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Current Opinion in Food Science

Growth, dormancy and lysis: the complex relation of starter culture physiology and cheese flavour formation

Avis Dwi Wahyu Nugroho[1,2,3], Michiel Kleerebezem[1,3] and Herwig Bachmann[1,2,4]

Fast acidification and growth are desired from lactic acid bacteria starter cultures during food fermentation to minimise the risk of spoilage and process failure. In addition, starter cultures play a predominant role in the formation of flavour volatiles. Recent studies in different microbial species have shown that high growth rates come at the expense of the expression level of metabolic enzymes and/or stress proteins. In starter cultures, such a trade-off would affect flavour formation, which depends on the level of flavour-forming enzymes and the prolonged survival of cells. Moreover, starter culture performance during cheese ripening could also be influenced by its cultivation history due to the low number of divisions during cheese manufacturing and limited proteome adjustment during ripening. These findings indicate that changes in (pre)-culture conditions can modulate proteome allocation and metabolic stability in starter cultures, and thereby provide novel approaches to steer flavour formation.

Addresses
[1] TiFN 9A, 6709 PA Wageningen, The Netherlands
[2] Health Department, NIZO Food Research, Kernhemseweg 2, 6718 ZB Ede, The Netherlands
[3] Host-Microbe Interactomics Group, Wageningen University & Research, De Elst 1, 6708 WD Wageningen, The Netherlands
[4] Systems Biology Lab, Vrije Universiteit Amsterdam, De Boelelaan 1108, 1081 HZ Amsterdam, The Netherlands

Corresponding author:
Bachmann, Herwig (herwig.bachmann@nizo.com)

Current Opinion in Food Science 2021, **38**:22–30

This review comes from a themed issue on **Food microbiology**

Edited by **Anderson de Souza Sant'Ana**

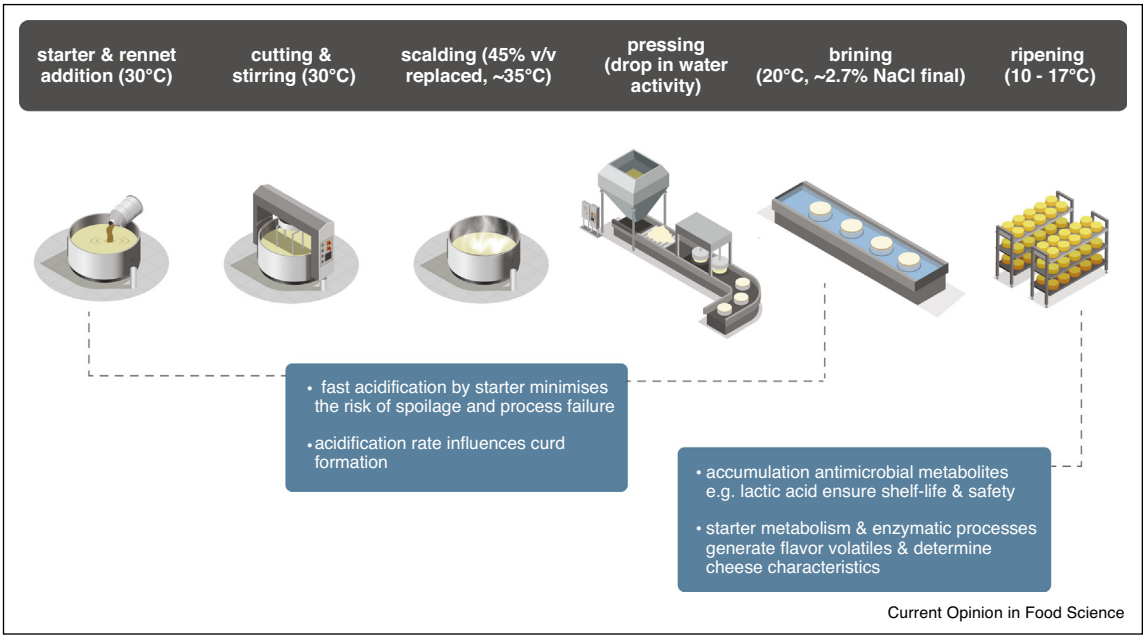
https://doi.org/10.1016/j.cofs.2020.12.005

Introduction

Dairy fermentation is a dynamic process that encompasses adverse and fluctuating conditions for the fermenting organisms. During cheese manufacturing, environmental parameters such as temperature, pH, osmolarity and lactose concentration change significantly and they can be stressful to starter bacteria (Figure 1). Rapid environmental shifts impose limitations to cell growth and adaptation. In a typical Gouda or Cheddar type cheese, starter cultures only divide up to 6 times [1,2] before entering a non-growing state that is accompanied by environmental changes such as carbon starvation, pH decrease, cold, and salt stress. It is during this ripening period that enzymatic conversion to flavour volatiles through the metabolism of starter cells is most prominent [2,3]. The low number of division during the initial cheesemaking combined with limited *de novo* protein synthesis of non-growing cells during ripening suggests that starter culture preparation might influence their functional properties in cheese. This suggestion is based on the notion that upon cell division proteins are divided over daughter cells and these proteins can contribute to cellular behaviour. For example in *Escherichia coli*, proteins of the *lac*-operon induced by exposure to lactose decreased over 10 generations after cells were transitioned to glucose, but its presence was sufficient to minimise growth delay in the subsequent lactose transition [4•]. It is conceivable that similar mechanisms could lead to a cell 'memory' that is determined during starter preparation and which would influence starter functionality in a product, but to date these are largely unexplored for food fermentations.

Furthermore, during starter culture selection, specific traits such as biomass yield, acidification rate, stress resistance, or production of specific metabolites are often analysed using standard environmental conditions that do not resemble 'in product' conditions. While these selection criteria are potentially relevant for starter performance in the application, disparate performance might be observed during the application due to environmental differences. For example in the propagation of dairy *Lactococcus lactis* strains, haem supplementation has been shown to significantly alter cellular robustness during freezing and freeze-drying [5] while galactose co-supplementation induces *lacS* expression and shortens lag phase in lactose-grown cultures [6]. During cheesemaking, strains prepared under different preculture conditions might therefore vary in their survival for example, after curd scalding, possibly resulting in altered flavour formation during the subsequent ripening process [7,8]. In *L. lactis*, retentostat experiments have also shown that aroma formation during cheese ripening is best resembled by bacteria at near-zero growth rates [9••,10]. This suggests that long-term cell integrity is of importance to flavour formation, but this is often overshadowed by the argument that lysis and release of cytoplasmic enzymes into

Figure 1



Schematic representation of Gouda-type cheese production including the changes in conditions that starter cultures are exposed to.

the cheese matrix is an important part of cheese ripening [11].

In the ideal situation, one might desire a strain that is optimised for all situations and functions throughout fermentation. However, the optimisation of a trait often comes at the expense of another due to biological and physical constraints which dictates the allocation of a cell's resources [12]. To some extent, these trade-offs may be explained from a protein economy perspective, where nutrient and energy resource allocation in cells is constrained by their proteome synthesis and adjustment [13••]. However, our fundamental and detailed understanding of mechanisms that govern the interplay of microbial growth and metabolism remains limited, especially in the context of microbes participating in food production processes [14].

This review summarises important conceptual advances in our understanding of the effect of various growth modulations on the stress tolerance and metabolic capacities of bacteria. While growth optimisation of a bacterial population gives more offspring and leads to predomination in natural habitats, it does not guarantee optimised starter functionality. We will highlight how the functionality of lactic acid bacteria (LAB) starter cultures might be affected through culturing and fermentation conditions, especially during the non-growing phase for example, throughout the ripening of cheese. A better understanding of the constraints that shape trade-offs in industrially relevant traits will open new avenues to modify process conditions and to steer functionalities of starter cultures. Such adjustments can influence application performance and product properties through modulation of a variety of traits, including lag-time, flavour formation and processing robustness.

Growth rate optimisation and the evidence of trade-offs

Fast microbial growth is often desired since it enables shorter test and trial time in research and development, as well as the higher production rate and cost-efficiency of manufacture [15]. In the dairy industry (Figure 1), high acidification rate as a result of fast growth is considered an important physiological trait of starter LAB, based on the important role of acidification rate in determining texture properties of fermented milk [16,17] and the effective prevention of growth of undesired spoilage bacteria. The rapid accumulation and high concentrations of metabolic end-products like lactic acid and acetic acid ensures a long shelf-life and safety of the products [18]. Consequently, research has aimed to select and investigate specific strains that display naturally high growth rates in standard production conditions. For example, studies in *Streptococcus thermophilus* have focused on understanding the genomic requirements for fast acidification [19] and investigating the role of specific enzymes such as protease [20] and urease [21]. Nonetheless, we are not aware of comprehensive reports on the consequences of culture

optimisation towards rapid growth and acidification affecting other relevant metabolic traits such as flavour formation.

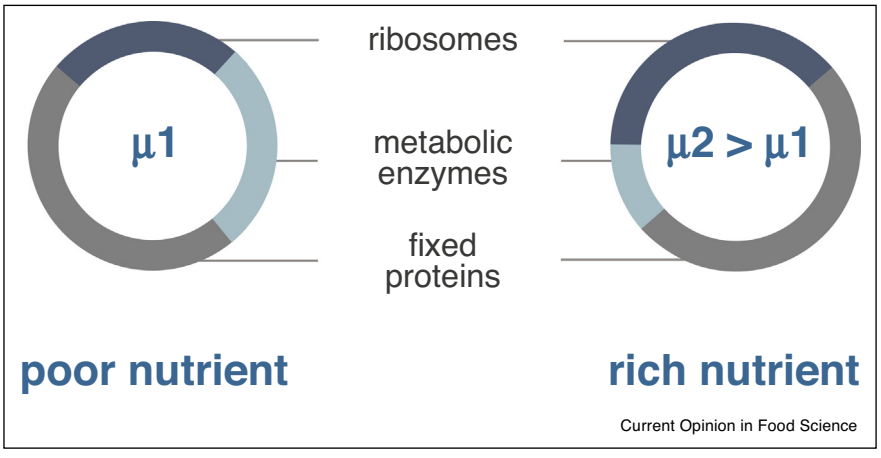
In contrast, Systems Biology approaches in model microorganisms have defined quantitative links and interactions between growth rate and physiological subsystems using coarse-grain analysis [22•] of global proteome data. Studies in *E. coli* have underpinned the robust relation between growth rate and the relative abundance of the protein synthesis machinery (i.e. ribosomal fraction) [23]. By varying carbon and energy source, increasing growth rates were found to coincide with increasing relative abundance of the ribosomal proteins within the overall cytoplasmic proteome. Importantly, increased proteome allocation toward ribosomes also coincided with decreased proteome allocation towards metabolic proteins [24]. This trade-off could be constrained by the total cytoplasmic proteome 'size' and global regulatory processes that govern proteome adjustment to support maximal growth rate under a specific condition (Figure 2). Although not explicitly addressed in these studies, growth-rate mediated modulation of the abundances of enzymes relevant for biotechnological processes is plausible, since these enzymes are part of the metabolic-protein fraction.

Similar results were also obtained in yeasts under steady-state growth conditions limited by carbon, nitrogen, phosphorus, and sulphur. A linear correlation was found between growth rate and the expression level of more than one quarter of all yeast genes (1470 out of 5537 imputed genes) independent of the limiting nutrient [25]. In a similar study [26], 493 protein coding genes were upregulated and 398 protein-coding genes were downregulated with increasing growth rate. Positively correlated genes mainly corresponded to the production of growth-related functions for example, protein translation, macromolecules synthesis and organelle production, whereas negatively correlated genes were enriched in stress-related functions [25,26]. Taken together, contemporary 'omics studies in *E. coli* and yeasts suggest that optimisation towards fast growth may reduce desired metabolic capacity as well as stress robustness. Arguably, the constant environmental parameters of chemostats, where most of the evidence was obtained, may select for proliferation rather than protection and robustness. Nonetheless, the relation between growth rate and metabolic functions was also observed in bacterial cells grown in batch cultures, albeit at slightly lesser extent [27]. Therefore, these trade-offs associated with growth rate were suggested to apply universally and they are suggested to describe a bacterial growth law [23].

Growth rate optimisation and LAB starter functionality

The observed trade-offs in *E. coli* and yeast raise the question whether growth rate influences the performance of dairy starter cultures. Although we are not aware of dedicated investigations in LAB, empirical observations in *L. lactis* reported that strains with ssp. *lactis* phenotype generally grow fast but generate less flavours during cheese ripening in comparison to the slower growing strains that have a ssp. *cremoris* phenotype [28]. Intriguingly, the lower flavour generating properties of the ssp *lactis* phenotype strains seem to correlate with their ability to utilise alternative ATP sources, like arginine and maltose, but also with their higher temperature and salt tolerance [29]. Hence, a relation between growth rate and starter functionality seem to be apparent when comparing

Figure 2



Schematic simplification of bacterial proteome allocation. The cellular proteome can be partitioned into a fixed, metabolic, and ribosome-related fraction. Growth rate (μ) is finely tuned to the fraction of ribosomal and metabolic proteins. Faster growth due to for example, a better carbon source in the medium requires more ribosomes to ensure fast synthesis of proteins needed for cell division. Because of a cytoplasmic proteome constraint this is suggested to come at the expense of metabolic enzymes and proteins. Figure adapted from Scott *et al.* [23].

the two *L. lactis* subspecies. While the fast growth of ssp. *lactis* seems to be a consequence the evolutionary history of the strains and the adaptation towards dairy environments [14,29], there is evidence that growth rate modulation within a strain correlates with changes in its metabolic capacity and/or stress resistance. For instance, upon valine or isoleucine starvation, *L. lactis* cultures displayed a 3 hours extended lag-phase and an approximate 30% reduced maximum cell density when reaching the stationary phase of growth, while at the same time increasing their formation of flavour compounds by 1.5–2.0-fold [30]. However, it cannot be concluded to which extent altered flavour formation can be attributed to differences in growth rate or amino acid availability in this experiment. In a better controlled study, cells cultivated at low dilution rate (D = 0.1/h) showed only 3% of damaged cells after freezing, which is quite different compared to cells cultivated at high dilution rate (D = 0.8/h) that showed approximately 15% of damaged cells after freezing [5]. Taken together, these observations appear to support that, analogous to the observations in *E. coli* and yeast, the growth rate of *L. lactis* also affects cellular metabolism, physiology and robustness.

On the other hand, the adaptation of *L. lactis* and other LAB to nutrient-rich environments such as milk has led to them being remarkably fastidious and metabolically squandering in comparison to *E. coli*. For example, during growth, glycolysis in *L. lactis* cells runs close to its maximal capacity and is barely controlled by ATP demand [31], which is in clear contrast to *E. coli* [32]. Moreover, while the interplay of growth rate, glycolytic flux and redox-balance in *L. lactis* has been proposed to control the metabolic shift from homo-lactic (ATP-inefficient) to mixed-acid (ATP-efficient) metabolism [2], the proteome associated with these two metabolic 'states' hardly differs [33•], which appears to disagree with the cytoplasmic proteome constraints that are predicted by the proposed bacterial growth law. Alternatively, a scenario where growth rate is primarily restricted by membrane proteome capacity rather than a cytoplasmic proteome capacity may control growth-rate associated metabolic adaptations in *L. lactis* [34,35].

Taken together, evaluating the empirical observations in *L. lactis* and some other LAB in the light of the robust scientific evidence available for *E. coli*, it is tempting to speculate that similar proteome-constraint dependent trade-offs are applicable in these species. However, some of the observations in *L. lactis* appears to contradict such similarity, and it is important to realise that there are major differences between *E. coli* (Gram-negative proteobacteria) and *L. lactis* (Gram positive low G + C-content Firmicutes). It remains to be seen whether similar trade-offs are applicable in these bacteria. Notably, unravelling of the constraints that are applicable under various environmental conditions in *L. lactis* and other LAB is a requirement to further understand the relations between growth rate and physiological and metabolic adaptations, and to subsequently employ such knowledge for the optimisation of starter culture preparations.

Cellular adaptation to (stationary) non-growing state

In various fermentation processes, starter cultures enter a non-growing state during ripening or product maturation where viability and metabolic activity are maintained for a prolonged period. The environmental causes for growth cessation include nutrient starvation, environmental stresses, and the presence of inhibitory compounds such as organic acids [36]. In applications such as cheese ripening by LAB [37], wine fermentation by yeasts [38], or sausage ripening by *Staphylococci* [39,40], the metabolism of non-growing cells are desired for metabolite production [3] as well as safety for example, through the conversion of biogenic amines [40] or the depletion of nutrients. The metabolic activity of non-growing cells is also important in biotechnology applications since metabolic resources allocated to growth can be diverted to the production of desired metabolites [41] and allows its continuous production, for example, by cell immobilisation [42], retentostat [43] or partial cell recycling chemostat cultivation [44]. While there is little quantitative data available for the length of time that enzymatic conversions can proceed during ripening of fermented products, it was shown that *L. lactis* stored in a sugar-depleted buffered medium for over 3.5 years was still able to catabolise amino acids [45••]. Recently, a novel method was described that allows to follow metabolic activity continuously in translationally blocked/non-growing cells for weeks to months, offering new approaches to study metabolism, enzyme decay and functional properties in non-growing cells to mimic the roles of starter culture cells during ripening [46]

As cells enter a non-growing state, various metabolic changes occur to aid in cell survival under non-optimal conditions. In the case of carbon exhaustion, *L. lactis* gradually lose its ability to grow on solid media within weeks, but remarkably maintained intact cell membrane and metabolic ability for over 3.5 years [45••]. Under such starvation, cell response might vary between species, but stringent responses are generally observed. Overall, transcription and translational machineries, DNA replication, and cell division are strongly repressed [47]. At the same time, general stress resistance including heat, acid, and oxidative stress are significantly induced [47,48], while autolytic genes are significantly repressed [45••]. Cells consequently redirect their catabolic activity from the exhausted carbohydrate by upregulating the activity of alternative pathways that can provide ATP [49], which may include other carbon or nitrogen sources. These are indicated by the alleviation of repression by central

transcriptional regulators such as the catabolite repression protein CcpA and the global nitrogen metabolism regulating protein CodY which together affect roughly 200 genes in *L. lactis* [47,50,51]. Interestingly, amino acids such as arginine [51,52], branched-chain amino acids [45••], and citrate [53] are commonly found to be the alternative source of ATP generation under carbohydrate starvation. [50]. Their catabolic pathways are directly responsible for the production of potent flavour volatiles in (dairy) fermentation [3]. Hence, the flavour forming capacity of LAB seem to be highly dependent on the adaptation toward nutrient starvation and its corresponding non-growing state.

Metabolic stability of non-growing LAB during cheese ripening

A number of studies assessed the transcriptional response of lactococci during cheese ripening [54–56]. *L.* lactis typically reaches stationary phase of growth within 24 hours after milk inoculation, which is accompanied by the downregulation of ribosomal protein levels [55]. While the total residual lactose concentrations vary in different cheese types [57], the limited diffusion and exhaustion of lactose in a cell's proximity is described to induce a carbon starvation response [55]. General stress responses, including increased expression of acidic and oxidative stress proteins, are induced at the early stage of cheese ripening and support long term cell survival [55]. Although starter culture colony forming unit recovery declines quite drastically during cheese ripening, for example, 20-fold in 28 days [1], cell membranes of these non-growing starter cultures are reported to remain mostly intact for extended periods [37,58], despite a higher level of observed permeability [59,60]. These apparently intact but non-growing/dormant cells [2,37] are probably of key-importance for the flavour volatiles that keep accumulating throughout a year of ripening, particularly those derived from amino acids [61].

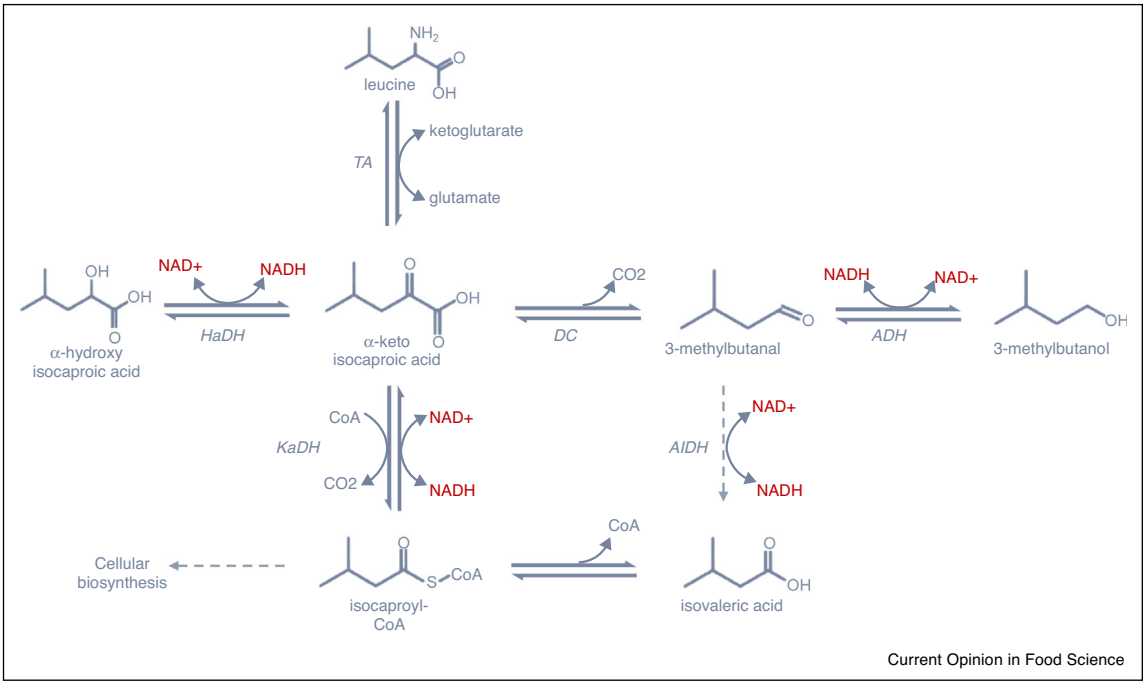
It is remarkable that the production of volatiles as a result of cell metabolism can be observed for prolonged periods in non-growing cells because proteins are considered to be notoriously instable and at constant risk of misfolding, damage, and aggregation [62]. However, intracellular macromolecular crowding is suggested to prolong enzyme functionality and stability by interactions between proteins [63] that can even contribute to renaturation of partially misfolded proteins. In contrast to the observed stability of amino acid conversion in intact cells for up to years [45••], *in-vitro* activity of enzymes typically declines dramatically within a few hours of conversion at maximum rate. In intact cells, protein integrity is ensured by chaperons [64] as well as the recycling of denatured and damaged proteins after active degradation through, for example, Clp proteins [65]. These mechanisms that minimise enzyme decay is not only crucial for general survival and adaptability [66] but also potentially for prolonged metabolic conversion.

Taken together, we hypothesise the proteome changes, turnover, and repair and renaturation processes in starter cultures are important for their functionality throughout cheese manufacturing and ripening. However, little is known about the protein dynamics under these conditions. This lack of investigation is likely due to technical challenges of working in food matrices. Because of the instability of mRNA, transcriptome data does not reflect the actual level of enzymes well [67] especially after prolonged incubation where translation is hampered by energy and resource deprivation. The combination of the very few generations made in cheese with the limited protein turnover during non-growing cheese ripening, raises the question whether at least part of the cellular functionality established during starter preparation is retained throughout ripening. In the absence of active protein degradation, growth of 4–6 cellular divisions during cheese production implies that the initial starter culture proteome may still constitute 1.5–12.5% of the cellular proteome during ripening [1]. This suggests that proteins that are expressed at a low level during starter preparation might disappear or get reduced to a level that has no impact on a functional property. On the other hand, highly expressed proteins may still be present in a small but adequate amount to influence metabolite formation over the extended period of ripening time. Understanding whether such a lasting impact of biological response exists and if it has functional consequences is relevant for the optimisation of starter cultures through altering preculture conditions.

Flavour formation and the role of cell lysis during cheese ripening

In cheese and other fermented foods, volatile formation is most prominent during the ripening or maturation stage. The use of processes or strains that result in cell lysis during cheese ripening has been widely investigated in the last few decades and considered as an important factor [8,68,69]. The underlying reasoning is that cell lysis leads to the release of intracellular enzymes into the food matrix, and consequently enhance macromolecule degradation for example, proteins [8,70] and fats [69]. Their degradation products subsequently become the precursors for the following cascade of reactions that lead to flavour volatiles. For example, the formation of key volatiles in cheese such as 3-methylbutanal, phenylacetaldehyde, and methanethiol is derived from amino acid conversions [3,71]. Therefore, it is believed that enhanced lysis leads to increased flavour formation. However, we consider that the evidence available for a direct and quantitative relation between cell lysis and specific production of flavour compounds remains unconvincing. The main reason for our doubts arises from the fact that many of the conversions required to achieve volatile

Figure 3



Metabolic pathway of leucine degradation to important volatile compounds for example, 3-methybutanal, 3-methylbutanol, and isovaleric acid. Reactions that rely on NAD + and NADH are highlighted in red. TA = transaminase, HaDH = hydroxy acid dehydrogenase, DC = keto acid decarboxylase, ADH = alcohol dehydrogenase, AlDH = aldehyde dehydrogenase and KaDH = keto acid dehydrogenase. Adapted from Smit *et al.* [71].

formation depend on complete pathways involving multiple co-factor dependent enzymes (Figure 3). Without the efficient co-factor regeneration that can occur within an intact cell, these reactions are not likely to be sustained and thus would rapidly decline in extracts of lysed cell that are released in the cheese matrix. Moreover, compared to freely dispersed enzymes, intracellular enzyme activity could persist much longer due to renaturation and chaperone-mediated enzyme maintenance and repair [72].

Nevertheless, enhanced lysis was shown to consistently correlate with increased abundance of peptides and amino acids [8,68,70,73] and fatty acids in the cheese matrix [69], and has also been reported to enhance removal of bitter-peptides [68,70,73]. However, the relation to product-flavour was mostly demonstrated by descriptive sensory studies [73], without actual measurement of volatile compounds using GC–MS analysis or similar approaches. In addition, these *in situ* lysis studies compared different bacterial strains with highly different flavour forming phenotypes [69,70] or mixtures of different starter strains [68,73] with highly variable genomic content and physiological characteristics [29]. These variations, disallow generic conclusions about flavour formation resulting from increased cell-lysis rather than from confounding factors such as metabolic diversity of genotypically distinct strains [69,70], microbial interactions in mixed starters [68,73] and/or distinct physico-chemical characteristics (e.g. pH or protein degradation) resulting from differences in acidification rates or proteolytic activity [70,73]. Despite our doubts concerning the importance of cell-lysis in flavour volatile formation during ripening, there may be a prominent role of subpopulation lysis especially in mixed-culture starters. In such mixed starter cultures, the lysis of one of the community members may contribute to milk-protein degradation and increased peptide and amino acid availability for other members of the starter culture that remained intact and effectively convert these compounds to their flavour volatile derivatives. However, to the best of our knowledge such cross-feeding interactions or syntrophic chains that lead to flavour compound production have not been reported to date [68,73].

In conclusion, at present, the evidence favouring a prominent role of cell lysis in flavour volatile formation during cheese ripening appears circumstantial, and more importantly, the majority of key-volatiles that determine cheese flavour can only be formed through an intact multi-enzyme pathway that in our opinion depends on cellular integrity. It is likely that volatile formation relies on the

interplay and the balance between lysed, intact non-growing, as well as (slow-) growing cells throughout cheese ripening.

Modulation of prolonged biocatalysis processes

For semi-hard cheeses, the ripening step can take place from 4 weeks to >1 year at controlled temperature <20°C. This process is relatively slow and expensive, and thus ways to modulate (i.e. accelerate) ripening is of economic interest. Current technologies to accelerate volatile formation include elevated temperature, high pressure, as well as the addition of exogenous enzymes [74]. Such technological development mostly focuses on the quality [75–77] and safety aspect of cheese rather than on the biological understanding of starter culture's behaviour which remains largely unexplored even in the standard conditions. Recent publications have trended to study the expression of genes important for flavour development and adaptation towards ripening conditions by employing techniques such as RT-qPCR [78,79], RNA-seq [55] and metatranscriptomics [80,81]. However, the eventual accumulation of flavour volatiles produced by non-growing starter cells heavily relies on the actual catalytic activity rate of the enzymes present in a cell which is typically not the subject of such studies. A remaining key-question is related to the mechanisms that underlie such long-term activity and its maintenance and whether it is possible to modulate and steer these mechanisms to control the kinetics and persistence of *in-situ* flavour formation. The aforementioned method that allows the determination of long-term measurement of metabolic activities in non-growing bacterial cells [46] can provide information about the stability of product formation, which has been reported to vary between (pre)-culture conditions and strains [7,45••,46,82].

Summary

Resource allocation theory suggests that growth modulation for example, during starter preparation might result in starter cells with altered level of metabolic enzymes. Because of limited divisions in cheesemaking, the impact of cultivation history during starter preparation might eventually influence cheese manufacturing since small differences in proteome composition can have significant functional consequences in a non-growing state. We think that the cell's ability to maintain membrane integrity and retain metabolic activity is important for the accumulation of flavour volatiles and that this is predominantly dictated by (the lack of) time-dependent decay of enzymatic conversions. The postulated importance of culture history for *in situ* starter functionality raises the question whether biomass-optimised, growth-rate-optimised and/or acidification-rate-optimised starter culture production regiments could have trade-offs for the downstream starter functionality. Alternatively, adjusting starter production regiments to optimize for cellular robustness and flavour pathway expression could lead to starter cultures with improved functional properties during application in cheese production (and ripening).

Funding

This study was funded by TiFN (Program 16MF01, Wageningen, The Netherlands).

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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