the other hand, is associated with low specific growth rate, carbon limitation, and low glycolytic flux (11, 14).

Aerobic conditions appear to lead to improved growth yields in most lactic acid bacteria (20). However, while six out of six strains of *L. lactis* displayed a respiration-associated metabolic shift upon aeration and addition of heme (21), only about 20 % of 76 screened strains of *Lactobacillus*, *Leuconostoc* and *Weissella* species showed respiratory phenotypes (20).

We used chemostat cultures under anaerobic and respiration-permissive conditions to enable quantitative analysis of the metabolic shifts between homofermentative fermentation, mixed-acid fermentation, flavor formation and respiration in *L. lactis* CHCC2862 using industry-like rich medium. We found that *L. lactis* CHCC2862 remained homofermentative in anaerobic chemostats, regardless of the dilution rate. The acetate yield increased on lactose with decreasing dilution rate but the overall yield of lactate on consumed lactose was above 96 % in all anaerobic chemostats.

In contrast, under respiration-permissive conditions the metabolite production profile varied with the dilution rate. In conjunction with respiration, the flavor-forming and mixed-acid pathways were active in...
L. lactis CHCC2862 at all growth rates, but clear differences were observed in lactate formation. The lactate concentration was below the HPLC detection limit at the lowest dilution rate, while a mixture of lactate and acetoin, acetate, and CO₂ was produced at the two higher dilution rates. Several enzymes, primarily LDH, ALS, PDH, and PFL, compete for pyruvate as substrate, and LDH also competes with e.g. NADH oxidase for NADH. It was clear from the overall yields that, at the highest dilution rates the most active enzymes were ALS and LDH, as the pyruvate flux was directed towards acetoin and lactate. Acetate was also formed but the specific rate of acetate formation plateaued, along with decreasing yield of acetate on lactose. This may be attributed to allosteric regulation of several enzymes by fructose-1,6-bisphosphate (FBP). The FBP level can be expected to increase at high specific growth rates (13, 14, 22). FBP increases the affinity of LDH towards both pyruvate and NADH (23) and it is known to inhibit PTA and ACKA, involved in acetate formation (22, 24). Together, this would mean that carbon flux is redirected away from acetate towards lactate at high dilution rate. It is, however, interesting that such a shift was not clearly seen under anaerobic conditions in this strain which showed similar yields at all anaerobic steady states. Tentatively, this could be due to regulation via the NADH/NAD⁺ ratio, which should generally be higher under anaerobic than under aerobic conditions. Moreover, at $D = 0.1 \text{h}^{-1}$ the specific lactose uptake rate, which translates into the glycolytic rate, was almost three times higher under anaerobic conditions ($q_S = 3.25 \pm 0.41 \text{mmol·gDW}^{-1}·\text{h}^{-1}$) than under respiration-permissive conditions at the same dilution rate ($q_S = 1.22 \pm 0.09 \text{mmol·gDW}^{-1}·\text{h}^{-1}$).

Previous studies have shown that only few changes in transcription and protein synthesis are direct results of respiration-permissive conditions (7, 25). It has been suggested that NADH is a key regulator in carbon metabolism, as a low NADH/NAD⁺ ratio could allosterically, or via substrate concentration-related effects, redirect metabolism in favor of enzymes using NAD⁺ or pyruvate as substrate, without necessarily changing the gene expression levels or enzyme concentrations (12, 26). Recently, Cesselin...
and colleagues analyzed the transcriptional response in early stationary phase under respiration-permissive conditions and found that late changes in the transcriptome included significant upregulation of several genes in the acetate (pdhABCD, poxL and pflB) and acetoin pathways (als, aldB, butA and butB) (21). Using mutants with inactivated pdhA, pfl, pta and als, they concluded that acetate production is necessary for the increased biomass yield, while the acetoin production, being pH neutral, alleviates acid stress and prolongs survival. Moreover, pyruvate accumulation was identified as the main driver for als activity (21). The kinetic properties of ALS, PDH and LDH also favor acetoin production at high intracellular pyruvate concentrations (27).

Our results imply that, in addition, acetoin production is required to compensate for the NADH consumed in respiration. We calculated that there was a net production of NADH from catabolism of lactose in respiratory cultures. This is related to the higher levels of flavor-forming and mixed-acid products. Under respiration-permissive conditions at the lowest dilution rate almost no lactate was produced, and acetoin and CO₂ were produced instead. If no lactate is formed, NADH must be oxidized either via ethanol formation, or via the ETC. Only traces of ethanol were observed, regardless of the dilution rate, which leads us to the conclusion that NADH is oxidized via the ETC. We measured the oxygen uptake rate and found that the respiratory NADH consumption aligned with the net catabolic NADH production to within ± 8 %, indicating that the net NADH production in catabolism is well balanced with the NADH consumption due to respiration.

The increase in biomass yield is a clear result of heme-activated respiration, whether additional energy is released, or the energy demand is reduced. Under anaerobic conditions, the H⁺-ATPase transfers protons across the cytoplasmic membrane at the expense of ATP to maintain the intracellular pH (8, 16). Under respiration-permissive conditions, the flow of electrons through the ETC extrudes protons without any additional need for ATP, thereby reducing or eliminating the cost of cellular ATP proton transfer (8).
However, respiration in *L. lactis* is different from that in other respiring organisms such as *E. coli* and *B. subtilis*, which use the Krebs cycle to produce NADH for full respiration. *L. lactis* does not possess all the enzymes required for a complete Krebs cycle, and thus generates NADH via fermentation. During respiration, the re-oxidation of NADH takes place via the ETC, and the accumulated pyruvate must therefore be consumed via NADH-producing or redox-neutral reactions, such as acetoin formation, to support respiration, which is completely in line with our results and previously reported data (21).

**Cellular economy**

Respiration capacity is often related to both the NADH/NAD\(^+\) and the ATP/ADP ratios, and affects biomass production. However, NADH oxidation and ATP production are only weakly connected in *L. lactis*, as most of the ATP is generated by substrate-level phosphorylation (15). The function of H\(^+\)-ATPase in relation to the ETC has not yet been fully elucidated, and the question is whether H\(^+\)-ATPase contributes with additional ATP besides that produced in glycolysis and mixed-acid fermentation (15).

We estimated the substrate-level phosphorylated ATP (ATP\(_{SLP}\)) from the metabolite yields (Table 2). Anaerobic conditions led to higher ATP\(_{SLP}\) values than respiration-permissive conditions did. Nevertheless, the biomass yield was significantly increased under all respiration-permissive conditions studied. At the lowest dilution rate, the biomass yield increased 165 % compared to anaerobic conditions, whereas it increased 36 % and 23 % at \(D = 0.5\) h\(^{-1}\) and \(D = 0.8\) h\(^{-1}\), respectively (Table 1).

Heme-activated respiration undisputably increases biomass yield. However, it is not clear if this is because additional ATP is formed, or because the cellular ATP requirement is reduced. When environmental conditions are favorable and nutrient supplies are abundant, micro-organisms direct nutrient and energy resources primarily towards growth-related cellular processes (28, 29). In contrast, when nutrient and energy supplies are limited, microorganisms predominantly employ metabolic energy for maintenance-related processes, which can lead to shifts in metabolic pathways, including changes in
energy storage and utilization pathways (30). The maintenance coefficient obtained under anaerobic
conditions (Figure 4A) corresponds to previously reported values of comparable \textit{L. lactis} strains, such as
\textit{L. lactis} subsp. \textit{lactis} IL1403 (31, 32). The maintenance energy demand is the likely reason for the lower
biomass yield at the lowest dilution rate under both anaerobic and respiration-permissive conditions
(Table 1).

The \( m_s \) obtained from respiration-permissive conditions was only 10\% of the anaerobic value. This
explains the large difference between anaerobic and aerobic biomass yields at \( D = 0.1 \text{ h}^{-1} \), as a larger
fraction of the lactose consumption would be used for maintenance energy requirements under
anaerobic conditions, instead of providing energy for biomass formation. In addition, when excluding the
biomass formation, the carbon recovery was below 90\% for all the respiratory cultures, and around
100\% for the anaerobic cultures. Since the calculated carbon recoveries did not include carbon
potentially incorporated into biomass, the low carbon recoveries suggest that lactose was assimilated
into biomass under respiration-permissive conditions.

These observations indicate either that additional ATP formation could take place from the available
lactose due to respiration, or that respiration-permissive conditions cause a lower energy requirement
for maintenance. Our data does not allow us to clearly distinguish between these mechanisms, but both
would lead to fulfilment of the maintenance energy demand at a lower substrate consumption rate.

Cellular robustness

In industry, cells are usually cultivated in batch fermentation and harvested in the stationary phase.
Batch cultivations are characterized by dynamic changes throughout the process time. Early in the batch,
the substrate concentration and the specific growth rate are high. Towards the end, substrate is
depleted, product concentrations accumulate and the specific growth rate decreases. This activates
various stress resistance mechanisms due to nutrient limitations, leading to an increased tolerance to
other stress conditions, such as freezing and freeze-drying (1). Previous studies have shown that fermentation conditions and harvest time influence the cellular robustness of starter cultures (3). In the present study, cells were instead harvested at steady state in chemostat cultures to investigate how the physiological state of cells affects frozen and freeze-dried products. At steady state, growth occurs at constant specific growth rate below $\mu_{\text{max}}$ and all culture variables remain constant. This offers the possibility to examine the cell biology in a quantitative and controlled manner. Our results showed that at high dilution rates, a higher degree of cell damage was observed under anaerobic conditions than under respiration-permissive conditions, indicating that respiration leads to more robust cells as long as it occurs in parallel with lactate production at intermediate or high specific growth rate. The reasons for this are not clear, but the lower acid stress and modulation of the membrane composition in the presence of oxygen may affect the robustness of the final product (21, 33).

The acidification activity is an important technological property of industrial dairy starter cultures. The adaptation of cells to a milk medium after their production is crucial for successful acidification in standard dairy production processes. Under anaerobic conditions, the initial acidification activity, here expressed as the specific acidification time ($t_{\text{sp}}$), did not differ significantly between the three types of product formulations (fermentation broth, frozen and freeze-dried). However, in respiration-permissive conditions freeze-dried products had a slightly longer specific acidification time at $D = 0.5 \, \text{h}^{-1}$, and much longer at $D = 0.1 \, \text{h}^{-1}$, than at $D = 0.8 \, \text{h}^{-1}$. These observations imply that the metabolic shift towards flavor-forming pathways affects the initial ability of freeze-dried cells to acidify milk. They also highlight that the freeze-drying process may have a huge impact and should be included in investigations of the robustness of starter cultures.

The maximum acidification rate ($r_{\text{pH, max}}$) was not significantly affected by any of the cultivation conditions, except by anaerobic growth at $D = 0.1 \, \text{h}^{-1}$, after which it was on average about 13 % lower.
than in the other cases (p < 0.05). The reason for the low value of $r_{pH,max}$ at low specific growth rate under anaerobic conditions may potentially be the high maintenance energy demand, hypothetically leading to loss of function resulting from energy limitation. The relatively high values of both $r_{pH,max}$ and $t_{spe}$ in the freeze-dried product prepared after respiration-permissive cultivation at $D = 0.1 \, h^{-1}$ indicate that the shift of metabolism does not change the potential metabolic acidification rate, but does affect the ability of the cells to adapt and initiate growth and milk acidification. The physiological state of cells under respiration-permissive conditions at $D = 0.1 \, h^{-1}$ suppresses lactate formation, which is the principal acidification activity. Furthermore, in respiration-permissive conditions at $D = 0.1 \, h^{-1}$ high levels of acetoin were present which may have contributed to the poor adaptation, since acetoin has been shown to inhibit cell growth (34).

$L. lactis$ starter cultures are produced in batch cultivations striving for high biomass yield of robust high-performance cells. Chemostat cultivations represent snapshots of what takes place at different points in a batch cultivation. Clearly, the respiratory metabolism was quite flexible. Low specific growth rate resulted in undetectable lactate formation and high acetoin formation. The frozen products of $D = 0.5 \, h^{-1}$ and $0.8 \, h^{-1}$ and the freeze-dried product of $D = 0.8 \, h^{-1}$ exhibited the highest acidification activity among all tested conditions, whereas cells produced under respiration-permissive conditions at the lowest dilution rate performed very poorly after freeze-drying. This raises the question whether the activity of the LDH pathway is beneficial or if the high level of acetoin is detrimental for cellular robustness during storage of starter cultures. The harvest point in current starter culture production setup takes place in the stationary phase where the specific growth rate is very low. To achieve high $L. lactis$ biomass yield and high-performance freeze-dried starter cultures one may want to reconsider this harvesting procedure.
In conclusion, this study provides a quantitative analysis of chemostat cultures of *L. lactis* under respiration-permissive and anaerobic conditions. Respiration-permissive conditions gave clearly higher biomass yield, ranging from 0.29 – 0.33 C·mol·C·mol\(^{-1}\), compared to anaerobic conditions, 0.11 – 0.26 C·mol·C·mol\(^{-1}\). Lactate formation was eliminated under respiration-permissive conditions at dilution rate \(D\) \(= 0.1\ \text{h}^{-1}\). Respiratory cultures have a very low maintenance requirement compared to anaerobic cultures, which is probably the main reason for the increased biomass yield. Respiration-permissive conditions have been reported to have several advantages in previous studies and our findings confirm their superior performance at high dilution rates but not necessarily in freeze-dried products obtained at lower dilution rates. This work thus underlines the importance of systematically studying the upstream as well as the downstream aspects of production processes.

### Materials and Methods

#### Upstream procedure

**Organism and pre-culture preparation**

The strain used in this study was *Lactococcus lactis* subsp. *lactis* CHCC2862, an industrial strain used in cheese-making (Chr. Hansen Culture Collection, Hørsholm, Denmark). Strain material was transformed into glycerol stocks by overnight static cultivation in M17 medium (Oxoid) and 5 g·l\(^{-1}\) lactose (Merck). The working volume was 100 ml, the temperature 30 °C, and the starting pH 6.5. At harvest, the broth was mixed with glycerol solution to a final volume ratio of 15 % (vol/vol), divided into vials of 1.0 ml and stored at -50°C. For each cultivation condition a new glycerol stock vial was used to inoculate static pre-cultures containing M17 medium and 5 g·l\(^{-1}\) lactose, in a working volume of 100 ml, at a temperature of 30 °C and starting pH 6.5. Cells were harvested in the late growth phase before lactose depletion and added to the bioreactor to achieve an initial optical density (OD\(_{600}\)) of 0.06.
Fermentation procedure

The fermentation medium was an M17-based medium modified to support high sugar concentration, consisting of the following (g·l⁻¹): yeast extract, 7.5 (Oxoid); MgSO₄·7H₂O, 0.75 (Merck); tryptone, 15 (BD); soya peptone, 15 (Oxoid); lab Lemco powder, 15 (Oxoid); ascorbic acid, 0.7 (VWR); and lactose, 40 (Merck) (35). Hemin stock solution was added to a final concentration of 10 mg·l⁻¹ to obtain respiration-permissive conditions. Hemin (Sigma) stock solution, 0.5 g·l⁻¹, was prepared in alkaline water (containing 0.05 M NaOH), and sterile filtered with a 0.8/0.2 µm Supor membrane filter. A fresh stock solution was prepared before every fermentation experiment. It should be noted that heme and hemin correspond to ferrous (reduced) and ferric (oxidized) iron in the protoporphyrin ring, respectively. In this work we refer to ‘heme’, regardless of the iron redox state.

All fermentations were carried out in a 1.5 liter Sartorius Biostat B-Twin tower with a 1 liter working volume. Initially, 0.5 ml·l⁻¹ antifoam (suppressor 3130, Hydrite) was added. The temperature was 30 °C and the pH set point was 6.0, adjusted with 24 % ammonium hydroxide. Anaerobic conditions were established by continuously flushing the headspace with nitrogen gas at 0.1 l·min⁻¹ at an agitation rate of 300 rpm. Under respiration-permissive conditions, the bioreactors were sparged with air. A minimum threshold of 60 % dissolved oxygen was ensured by cascade control, i.e. gradually increasing the stirrer speed and the aeration rate using Sartorius control software. The initial airflow was set to 0.5 l·min⁻¹, and the agitation rate was 400 rpm. The outlet gas was monitored with a Thermo Scientific™ Prima BT Bench top Mass Spectrometer (Thermo Fischer Scientific).

All chemostat fermentations were initially run in batch mode. Batch operation was switched to chemostat mode at the late exponential growth phase before lactose depletion, using the same cultivation conditions. We aimed to achieve the three dilution rates, 0.1, 0.5, and 0.8 h⁻¹, for both anaerobic and respiration-permissive conditions. However, the actual dilution rates were 0.11, 0.52 and
0.74 and 0.78 h⁻¹ for anaerobic conditions. For respiration-permissive conditions the actual dilution rates were 0.11, 0.52 and 0.8 h⁻¹. Steady states were verified after a minimum of eight residence times by repeated measurements of CO₂, OD₆₀₀ and metabolites. All fermentations were performed in biological duplicates, and each steady state originated from an independent pre-culture and chemostat start-up. The results given are based on 2 measurements at each steady state and each biological replicate, separated by at least one residence time.

**Sampling**

Steady state sampling was performed by withdrawing approximately 15 ml broth and transferring it directly to a 50 ml Nunc™ tube containing stainless steel beads (40 g, 4 mm diameter), precooled to -30 °C. The sample did not freeze and was quickly distributed to further analysis tubes (36).

**Downstream procedure**

**Preparation of frozen and freeze-dried product**

Figure 2 illustrates the downstream process steps performed in this study. Cells were harvested from each fermentation experiment after steady-state samples had been collected. The broth was divided into two equal parts; one was used to produce the frozen product (FP) and the other the freeze-dried product (FDP). Both were concentrated by centrifugation (Heraeus multifuge x 3 FR, Thermo Fisher Scientific) at 4000 rpm for 20 min at 4 °C. The supernatant was discarded, and the concentrate was resuspended. A cryo-additive was added to the cells intended for freeze-drying at a ratio of 1:1 (1 g dry matter of concentrated cells per 1 g dry matter of cryo-additive) prior to freezing. The cryo-additive contained 6 % skim milk, 8 % trehalose, and 4 % sodium ascorbate, and was sterilized at 110°C for 20 min.
The resuspended concentrates (with or without cryo additive) were pelletized by drop-wise freezing in liquid nitrogen using a 25 ml single-use pipette. The frozen products (FP) were subsequently stored at -50 °C until analysis or freeze-drying (FDP).

Freeze-drying of a known weight of frozen pellets in pre-weighed containers was performed on shelves precooled to -50 °C in a Telstar LyoBeta 15 freeze-dryer. After a holding step of 8-9 h at -50 °C, the chamber pressure was decreased to 0.3 mbar, and the shelf temperature was increased from -50 to 30 °C to initiate sublimation. The drying process continued until there was no further weight loss of the product, after which the vacuum was released by injecting air. FDP samples were packed in aluminum foil packaging bags, sealed and stored at -50 °C until analysis.

Analytical methods

Biomass measurements

Optical density was measured at 600 nm (OD\textsubscript{600}) with a Hitachi U-1900 spectrophotometer after appropriate dilution with 0.9% NaCl. The dry weight of the fermentation samples was measured gravimetrically using 0.2 µm polyethersulfone membrane filters. The correlation between OD\textsubscript{600} and cell dry weight (DW) was estimated to be 0.35 OD\textsubscript{600} gDW\textsuperscript{-1}. The moles of carbon (C-mol) of biomass was calculated by assuming the same biomass composition under all fermentation conditions and using the biomass composition of \textit{L. lactis} grown in M17, CH\textsubscript{1.70}O\textsubscript{0.51}N\textsubscript{0.22} (37).

Substrate and metabolite analysis

\textit{Lactose and lactate}

Samples were prepared by adding 200 µl 4 M sulfuric acid (H\textsubscript{2}SO\textsubscript{4}) to a 1 ml sample to arrest metabolic activity. Prior to analysis, 1.8 ml perchloric acid and 2.0 ml internal standard solution was added, followed by centrifugation for 30 min at 4°C and 2800 × g. Final dilutions were made using MilliQ water.
to ensure that the concentration was within the analysis limits/range of the equipment. Measurements of lactose and organic acids were performed using an ICS-3000 Ion Chromatography dual system. Lactose was analyzed with a CarboPac PA 20 3 × 150 mm column, using an eluent gradient of KOH in MilliQ water. The initial concentration was 0.19 mM KOH, increasing linearly to 100 mM KOH at 10.5 to 19 min, then decreasing instantaneously back to 0.19 mM at 27 to 27.5 min. The flow was 0.4 ml·min⁻¹, start pressure between 2500-2900 psi and the column temperature was set to 25 °C. Lactate was analyzed with an IonPac ICE As6 9 × 250 mm column, using 0.4 M heptafluorobutyric acid as eluent. The flow was 1.0 ml·min⁻¹, the start pressure 750-900 psi and the column temperature was set to 26 °C.

**Acetoin and ethanol**

Acetoin and ethanol were measured using headspace gas chromatography with a Perkin Elmer flame ionization detector. Samples were prepared by transferring 1 ml fermentation broth directly into a headspace vial containing 200 μl 4 M H₂SO₄ to stop metabolic activity, after which the vial was sealed with a crimped Teflon-lined aluminum cap. The headspace vials were heated to 70 °C for 36.5 min prior to analysis. The gas was analyzed using a polar column: FFAP 25 m × 0.2 mm × 0.33 μm (Agilent technologies). The oven temperature was held at 80 °C for 2 min, and then ramped to 230 °C and held for 0.5 min.

**Acetate and formate**

The analysis of acetate and formate was like that of acetoin and ethanol, although sample preparation differed: 1 ml of fermentation broth was transferred to a headspace vial containing 1 ml saturated sodium bisulfate solution and 1 ml 96 % ethanol was added before the vial was sealed with a Teflon-lined aluminum cap. Ethanol reacts with acetate and formate, forming ethyl esters with a lower boiling point, which were analyzed using headspace gas chromatography as describe above.
Acidification activity

The acidification activity was measured using the Cinac system (Alliance Instruments, France). The method measures the decrease in pH and estimates the acidification activity and how fast cells adapt to the applied conditions. The acidification assay was carried out in bottles containing 200 ml standardized milk (autoclaved skim milk powder and water with a dry matter content of 9.5 %) at 30°C for 16 hours. The bottles were inoculated with the selected culture product at different inoculation percentages (wt/wt): 0.1 % for fermentation broth samples; 0.02 %, 0.01 %, and 0.005 % for frozen products, and 0.005 %, 0.0025 %, and 0.00125 % for freeze-dried products. Before starting the pH measurements, equilibrium was ensured between the pH of the milk and the electrode. The pH was continuously monitored in the inoculated milk and led to the determination of the maximum acidification rate, \( r_{\text{pH, max}} \), and the time necessary to obtain a decrease of 0.08 pH units, \( T_{0.08} \) (min). The higher the \( T_{0.08} \), the longer the latency phase and the lower the acidification activity. The specific acidification time, \( t_{\text{spe}} \) (min \( \cdot \log(\text{cells} \cdot g^{-1}) \)), was defined as the ratio of \( T_{0.08} \) to the corresponding log of cell concentration, and was calculated according to Equation 1 (38).

\[
t_{\text{spe}} = \frac{T_{0.08}}{\log(\text{cells} \cdot g^{-1})} \tag{Eq. 1}
\]

Membrane potential

Flow cytometry was used to analyze cell counts and distinguish the ability of cells to maintain a membrane potential. Prior to analysis, cells were re-activated in MRS medium for 30 min at 40°C. The cells were then added to a stain mix, the temperature was lowered to 20 °C, and the mixture allowed to stand for 30 min. The stain mix contained 210 μl 50 % (wt/wt) glucose solution, 210 μl 1.5 mM 3,3′-diethyloxocarbocyanine iodide (DiOC 3.3’) solution in dimethylsulfoxide, and 50 g bead mix which was used as internal standard in the flow cytometry. After staining, the samples were analyzed with a BD...
LSRFortessa Flow Cytometer, using a blue laser at 488 nm. The conversion of sugar to lactate produces protons inside the cell which are actively pumped into the environment. This creates an electric gradient over the cell membrane, which drives the diffusion of DIOC 3.3’ across the cell membrane into the intracellular space, resulting in red fluorescence. The increase in intensity of the red fluorescence provides a measure of the membrane potential and was detected by the far-red parameter. The far-red parameter differentiates cells into two separated groups, those that are able to create a membrane potential (intact cells) and those that are not (damaged cells).

**Calculation of energy parameters**

**Substrate-related maintenance**

The substrate-related maintenance coefficient \( m_s \) and maximum biomass yield on lactose \( Y_{SX}^{\text{max}} \) were predicted by linear regression of the specific substrate consumption rate \( q_S \) and the dilution rate \( D \) (39). The slope represents \( 1 / Y_{SX}^{\text{max}} \), and the intercept on the y-axis gives \( m_s \).

**NADH-NAD⁺ balance**

The overall NADH-NAD⁺ balance was evaluated from the metabolite yields according to Equation 2-5, where \( Y_{Si} \) is the yield of metabolite \( i \) on consumed lactose \( S \). Under anaerobic conditions, it was assumed that acetate was formed via PFL. The overall net NADH formation in catabolism \( (Y^{\text{SNADH, catabolism}}) \) was therefore calculated according to Equation 2.

\[
Y^{\text{SNADH, catabolism}} = Y_{SAcetate} - Y_{SEthanol} \quad (\text{Eq. 2})
\]

Under respiration-permissive conditions, it was instead assumed that acetate was formed via PDH as no formate was detected. Furthermore, NADH consumption in the ETC was estimated from the oxygen uptake. The net NADH formation in catabolism \( (Y^{\text{SNADH, catabolism}}) \) and net NADH consumption due to respiration \( (Y^{\text{SNADH, ETC}}) \) were therefore calculated according to Equation 3 and 4.
YSNADH, catabolism = 2YSAcetate + 2YSAcetoin + 2YSDiacetyl – YSEthanol (Eq. 3)

YSNADH, ETC = 2YSOxygen – YSDiacetyl (Eq. 4)

The overall net NADH formation was calculated as the difference between the NADH formed in catabolism and consumed in the ETC,

YSNADH = YSNADH, catabolism – YSNADH, ETC (Eq. 5)

the second term being zero under anaerobic conditions.

Substrate-related ATP yield

The net amount of ATP produced by substrate-level phosphorylation per consumed lactose, ATP_{SLP}, was calculated from the product yields according to Equation 6.

ATP_{SLP} = YSLactate + 2 YSAcetate + YSEthanol + 2 YSAcetoin + 2 YSDiacetyl (Eq. 6)

Data analysis

The impact of each cultivation condition and product performance was statistically analyzed by the two-sample t-test where statistical differences between means were tested at 5 % level of significance.

Where applicable, values are reported as 95% confidence intervals (average ± stdev - t_{0.025, df}).

The significance of regressions of the linear rate equations, used for estimation of the maintenance coefficients, was assessed using the F-test by calculating the test statistic $F_{\text{obs}}$ while the 95% confidence intervals of individual parameters were estimated using the t-test.

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Figure legends

**FIGURE 1** Homofermentative, mixed acid and flavor-forming metabolism of lactose in *L. lactis*. LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; PDH, pyruvate dehydrogenase; ADHE, acetaldehyde dehydrogenase; ADHA, alcohol dehydrogenase; PTA, phosphotransacetylase; ACKA, acetate kinase; ALS, acetolactate synthase; ALDB, acetolactate decarboxylase. Stoichiometries assume complete degradation of lactose into each metabolite. Adapted from (40, 41).

**FIGURE 2** Overview of experimental procedures, showing the upstream and downstream processes included in this study: fermentation, separation, freezing and/or freeze-drying. For each condition resulting in frozen product (FP) and freeze-dried product (FDP). For anaerobic operation the head space was flushed with N₂. For aerobic conditions, air was sparged under the impeller. Steady state was obtained at dilution rates (*D*) of 0.1 h⁻¹, 0.5 h⁻¹, and 0.8 h⁻¹ from individual startup cultures. Samples were withdrawn directly from the bioreactor and quenched on cold stainless steel balls.

**FIGURE 3** Overview of concentrations and rates of consumption and production of main substrates, products and biomass in chemostat cultivations. A and C, anaerobic conditions; B and D, respiration-permissive conditions. A and B, concentrations; C and D, specific consumption and production rates determined at steady state. Symbols: ←, Lactose; □, Lactate; ⬤, Biomass; ✷, Acetoin; ←, Acetate; ←, Formate; ←, Ethanol; △, CO₂, ↗, O₂. Data points represent the average of biological replicates, and error bars the 95% confidence interval from measurements of two separate steady state samples of each cultivation.
**FIGURE 4** Determination of substrate-related maintenance. **A.** Plot of the specific lactose consumption \( (q_s, \text{C-mmol-gDW}^{-1}\cdot\text{h}^{-1}) \) versus the dilution rate \( (D, \text{h}^{-1}) \), to calculate the substrate-related maintenance coefficient and maximum biomass yield. Filled circles, anaerobic (AN); open circles, respiration-permissive (RES) conditions. **B.** Distribution \( (C-\text{mmol-C-mmol}^{-1}) \) of specific lactose consumption rate between maintenance- (gray) and growth- (white) related processes under anaerobic (AN) and respiration-permissive (RES) conditions. The error bars represent the 95 % confidence interval.

**FIGURE 5** Flow cytometry of cells in the fermentation broth (FB, black), frozen product (FP, gray), and freeze-dried product (FDP, white). **A.** Total number of cells; **B.** Percentage of damaged cells of *L. lactis* CHCC2862 in each process step and dilution rate after harvesting from anaerobic (AN) and respiration-permissive (RES) chemostats. The error bars represent the 95 % confidence interval from measurements of two biological replicates.

**FIGURE 6** Acidification activity of cells in the fermentation broth (FB), frozen product (FP), and freeze-dried product (FDP) after cultivation under anaerobic (AN) and respiration-permissive (RES) conditions. **A.** Maximum acidification \( (r_{\text{pH, max}}) \). **B.** Specific acidification time \( (t_{\text{spe}}) \). The error bars represent the 95 % confidence interval from measurements of two biological replicates.
### TABLE 1 Metabolite yields (C-mol∙C-mol⁻¹) on lactose under anaerobic conditions (AN) and respiration-permissive (RES) conditions†

| Dilution rate | Biomass | Lactate | Acetate | Acetoin | Diacetyl | Ethanol | Formate | CO₂ | O₂ | C recovery excl. biomass (%) |
|---------------|---------|---------|---------|---------|----------|---------|---------|-----|----|-----------------------------|
| AN 0.1 h⁻¹    | 0.11 ± 0.01 | 0.96 ± 0.03 | 0.03 ± 0.00 | n.d. | n.d. | 0.01 ± 0.00 | 0.03 ± 0.00 | t.a. | n.d. | 104 |
| AN 0.5 h⁻¹    | 0.24 ± 0.03(*) | 0.99 ± 0.13 | 0.02 ± 0.00(†) | n.d. | n.d. | 0.01 ± 0.00(†) | 0.02 ± 0.00(†) | t.a. | n.d. | 104 |
| AN 0.8 h⁻¹    | 0.26 ± 0.04(†) | 0.96 ± 0.09 | 0.01 ± 0.00(†) | n.d. | n.d. | 0.00 ± 0.00(†) | 0.02 ± 0.00(†) | t.a. | n.d. | 100 |
| RES 0.1 h⁻¹   | 0.29 ± 0.03 | 0.00 ± 0.00 | 0.07 ± 0.00 | 0.39 ± 0.03 | 0.01 ± 0.00 | t.a. | n.d. | 0.30 ± 0.05 | 0.15 ± 0.02 | 78 |
| RES 0.5 h⁻¹   | 0.32 ± 0.03 | 0.22 ± 0.02(*) | 0.07 ± 0.00 | 0.34 ± 0.03 | 0.01 ± 0.00 | t.a. | n.d. | 0.22 ± 0.02(†) | 0.12 ± 0.00(†) | 87 |
| RES 0.8 h⁻¹   | 0.33 ± 0.04 | 0.23 ± 0.02(†) | 0.05 ± 0.00(*) | 0.29 ± 0.04(†) | 0.01 ± 0.00 | t.a. | n.d. | 0.20 ± 0.02(†) | 0.11 ± 0.01(†) | 78 |

† Values given are the average of biological duplicates and the 95 % confidence interval from measurements of two separate steady state samples of each cultivation. The cell biomass was not included in the carbon recovery (C recovery) calculation. (t.a. - trace amount; n.d. - not detected). Data in parentheses indicate significance levels at p > 0.05 (†) and p < 0.05 (*) of the yields compared between dilution rates for anaerobic and respiration-permissive conditions. For D = 0.5 h⁻¹, the entry indicates statistical significance compared to D = 0.1 h⁻¹. For D = 0.8 h⁻¹, the first entry indicates statistical significance compared to D = 0.1 h⁻¹, and the second compared to D = 0.5 h⁻¹. At each dilution rate, all metabolite and biomass yields were significantly different between anaerobic and respiration-permissive conditions with p < 0.05.
TABLE 2 The overall NADH-NAD$^+$ balance ($Y_{SNADH}$) and substrate-level phosphorylated ATP ($ATP_{SLP}$) estimated from metabolite production under anaerobic (AN) and respiration-permissive (RES) conditions†

| Dilution rate (h$^{-1}$) | $Y_{SNADH}$, catabolism (mol · mol$^{-1}$) | $Y_{SNADH}$, ETC (mol · mol$^{-1}$) | $Y_{SNADH}$ (mol · mol$^{-1}$) | Estimated $ATP_{SLP}$ (mol · mol$^{-1}$) |
|--------------------------|--------------------------------------------|-----------------------------------|-------------------------------|----------------------------------------|
| AN 0.1                    | 0.16 ± 0.02 Eq. 2 / Eq. 3                  | n.a.                             | 0.16 ± 0.02 Eq. 4             | 4.28 ± 0.1 Eq. 5                      |
| AN 0.5                    | 0.08 ± 0.02 Eq. 2 / Eq. 3                  | n.a.                             | 0.08 ± 0.02 Eq. 4             | 4.27 ± 0.6 Eq. 5                      |
| AN 0.8                    | 0.03 ± 0.02 Eq. 2 / Eq. 3                  | n.a.                             | 0.03 ± 0.02 Eq. 4             | 3.97 ± 0.4 Eq. 5                      |
| RES 0.1                   | 3.34 ± 0.17 Eq. 4                          | 3.56 ± 0.58 Eq. 5                | -0.22 ± 0.67 Eq. 6            | 3.36 ± 0.16 Eq. 7                      |
| RES 0.5                   | 3.00 ± 0.18 Eq. 4                          | 2.75 ± 0.11 Eq. 5                | 0.25 ± 0.18 Eq. 6             | 3.87 ± 0.23 Eq. 7                      |
| RES 0.8                   | 2.34 ± 0.23 Eq. 4                          | 2.51 ± 0.20 Eq. 5                | -0.17 ± 0.11 Eq. 6            | 3.28 ± 0.26 Eq. 7                      |

†Values given are the average of biological duplicates and the 95% confidence interval from measurements of two separate steady state samples from each cultivation. (n.a. - not applicable).
