Invited review: Starter lactic acid bacteria survival in cheese: New perspectives on cheese microbiology

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ABSTRACT

The importance of starter cultures to cheese manufacture and ripening is well known. Starters are inoculated into cheese milk at a level of $\sim 10^6$ cfu/mL either from a bulk culture or using commercial direct-to-vat cultures. Before ripening, starters grow in the milk to reach populations of 10^7 to 10^9 cfu/g of curd depending on processing variables such as cook temperature, inclusion of washing steps, degree of partitioning with curds and whey, and importantly salt addition rate. Inherent strain-related properties also determine final populations in the curd following manufacture and include temperature sensitivity, salt sensitivity, presence of prophage, autolytic and permeabilization properties (which are influenced by processing steps), presence and type of cell envelope proteinase, and metabolic activity. Ripening of important industrial cheese varieties such as Cheddar, Dutch, Swiss, and Italian-type cheese varieties is characterized by extended storage under temperature-controlled conditions enabling characteristic flavor and texture development to occur. Over ripening, microbiological, biochemical and enzymatic changes occur with a decline in starter viability, release of intracellular enzymes, hydrolysis of proteins, carbohydrates and lipids, and formation of a range of volatile and nonvolatile flavor components. Recent reports suggest that starter strains may be present during the later stages of ripening and therefore their potential role needs to be reconsidered. This review will focus on our current understanding of starter viability and vitality during cheese ripening and will also review the area of starter permeabilization, autolysis, and enzyme release.

Key words: starters, autolysis, permeabilization, cheese, ripening, enzymes

STARTER LOCATION WITHIN THE CHEESE MATRIX

In cheese manufacture, starter strains are added to the milk and generate lactic acid-enabling gel syneresis, whey expulsion, and curd formation. Starters are entrapped within the curd ($\sim 90\%$) at the whey drainage stage of Cheddar manufacture (Doolan et al., 2014). Spatial location and colony distribution within the cheese have been foci of current research. Jeanson et al. (2011) used a gel cassette model system to examine the effect of inoculum level on colony distribution. The system used curd manufactured by UF and subsequently inoculated over the range 10^4 to 10^7 cfu/g before cheese manufacture and storage. Overall colony distribution in the cheese appeared to be random. Increasing inoculum level by a factor of 1,000 (from 10^4 to 10^7 cfu/g) resulted in colonies being 10-fold closer to each other in distance within the matrix. Average colony size also decreased when the number of colonies increased along with inoculum size. Colonies in cheeses arising from standard inoculum levels ($\sim 10^6$ cfu/g) were generally separated by 30 to 50 μm . However, increasing inoculum size from 10^4 to 10^7 cfu/g resulted in a 7-fold increase in interfacial area of colonies (cm^2) per cheese volume (cm^3). This finding is significant for estimation of contact area of bacterial enzymes to cheese matrix substrates. Le Boucher et al. (2015) monitored production of metabolites in UF, nonfat model cheeses having modified spatial distribution of cells with either large or small colony sizes. Viability and autolysis in cheeses were unaffected by colony size and autolysis did not appear to occur over 27 d of ripening. Diameter differed significantly between small ($\sim 8 \mu\text{m}$) and large colonies ($\sim 46 \mu\text{m}$) with a 5-fold larger surface area presented by the small colony bacteria to the matrix compared with the large colony cheese. Interestingly, significantly higher free amino acid (FAA) levels were generated from small colony cheeses. Generation of peptides (6 to 45 residues) indicated that $\sim 98\%$ of discriminant peptides were present in higher abundance in small colony cheeses by d 2 of ripening, many of which originated from β -CN. Likewise, volatile flavor compounds such as diacetyl were more abundant in cheeses containing

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small colonies. The authors concluded that the production of metabolites originated from live nonlysed cells and that metabolite abundance was influenced by spatial distribution of colonies within the cheese. Small colony cheeses presented a larger interfacial area for cell envelope protease (**CEP**) or lactocepin compared with larger colony cheeses. The authors suggest that diffusion of substrates did not appear dependent on colony size, but rather on other intrinsic properties.

Previously, dextrans of 4 to 155 kDa molecular mass were shown to diffuse into colonies, whereas BSA, lactoferrin, and α_{S1} casein were shown to diffuse up to the colony surface but were excluded from entering within the colony (Floury et al., 2015). Hence, Le Boucher et al. (2015) proposed that colonies may be either resistant to diffusion or that molecules are subjected to a process of selective entry into the colony. Laloy et al. (1996) indicated that location of colonies within cheese during ripening was affected by fat content. First, the level of starter bacteria populations retained in cheese increased with fat content. Subsequent electron micrographs showed 85% of cells were located on the periphery of the milk fat globule membrane (**MFGM**). A potential interaction between bacterial cells and MFGM was also noted during ripening whereby bacterial enzymatic activity or mechanical pressure appeared to have altered the thickness of the MFGM. Most importantly, although viable cells appeared to be located on the external face of the MFGM, lysed or “ghost” cells after 2 mo ripening appeared to be included into the MFGM or located on the inside of the MFGM. Lopez et al. (2006) examined the interaction between bacteria and fat globules in Emmental cheese. Confocal laser scanning electron microscopy followed bacterial localization during ripening and showed colonies located at the fat/protein interface while coalesced and free fat and bacterial colonies were entrapped within the casein matrix. Additionally, fat globules were also surrounded by whey pockets and enclosed by casein. This aqueous whey phase may contain proteins, sugars, and lipolytic breakdown products with bacterial colonies also present. Hannon et al. (2006) examined the effect of varying pH (6.6 to 5.2) at the renneting stage of UF cheese containing milkfat on autolysis of *Lactococcus lactis* subsp. *cremoris* AM2. Release of autolytic marker enzymes lactate dehydrogenase (**LDH**) or peptidase X (**PepX**) indicated an earlier and more extensive lysis at higher renneting pH values (6.6 and 6.2) compared with cheese renneted at pH 5.2. Cheese renneted at pH 6.6 had a coarse grainy texture different from the smooth texture of cheese renneted at 5.2. Renneting pH significantly affected cheese microstructure. In the former cheeses, large colonies were present with an uneven distribution, while at lower renneting pH,

small colonies or single cells were present and evenly dispersed within the protein matrix.

Locating bacteria in the cheese matrix and revealing their metabolic status will contribute to understanding the spatial variation in cheese ripening.

SINGLE STRAIN VERSUS MIXED STRAIN STARTER VIABILITY

Researchers in New Zealand in the 1960s and 1970s classified single strain starters based on differences in their viability during ripening. Studies demonstrated a relationship between starters with high viability over ripening and development of bitterness. Conversely, starters with low viability did not generate bitter flavor notes above threshold levels. This work led to the terms “bitter” and “non-bitter” being applied to various starter strains. Further examination of these starter groups indicated differing responses to Cheddar cooking temperature (typically 38.5°C) and tolerance to salt added to curds before pressing (typically at a rate of 2.7% wt/wt) (Martley and Lawrence, 1972; Lowrie et al., 1972). Starters that developed bitterness were associated with tolerance of both of the above parameters whereas nonbitter starters were associated with sensitivity to cooking temperature and salt, leading to a reduction in cell numbers from the cooking stage onward. Hence, the latter starters had curd populations on d 1 of ripening at least 1 to 2 log below those found for bitter strains. Nonbitter strains were typically *Lc. lactis* subsp. *cremoris* and included strains such as SK11 and AM2, whereas many of the bitter strains were found to be *Lc. lactis* subsp. *lactis* (Lowrie and Lawrence, 1972). The relationship between viability and bitterness was also investigated using a bitter strain exposed to phage during cheese manufacture, which resulted in lower than normal populations on d 1 of ripening and a reduction in bitterness in the cheese (Lowrie et al., 1974). Mills and Thomas (1980) provided evidence for the basis of bitterness based on the activity of CEP present at high levels in bitter starters and the associated inability to remove bitter peptides during ripening. High populations of lactococci having a CEP (Prt⁺) compared with the same strain without the CEP (Prt⁻) generated bitterness. Hence it was postulated that heat- and salt-tolerant starter strains having an active cell wall proteinase would generally lead to development of bitterness during ripening.

In the Netherlands, Exterkate and Visser from the 1970s to the 1990s demonstrated clear differences in specificity of CEP from bitter and nonbitter strains. The latter strains had CEP III type enzymes acting preferentially to hydrolyze α_{S1} -CN and κ -CN fragments during ripening, whereas bitter strains possessed CEP

I-type enzymes acting preferentially on β -CN. The latter casein fragment is a greater source of bitter peptides than either of the other casein substrates. Børsting et al. (2015) brought together the more detailed CEP classification provided by Exterkate et al. (1993) with an extension to 8 groups (a to h) by Broadbent et al. (1998) and further modeled the PrtP sequence variation to propose amino acid residues significant to the peptide cleavage activity. Groups a (example strain SK11) and b (strain AM2, S2) CEP proteins are associated with nonbitter strains, whereas groups g (strain HP) and h (strain S3) have been associated with bitter peptide production. Broadbent et al. (2006) showed that autolysis was unaffected by allelic exchange of catalytic sites among groups b and h, but detection of LDH was done on 24-h LM17 culture supernatants, which may have mitigated any differences among the strains. Viability of bitter strains in Cheddar cheese curd was higher on d 1 of ripening than that of nonbitter starter strains, whereas the latter subsequently showed greater loss of viability during extended ripening (Martley and Lawrence, 1972; Wilkinson et al., 1994).

Strain and species differences in viability have been noted for thermophilic strains including *Lactobacillus* and *Streptococcus* starters and *Propionibacteria* in Emmental type cheeses (Valence et al., 1998). These workers monitored individual autolytic profiles of *Lb. helveticus* and *Propionibacterium freudenreichii* using immunoblotting of PepD dipeptidase or methylmalonyl coenzyme A mutase, respectively. Autolysis of *Lb. helveticus* initially occurred following pressing of the cheese and increased thereafter from entry into the cold room to the end of ripening. In contrast, *P. freudenreichii* appeared to autolyze to a much lesser extent and much later during cold room storage (d 92). Analysis of commercial Comte, Parmesan, and Beaufort cheeses indicated the presence of PepD, suggesting a generalized autolysis of *Lb. helveticus* also occurred within these varieties. Valence et al. (2000) examined autolysis of 2 *Lb. helveticus* strains in brined or unbrined Emmental cheeses using both PepD and LDH as markers of autolysis. Significant differences were noted between both strains, one of which exhibited an earlier and more extensive lysis. The absence of brining did not have an effect on autolysis of either strain, and while autolysis differed substantially between strains (5- to 7-fold difference in LDH), differences in proteolysis were less evident (~2-fold), indicating factors such as enzyme and substrate diffusion may also influence proteolysis. Mesophilic species such as *Leuconostoc* play a role in ripening of Dutch and Danish varieties where they generate aromatic compounds from citrate. Cibik and Chapot-Chartier (2000) examined the autolysis of 59 strains of *Leuconostoc* sp. under starvation conditions

in buffered systems held for 24 h at 30°C. A wide variation ranging from ~10% to 50% autolysis (measured by reduction in optical density at 600 nm) was noted between strains. Carbon source appeared to affect autolysis of one strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* that was chosen for further study, with highest autolysis detected when glucose was present. Peptidoglycan hydrolase activity was present in 18 of 29 strains examined. This initial evaluation suggests strain-related differences in autolysis for *Leuconostoc*, which requires further evaluation in cheese trials.

Viability of mixed strain starters during ripening suffers from the difficulty of assigning loss in viability to one particular strain. This is especially true when monitoring viability by plate counting on media that support the growth of several strains within the blend, which cannot be differentiated from each other. Wilkinson et al. (1994) examined viability of a 2-strain combination of G11/C25 starter compared with single strains of AM2 and HP during Cheddar cheese ripening. High viability was noted for the 2 strain blend; however, release of intracellular enzymes in the cheese indicated that one of the strains had a significant degree of autolysis (and presumably a loss in viability) that was not reflected in viable plate count data and was likely masked by higher survival of the other strain in the blend. This illustrates the limitations in monitoring strain-dependent responses and the necessity for alternative methods for distinguishing among strains.

METHODS TO ASSESS STARTER VIABILITY IN CHEESE

Ongoing research in starter viability measurement combines or even compares and contrasts both culture-dependent and culture-independent approaches. The most common media used for culture-dependent methods include M17 supplemented with lactose for enumeration of mesophilic lactococci, de Man, Rogosa, and Sharpe (MRS) for lactobacilli, and Lactobacillus Selective (LBS) Agar for monitoring of thermophilic lactobacilli and M17 for *Streptococcus thermophilus*. A cheese homogenate is prepared in a protein-solubilizing buffer (2% trisodium citrate) followed by dilution to levels that enable populations of between 20 and 200 colonies to be counted on plates following incubation. However, lactococci form chains, and in some cases, it may be necessary to include a dispersion step to ensure single cells are counted and indeed not all cells go on to form colonies. Plate counting has the advantage of being acceptable by regulatory agencies, often seen as the “gold standard” against which all other methods are judged (Wilkinson, 2018). One major disadvantage of viable plate counting is the general inability to

distinguish single strains of the same species within a starter blend. A key point is that although starters are dominant during early ripening, growth of nonstarter lactic acid bacteria (**NSLAB**) from initial low levels, $\sim 10^2$ to 10^3 cfu/g, reaching 10^6 to 10^8 cfu/g may limit usefulness of starter plate count data to 6 to 10 wk of ripening because overgrowth by NSLAB on starter media may render data unreliable (Crow et al., 1995a; O'Donovan et al., 1996). Nonetheless, this methodology is still widely used in the dairy industry, but more rapid and precise methods are clearly required.

Culture-independent methodologies can be divided further into (1) genetic methodologies based on monitoring of DNA/RNA release, (2) immunological labeling, and (3) flow cytometry including differential staining.

(1) Genetic methodologies employed by Ohmiya and Sato (1970) showed that in aseptic rennet curd, autolysis of cells was reflected by an increase in cell-free DNA. The use of genetic technologies was initially hampered by obstacles including degradation of DNA/RNA by nucleases, a lack of specificity as to whether it originated from starter or NSLAB, an inability to determine its origin from a particular strain within a mixed culture, and difficulty in assigning its source as from viable, viable but nonculturable (**VBNC**), or dead cells. A significant number of these technical issues have now been overcome through advances in molecular biological techniques. Treimo et al. (2006a) used a 16S rDNA microarray to quantify total bacterial DNA and species-specific DNA within mixed strain cultures. The limit of detection for total bacterial DNA was estimated as 10^4 cfu/mL, whereas species-specific probes enabled DNA content from *Lc. lactis* subsp. *lactis* and *P. freudenreichii* to be differentiated and quantified. This approach was used to study autolysis of mixed strains in buffer and in a liquid cheese model by quantifying free DNA released from *Lc. lactis* subsp. *lactis* and *P. freudenreichii* within a mixed culture (Treimo et al., 2006b). In buffers, an increase in free DNA corresponded to a decrease in absorbance at 600 nm and appeared to reflect autolysis of the strains. Degradation of free DNA was overcome by use of an internal DNA degradation standard, whereas species-specific 16S rRNA gene probes tracked autolysis of individual strains within a mixed culture. However, extended monitoring of autolysis was affected by DNA degradation and hence this method may be more suited to tracking of early autolysis.

The application of various formats of PCR has enabled major advances in all aspects of lactococcal molecular biology including its use in monitoring autolysis. Gatti et al. (2008) applied length-heterogeneity PCR to investigate autolysis of starter bacteria over 24 mo of ripening Parmigiano-Reggiano (**PR**) cheese. While

increases in DNA were noted in whole (unlysed) cells of strains from the natural whey starter during ripening, no growth was noted of these strains on agar media indicating the possibility that cells were in the VBNC state. However, while this technique did not quantify autolysis, it enabled study of population dynamics of different strains and species of a mixed culture during ripening. Lazzi et al. (2016) used length-heterogeneity PCR and activity of an intracellular peptidase to monitor autolysis in Grana Padano cheese and investigated the evolution of volatile flavor compounds during ripening. Between 2 commercial cheese manufacturing runs, one had an earlier and more complete autolysis than the other after 2 mo of ripening. Both starter autolysis and intact NSLAB cells were required for correct flavor development. Expression of the phage ϕ vML3 lysin gene in *Lc. lactis* indicated the importance of this gene product in lactococcal autolysis (Shearman et al., 1992). However, although viability of the construct strain declined rapidly, evidence of release of intracellular material was not provided. Overall, although direct detection of autolysis by genetic methods has been limited, these techniques have provided a deeper understanding of the molecular basis of autolysis.

(2) Immunological labeling: autolytic marker enzymes have been detected using polyclonal or monoclonal antibodies. Chapot-Chartier et al. (1994) used rabbit antisera to detect marker enzymes released by autolysis in St-Paulin cheese in a sandwich ELISA method. Antisera were conjugated to biotin with a detection limit of 4 ng/mL for both enzymes in cheese homogenate samples. *Lactococcus lactis* subsp. *cremoris* AM2 displayed an early and extensive release of PepX up to d 10, but which decreased thereafter. A slow continuous increase in quantity of PepC was detected. Generally, the ELISA method showed similar trends to activity found using synthetic substrates illustrating strain-related differences in autolysis. Overall, immunodetection is an interesting methodology for autolysis but detects a quantity of enzyme rather than activity at sampling. A true assessment of the release of marker enzymes requires measurement of both the quantity and activity of the enzymes. The quicker route toward immunodetection uses polyclonal antibodies; however, without extensive screening, these may cross-react with other cellular components. Development of monoclonal antibodies to marker enzymes or other cell epitopes is expensive and technically challenging and requires highly purified antigens that may not be conserved in all strains or even in the same strain under different environmental conditions. Immunomagnetic capture of particular cell subpopulations such as permeabilized cells is an avenue that has not as yet been applied to

the study of lactococci in cheese, but which may yield additional insights.

(3) Flow cytometry is a promising method for rapid bacterial viability (and nonviability) analysis. Individual cells within a liquid sample are counted based first on the differential light-scattering properties of cells (to distinguish cells from matrix interference), and second, differential labeling of intact or live cells from dead or damaged cells using a combination of permeant and impermeant fluorescent stains (Wilkinson, 2018). The most commonly used viability stains are a combination of SYTO9 and propidium iodide (**PI**). SYTO9 is a DNA stain that enters all cells within a sample and generates green fluorescence; thereafter, PI is used to counterstain the cells. Entry of PI only occurs in cells having damaged or compromised membranes, which then displaces SYTO9 green fluorescence. The amount of red fluorescence is related to the extent of cell damage. Cytographs of starter populations usually contain 3 regions: (1) SYTO9+, PI−, indicates a highly viable/intact cell population, (2) PI+ SYTO9− indicates a highly damaged/permeabilized or nonviable population, and (3) SYTO9+ PI+, a so-called double-positive population, indicates cells displaying a degree of damage or permeabilization whose ability to reproduce and grow is unknown. Data are typically displayed as a dot-plot on the screen, and spatial location of each data point is classified into the above 3 regions based on creation of control “gates” from live, dead, and damaged or permeabilized cells.

The lack of a general uptake by regulatory agencies for a flow cytometry-based method is an issue (although a flow cytometry-based method of probiotic viability has been accepted by the International Organization for Standardization and the International Dairy Federation, Wilkinson, 2018). Bunthof et al. (2001a) evaluated several stains for monitoring starter viability in cheese, including the fluorescent esterase substrate carboxyfluorescein diacetate cFDA, which indicates intracellular esterase activity, and the use of membrane impermeant stains TOTO-1 and PI for labeling dead cells. The authors concluded that cFDA was suitable for specific labeling of live cells within a population, whereas TOTO-1 was considered superior to PI for labeling of damaged or dead cells due to its high yellow fluorescence, larger size, and lower membrane partitioning. Culturability of strains as monitored by plate counts and cFDA with TOTO-1 staining indicated similar trends; however, a statistical correlation was not made between both sets of data. Overall, a combination of cFDA and TOTO-1 appeared suitable for starter viability monitoring. Bunthof and Abee (2002) applied this staining combination to determination

viability of lactic acid bacteria (**LAB**) strains including: *Lactiplantibacillus plantarum* cells grown in skim milk, a mixed strain commercial cheese starter at both mother culture and at bulk culture stages, and 2 yogurt starters containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (*Streptococcus salivarius* subsp. *thermophilus*). A commercial milk clearing solution reduced background interference by a factor of 10,000. The lower limit of detection for *Lactiplantibacillus plantarum* populations added to milk was 10^5 cfu/mL using the following stains for total cell counts; SYTO9 as a single stain, and a combination of cFDA and TOTO-1. A strong correlation for all stains ($r > 0.99$) with plate count viability data was found over the range 10^5 to 10^9 cfu/mL of added *Lactiplantibacillus plantarum*. Subsequently, cFDA and TOTO-1 were used for enumeration and differentiation of live from permeable or nonviable cells in commercial probiotic products for both. Interestingly, flow cytometry analysis of the commercial cheese and yogurt starter cultures gave cell counts 3 to 5 times higher than plate counting, possibly as flow cytometry staining counts all cells with intact membranes, but which may not necessarily grow on solid media. Indeed, up to 50% of cells in commercial starter cultures in the mother preparation were present in a permeabilized state.

The flow cytometry data do not always agree with plate count data because of additional factors such as exposure of cells to stressors during processing and changes in microbiota during storage and ripening. Cell sorting enables discrete subpopulations identified by flow cytometry to be physically sorted for further analysis including whole genome sequencing, proteomics, and microscopy. Erkus et al. (2016) used both fluorescence-activated cell sorting and genetic methods to monitor the proportion of live, injured (permeable), and dead *Lactococcus* cells over 6 wk ripening of Gouda cheese. The DNA was extracted from cells treated with propidium monoazide (**PMA**) followed by quantitative PCR (**qPCR**), preventing DNA amplification from damaged or dead cells. Only the live, viable population is subsequently quantified by qPCR. Validation of the PMA method was achieved using sorted cell subpopulations, and PMA-quantified populations had a 1.7-log decrease in DNA copy number over 6 wk of ripening, whereas the intact cells measured by cell sorting dropped from 90% to under 5%. These results supported a limit of detection using the PMA treatment that was not seen with the fluorescence-activated cell sorting method. The proportion of injured cells (double-positive stain) increased from about 25% at 2 wk to over 90% at 6 wk of ripening (estimated at 8 logs). These authors showed that survival rate differed among 7 genetic lineages of

Lc. lactis, using metagenomic mapping to corroborate the contribution of the lineages to the 6-wk-ripened cheese microbiota.

In summary, the combination of multiple methodological approaches continues to be suitable, given the distinctions in the types of cell properties that can be monitored, and their relation to cell structure and viability.

STARTER AUTOLYSIS DURING CHEESE RIPENING

Determination of starter LAB autolysis in cheese during ripening has long been a topic of research using several approaches including (1) direct microscopic visualization of cells; (2) tracking release of intracellular marker enzymes or intracellular materials such as DNA, RNA, or nucleotide compounds; and (3) gene or protein expression. Direct visualization, principally through electron microscopy, continues to be used in studies of starter autolysis in cheese. Visualization of colonies within the cheese matrix, and hence inferring autolysis, relies on quantifying to some degree a reduction in intact/live cells over ripening together with the appearance of spheroplasted/damaged or “ghost” cells generated during the nonviable and autolytic phase (Hickey et al., 2015). Through this work, it is clear that (1) cell morphology changes during ripening while a structural disintegration suggests autolysis, (2) strain-related differences in cell integrity and autolysis exist, and (3) colonies are located at the protein-fat-moisture interface with apparent integration of the bacterial membrane with MFGM during autolysis (Laloy et al., 1996).

Monitoring Autolysis Through Tracking the Release of Intracellular Components

Ohmiya and Sato (1970) monitored the release of nucleotide material in “cheese-like medium” to demonstrate autolysis of starters. Several studies used the release of intracellular enzymes to detect autolysis. Law et al. (1974) demonstrated release of an intracellular peptidase in Cheddar cheese extracts prepared using added starter culture and not in an aseptic cheese. Activity of the released intracellular dipeptidase increased with the decline in viable starter numbers and was detected over a 120-d ripening period.

Law et al. (1976) also monitored release of an intracellular peptidase in cheese extracts following treatment of starter cells by lysozyme, which rendered them more susceptible to autolysis on salting of cheese that was reflected in increased FAA production. The use of marker

enzymes such as PepX or LDH to monitor autolysis in cheese have demonstrated the strain-dependent nature of starter autolytic properties.

Wilkinson et al. (1994) monitored both enzymes in cheese “juice” extracted through hydraulic pressure over ripening in Cheddar cheese manufactured using single strains of *Lc. lactis* subsp. *cremoris* AM2 or HP and from a 2-strain starter system G11/C25. A lower viability and higher release of intracellular PepX and LDH was detected from the “non-bitter” temperature and salt-sensitive strain AM2 compared with the “bitter” HP strain. Production of FAA and low molecular mass peptide material was highest for the autolytic AM2 strain.

Chapot-Chartier et al. (1994) tracked and compared autolysis of strain AM2 with *Lc. lactis* subsp. *lactis* NCDO 763 in St. Paulin cheese during ripening. The PepX and the aminopeptidase PepC were used as marker enzymes in cheese extracts and strain AM2 was again demonstrated as highly autolytic. Autolysis of lactobacilli and *Propionibacteria* in a range of cheese varieties including Swiss type cheeses was demonstrated using marker enzymes (Lortal and Chapot-Chartier, 2005). The marker enzyme approach of monitoring of autolysis in cheese is highly useful but requires that the marker is normally present in the target strain, has an exclusive intracellular location, can be assayed by using highly specific substrates, is stable in cheese, and measurable activity can be recovered from cheese extracts.

A key issue is to what degree activity measured at a particular time point represents that actually released by cell autolysis on the day or is a result of cumulative residual activity and as such is related to enzyme stability. Permeabilized cells may contribute to released intracellular enzyme activity and hence it may be difficult to quantify actual net starter autolysis at a particular point.

Mechanisms of Autolysis in LAB

In the 1990s, workers in the Netherlands succeeded in isolating the gene coding for the peptidoglycan hydrolase or autolysin from *Lc. lactis* subsp. *cremoris* and has been reviewed by Lortal and Chapot-Chartier (2005).

The *acmA* gene codes for a 46-kDa precursor protein that is processed into an active ~40-kDa AcmA autolysin, able to cleave *N*-acetylmuramyl-1,4- β -*N*-acetylglucosamine bonds in the peptidoglycan. A *Lc. lactis* subsp. *cremoris* strain with a deletion of *acmA* had an absence of autolysis compared with the wild-type strain and altered morphology, resulting in the formation of very long chains compared with the wild-

type strain. In a later study, a wild-type strain carrying multiple copies of a plasmid containing *acmA* showed increased autolysis (Buist et al., 1995, 1997).

A relationship between autolysin activity and specificity of the lactococcal CEP was subsequently demonstrated by Buist et al. (1998). The AcmA was degraded by CEP, as proteinase negative cells possessed the 40 kDa AcmA, whereas cells expressing secreted or cell wall-anchored CEP did not show secreted autolysin enzyme. Cells expressing CEP I-type activity exhibited reduced autolysis, whereas cells expressing a hybrid proteinase with a specificity intermediate between CEP I or CEP III had maximum autolysis. Autolysin activity in culture supernatants was lowest when cells expressed the anchored form of CEP I, thus showing the significance of binding to the cell wall. The secreted form of CEP I exhibited highest hydrolytic activity toward AcmA, reducing autolysis but not inhibiting release of PepX.

Pillidge et al. (1998) studied the effects of deletion of the *acmA* gene on autolysis of 2 highly autolytic *Lc. lactis* subsp. *cremoris* strains in buffer, reconstituted skim milk (**RSM**), and cheese, showing a degree of correlation between the high degree of autolysis found in buffer and in RSM for both these strains. However, screening of autolysis of a range of other lactococcal strains indicated differences between these media in terms of the levels of released intracellular marker enzyme fructose-1,6-biphosphate aldolase or decrease in absorbance at 600 nm with certain strains showing higher autolysis in buffer compared with RSM and vice versa. A lactococcal strain (*Lc. lactis* subsp. *cremoris* 2250) with the *acmA* gene deleted exhibited lower levels of autolysis in RSM compared with the wild-type strain, but this mutated strain also showed higher levels of autolysis compared with many of the other strains.

In Cheddar cheeses made using the wild-type strain or the deletion mutant, little difference in autolysis up to 18 d of ripening was noted, suggesting that factors other than AcmA may influence autolysis in cheese.

Pillidge et al. (2002) used Western blotting with an AcmA antiserum to detect several autolysin activity bands present in lactococcal strains, including in an *acmA* deletion mutant, indicating the potential presence of additional cell wall hydrolase activities, which may influence autolysis.

The presence of 4 additional autolysin enzymes denoted as AcmA, AcmB, AcmC, and AcmD was demonstrated in *Lc. lactis* (Visweswaran, et al., 2013). The AcmD and AcmA show 58% homology between the protein sequences. The AcmD had peptidoglycan hydrolyzing activity only at pH 4.0, whereas AcmA had activity at pH 4 and 8. The AcmD appeared to be

involved in cell separation as deletion mutants lacking this gene formed long chains and sedimented to a greater extent than the wild-type parent strain. The enzyme was secreted from the cell at pH values between 6 and 7 but the secreted form was not active.

Interestingly, overexpression of *acmD* showed increased cell autolysis only in the presence of AcmA, suggesting potential differences in site specificity as well as the pH dependence of cell binding by the various autolysin enzymes.

Poquet et al. (2000) identified membrane-bound serine protease, which functioned as a surface housekeeping enzyme involved in processing of proteins including a nuclease and the AcmA enzyme. Inactivation of the *htrA* gene resulted in *Lc. lactis* becoming temperature sensitive as growth at 39°C was lower compared with 30°C with a significant decrease in cell viability, indicating the importance of this protease in enabling growth of *Lc. lactis* at higher temperatures.

At lower growth temperatures (30°C), HtrA appears to degrade abnormal proteins including cleavage of AcmA into smaller extracellular active proteins that affect peptidoglycan binding. The authors speculated that the latter active autolysins may also be active against competitor microorganisms because uncleaved AcmA was active against the lactococcal cell wall, whereas the degraded subunits were not.

Steen et al. (2003) elucidated the mode of action and binding of AcmA to peptidoglycan. The enzyme has an N-terminal active site domain required for autolysis and intracellular PepX release. The C-terminal region consists of 3 homologous repeats of 45 amino acids known as LysM domains that bind specifically to the cell wall peptidoglycan of lactococci at the septum and pole regions. D-Alanine depletion described by Steen et al. (2005) is another factor related to HtrA and its influence on lactococcal autolysis. D-Alanine is required by the cell for the D-alanine-D-alanine dipeptide cross-linking component of peptidoglycan. The gene *alr* codes for an alanine racemase that converts L-alanine to D-alanine, and in *alr* deletion mutants, rapid autolysis occurred when D-alanine was removed from the medium.

Lipoteichoic acid (**LTA**), a structural component of the cell wall, prevents binding of AcmA to sites other than the poles and septum of the dividing cell (Steen et al., 2003). Modification of LTA can also occur with D-alanine and is encoded by 4 genes within the *dlt* operon, where *dltD* codes for ligase and thioesterase activities involved in D-alanylation of LTA. Deletion of *dltD* resulted in increased autolysis, whereas a strain constructed without *dltD* and *acmA* also showed autolysis but with a reduced cell septum thickness in cells with only the *acmA* deletion. The importance of the

D-alanylation of LTA was indicated by slower growth rate and increased autolysis in the *dltD* deletion mutant, but without affecting binding by AcmA or septum thickness.

A role for HtrA in regulating the activity of AcmA was suggested by Steen et al. (2005) who noted that as HtrA degraded AcmA into 2 autolysins, which had reduced binding capacity to the cell wall, it may therefore provide a control mechanism for an enzyme that could otherwise prove lethal to the cell.

Pang et al. (2014) cloned and sequenced the *N*-acetylmuramidase (*mur*) gene from *Lb. bulgaricus* with a view to understanding its regulation and its relationship to the autolytic phenotype using flow cytometry analysis. Knocking out the *mur* gene resulted in a reduction, but not a complete loss, of autolysis compared with the wild-type strain. The experimental knockout strain was more filamentous and more connected to other cells compared with the wild type. This work indicated a significant role for the *mur* gene but also indicated that it was not entirely responsible for cellular autolytic activity. Indeed, genome analysis of the *Lb. bulgaricus* strain indicated the presence of sequences likely to code for other autolysin enzymes.

Pang et al. (2017) further investigated the effect of bacterial sensing via a 2-component system on the autolytic behavior of a *Lb. bulgaricus* strain. A series of gene knockouts of 2 histidine protein kinase (HPK) genes resulted in a reduced autolysis rate, increased cell density, and the presence of damaged cell walls as visualized by electron microscopy. However, evidence for a regulatory interaction of the 2-component system with the autolysin enzyme *N*-acetylmuramidase has yet to be provided.

The relationship between CEP specificity and autolysin activity is not clear; whereas CEP I appears to influence autolysis by cleavage of AcmA in certain strains (Buist et al., 1998), other studies have shown that this may not be the case.

The influence of CEP specificity on autolysin (AcmA) degradation was examined in a range of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* strains by Govindasamy-Lucey et al. (2000). No effect of CEP specificity on the rate of autolysis of the test strains was noted in RSM, and in the case of a CEP negative strain, the rate of autolysis was actually twice that of the parent strain (CEP+). Govindasamy-Lucey et al. (2000) also showed that a strain with the *acmA* gene deletion still displayed a significant rate of autolysis. The effects of genetic changes in CEP specificity on autolysis as monitored by LDH release was investigated by Broadbent et al. (2006), who did not find any difference between wild-type and altered strains.

Further evidence was recently provided by Huang et al. (2020) for a relationship between proteolysis, cell wall synthesis pathways and resultant autolytic phenotype of *Lc. lactis* subsp. *cremoris* MG 1363. Deletion of the gene coding for PepV, a dipeptidase involved in the terminal stages of peptide hydrolysis, resulted in reduced growth, a prolonged lag phase, with an altered morphology indicative of cell autolysis compared with the wild-type MG1363 strain. This mutant strain was denoted as MG Δ PepV.

Addition of alanine alone to MG Δ PepV restored growth to wild-type levels, which indicated a relationship between alanine levels, PepV activity, and peptidoglycan synthesis. The level of dead cells of the mutant strain was higher; however, this strain also recommenced growth with live cells increasing after an extended lag phase of ~40 h. Within the mutant strain, a faster growing subpopulation was detected with single isolates having a distinct phenotype denoted as MG Δ PepV*. Examination of MG Δ PepV* over repeated growth cycles indicated that its growth rate may have arisen from a stable gene mutation.

A microscopic comparison showed that MG Δ PepV had a higher degree of autolysis and some transient “ballooning” potentially caused by fusion of cell membrane material compared with MG Δ PepV*. Both mutant strains had a single point mutation (CGT to TGT) in the MG Δ PepV* strain compared with the parent MG Δ PepV strain. This single base change mutation resulted in the replacement of a single amino acid residue 218 in MG Δ PepV* within the pleiotropic repressor protein CodY, which replaced a charged arginine with a noncharged cysteine residue. This change may have affected binding of DNA to the repressor CodY protein with upregulation of the CodY regulon thus enabling re-growth of MG Δ PepV* in the presence of glycine compared with MG Δ PepV. The dipeptide transporter genes *dppA* and *dppT* were also upregulated in the MG Δ PepV* compared with MG Δ PepV.

This concerted upregulation may enable increased transport and intake of glycine-containing peptides (as an alternative source of alanine for cell wall synthesis) into MG Δ PepV* cells. This emphasizes the importance of D-alanine within the peptidoglycan structure, suggesting that any alterations to proteolytic pathways generating an adequate supply of D-alanine may have 2 consequences: (1) a cell wall structure more susceptible to AcmA autolysin, or (2) a reduction in D-alanylation of LTA resulting in decreased AcmA degradation and increased autolysis.

The role of PepV in peptidoglycan synthesis has not yet been elucidated, but the above evidence points to a stronger relationship between proteolytic and cell

Figure 1. Overview of interactions between autolysis, proteolysis, peptidolysis, peptidoglycan (PG), and lipoteichoic acid (LTA) synthesis in *Lactococcus lactis*. Autolysin AcmA is produced inside the cell as a 46-kDa precursor. Secreted mature autolysin AcmA (40 kDa) cleaves *N*-acetylmuramyl-1,4- β -*N*-acetylglucosamine bonds, weakening the peptidoglycan, promoting autolysis and release of intracellular peptidases such as PepX. Deletion of AcmA results in nonrelease of intracellular PepT. Secreted autolysins are subject to proteolytic hydrolysis by cell envelope protease (CEP, where CEP I-type enzymes have higher activity on AcmA than CEP III) as well as by the HtrA surface protease. Both sections of HtrA-cleaved autolysins show reduced cell wall binding activity. The N terminus region of AcmA influences PG binding, intracellular PepX release, and the interaction of mature AcmA with AcmD. The C-terminal region of AcmA contains LysM domains that bind to PG at the septum and pole regions of the cell. The LTA prevents AcmA from binding at these regions. Deletion of intracellular PepV reduced growth, prolonged lag phase, and altered morphology with increased autolysis. CodY is a pleiotropic repressor protein that is responsive to branched-chain amino acid levels in the cell and is the main regulator of proteolysis. However, a single point mutation in a faster growing subpopulation of PepV deletion mutants affected binding of DNA to CodY and upregulated peptide transporters to provide substrates for PepV, including D-alanine for PG and LTA synthesis. However, it appears this relationship may hold only for this faster growing subpopulation and hence the question mark for the effect of PepV on PG synthesis. Alr, alanine racemase, carries out alanine racemization, whereas DltD carries out D-alanylation of LTA. Opp refers to the oligopeptide permease; Dpp refers to the dipeptide permease.

wall biosynthetic pathways (Figure 1). It is interesting to speculate as to whether the 3 phenotypes (wild type, $MG\Delta PepV$, and $MG\Delta PepV^*$) bear an analogy to starter populations in cheese during ripening.

Wild-type strains have inherent responses to cooking temperature, salt, and prophage during cheese manufacture, resulting in characteristic viability profiles for either bitter or nonbitter starters. Thereafter, during cheese ripening, $MG\Delta PepV$ may represent strain-related patterns of permeabilized or autolyzing cells while $MG\Delta PepV^*$ may represent long surviving starters (Desfossés-Foucault et al., 2013; Ruggirello et al., 2016) who have adapted to the cheese environment by

using alternative pathways to secure alanine or other nutrients, enabling a degree of re-growth following cell wall repair or synthesis.

Following the detection of a new subpopulation of starter cells by Yanachkina et al. (2020), we can propose an overall view of LAB viability (Figure 2) showing strain-related differences in accessibility to PepX substrates following cooking temperature, but which persist during storage in cheese model systems. It is possible that this subpopulation has particular phenotypic advantages and may form the nucleus of the extended survivor cell subpopulation akin to the $MG\Delta PepV^*$ cells noted by Huang et al. (2020).

Studies have indicated that permeabilization of strains such as *L. lactis* subsp. *cremoris* HP is not accompanied by significant enzyme release (Sheehan et al., 2005; Yanachkina et al., 2020). These observations may indicate that cellular conservation mechanisms are at play to ensure a functioning cell membrane, preventing loss of key peptidases and associated peptide transport systems to ensure an adequate supply of key amino acids and other nutrients during dormancy in cheese ripening (Figure 2).

Cell Starvation

Loss in viability and subsequent autolysis of starters in cheese during ripening has been mainly attributed to a lack of carbohydrate substrates for glycolysis. The lack of suitable substrates leads to metabolism of other

carbon sources including nucleosides, lipids, peptides, and amino acids (Stuart et al., 1999).

Under starvation conditions, the lactococcal cell may survive by entering a VBNC state that allows continuation of basic metabolic activities without reproduction and growth. Therefore, the cell can still maintain cell membrane integrity, membrane potential, and undergo transcription of a range of genes necessary for survival under stress but not for growth. Survival of 2 *Lc. lactis* subsp. *lactis* strains and 1 *Lc. lactis* subsp. *cremoris* under starvation conditions and at pH 7.2 or pH 5.2 was studied by Ganesan et al. (2007). Data indicated a strain-related effect regarding the time before the onset of nonculturability, which was also affected by carbohydrate source and pH. On entering the VBNC stage, cells could remain in this state for up to 3.5 yr but were capable of generating ATP (despite lactose

Figure 2. Overall schematic view of lactic acid bacteria (LAB) viability and activity during cheese manufacturing and ripening. Cells with damaged membranes are indicated by dashed lines. Small dots represent dye molecules and enzyme substrates. Arrows represent the direction of movement of enzymes and dye into or out of cells. CEP = cell envelope protease; LDH = lactate dehydrogenase; AMC = amino methyl coumarin peptidase substrate.

depletion) at either pH value. Cells in the VBNC state also displayed oligopeptide transport activity along with the ability to catabolize branched-chain amino acids indicating the presence of an intact, functioning cell membrane. Changes included induction of genes for glycolysis to levels ~2 to 4 times that noted during starvation.

Genes coding for autolysins (*acmABCD*) were not expressed and indicated that cell division and autolysis is greatly reduced during the VBNC stage (Ganesan et al., 2007). Strong expression of PepX was noted during starvation, whereas PepN was not and activity of these aminopeptidase enzymes was reduced to undetectable levels during long-term starvation. This suggests that PepX activity found during ripening may originate exclusively from dead or damaged cells (Chapot-Chartier et al., 1994; Wilkinson et al., 1994).

A strain-dependent relationship has been noted between the onset of the VBNC state and the following factors: time, carbohydrate, and pH value of the medium (Ganesan et al., 2007). The prolonged survival of cells during carbohydrate starvation may have been repressed by catabolite control protein A (CcpA) followed by a switch from carbohydrate metabolism to amino acid metabolism.

Overall, Ganesan et al. (2007) stated that strains remaining in a VBNC state for up to 3 yr could be attributed to maintenance of cell membrane integrity and functionality with reduced expression of autolysin activity, cell repair genes, conservation of cellular nucleic acids, and a switch to amino acid metabolism.

Phage Activity

The contribution of prophage to the autolytic properties of starter LAB has been noted for some time (Feirtag and McKay, 1987). Studies in New Zealand demonstrated the influence of cooking temperature on induction of prophage during Cheddar cheese manufacture. The addition of prophage-coding lactococcal strains to cheese milk was undertaken to limit cell numbers, reduce the level of CEP and induce early autolysis. This work showed that bitterness could be reduced even when using the bitter starter strain, *Lc. lactis* subsp. *lactis* ML8 (Lowrie et al., 1974). However, although starter viability and flavor quality were monitored, data for autolysis were not given.

Feirtag and McKay (1987a) screened bitter and non-bitter strains in broth or RSM over a modified Pearce activity test and detected 2 major responses: (1) a rapid decrease in absorbance 2 h after exposure of nonbitter strains to Cheddar cheese cooking temperature indicating autolysis, and (2) minimal reduction in absorbance

by bitter strains indicating no autolysis due to cooking temperature. Induction of prophage following cooking temperature was demonstrated by the presence of phage particles in electron micrographs. This report suggested that the autolytic response of certain nonbitter strains may be due to the thermolytic induction of a prophage by exposure to temperatures of 38 to 40°C as used in Cheddar cheese manufacture.

In another study, Feirtag and McKay (1987b) generated thermolytic mutants of a parent strain of *Lc. lactis* subsp. *cremoris* C2 using a chemical mutagen. Thermolytic mutants had a greater release of intracellular phospho- β -galactosidase compared with the parent strain. In contrast to their previous report, the presence of prophage particles was not evident in culture supernatants despite the lytic response, suggesting a different mechanism was responsible for the rapid autolysis.

The thermolytic response of *Lc. lactis* subsp. *cremoris* SK110 during a temperature shift from 30 to 40°C over 3 min was examined by Meijer et al. (1998). The presence of phage particles in the medium was determined together with a decrease in viability and absorbance of the culture. However, it was not possible to determine whether the prophage was directly induced by the temperature shift or as an indirect effect of the sudden temperature change. Meijer et al. (1998) also monitored the effects of medium pH and growth rate on autolysis. Absorbance decreased to a greater extent at pH 6.0 to 6.3 than at pH values below 5.8 and higher rates of growth resulted in greater autolysis. Temperature-induced autolysis was also reflected by higher levels of released PepX, PepN, and LDH.

Lepeuple et al. (1998a) compared St. Paulin cheeses manufactured using either the autolytic parent strain *Lc. lactis* subsp. *cremoris* AM2 or its prophage-cured derivative denoted as strain AM2-C. Loss in viability and release of PepX, PepN and PepC was higher in the parent strain compared with AM2-C. Electron micrographs also showed that AM2-C appeared to be intact, whereas the parent strain was in a damaged or permeabilized state in cheese during ripening.

Lepeuple et al. (1998a) suggested that these trends could arise from curing of the prophage (without resultant autolysis) or from alterations in the cell wall structure, which presumably may have made the cells more resistant to autolysis. While large differences in activity of intracellular peptidase enzymes between the strains were noted, significantly higher levels of low molecular weight peptides and amino nitrogen were observed for the parent strain compared with its prophage cured derivative.

Autolysin activity in *Lc. lactis* subsp. *cremoris* AM2 was examined by Lepeuple et al. (1998b) who detected

the presence of a 46-kDa protein using renaturing SDS-PAGE. The protein, which was active on cells of *Lc. lactis*, was designated A2. This enzyme was not the same as the previously described AcmA hydrolase (active at lower pH with a somewhat higher molecular mass), and indeed it appeared that, in comparison to other strains examined, strain AM2 contained lower AcmA activity. Evidence that the A2 hydrolase was encoded by a prophage was shown by the presence of the enzyme following mitomycin C induction of the prophage (with the visual presence of phage particles). It also appeared that the A2 enzyme was not due to prophage induction but may have been present in cells during growth or to low level of spontaneous prophage induction. The A2 protein was not secreted and the authors suggested that phage holin activity may be required for access through the cell membrane.

A study of spontaneously autolytic *St. thermophilus* strains by Husson-Kao et al. (2000) found that a bacteriolytic enzyme of ~31 kDa, homologous to prophage-encoded endolysin (Lyt51), was only present in autolytic strains. Early autolysis of these strains occurred in response to stressors including lactose depletion (while transfer to a fresh medium containing lactose halted autolysis and enabled growth to recommence), exposure to organic solvents, and NaCl. This report suggested that autolysis of *St. thermophilus* is as a result of incomplete or “leaky” repression of the prophage genes working in tandem with holin proteins, which destabilize the cell membrane potential in response to adverse environmental conditions.

Over 7 d of ripening of UF cheeses, autolysin genes were not expressed by *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, and prophage gene expression was repressed, which was ascribed to the lack of environmental stress in the UF cheese system (Cretenet et al., 2011). In comparing 2 *Lc. lactis* strains lacking the *acmA* gene, one of the mutant strains could still undergo autolysis despite *acmA* deletion, which appeared to be due to the presence of a ~30-kDa protein also detected in *Lc. lactis* subsp. *cremoris* strains E8, HP, and Wg2 and in their PrtP⁻ variants (Visweswaran et al., 2017). Additionally, strains E8 and HP produced a 20-kDa protein, which may have been a cleavage product of AcmA by the action of extracellular HtrA. However, the genome of strain HP encodes 2 endolysins of ~20.5 kDa. The highest activity of the ~30 kDa protein was localized in the cell wall, but it was also detected in the cytoplasm and cell membrane and did not appear to have a signal sequence for extracellular secretion. This activity was mostly due to phage endolysin genes expressed during growth. The genes for phage endolysin were also accompanied by sequences coding for phage holins, which

may be required to enable secretion of the 30-kDa protein. However, data were not available on whether the holin genes were also expressed during growth.

The lytic cassette of the lactococcal prophage TP712, which codes for endolysin and holin activities among others, has recently been studied by Escobedo et al. (2019). Membrane depolarizing agents were required to induce expression of both the prophage endolysin gene and the holin gene and this combination of gene expression appeared toxic to the cell. If endolysin alone was expressed, cell growth was not stopped. The endolysin encoded by the prophage contained a signal sequence for secretion, and cleavage of this sequence was also required for lytic activity. Hence, it would appear that phage-mediated autolysis of lactococcal cells may be induced by loss of membrane proton motive force with subsequent induction of both prophage endolysin and holin genes coding for enzymes required for cell bursting.

Cell Morphology and Phage Resistance

Delcour et al. (1999) and more recently Chapot-Chartier and Kulakauskas (2014) reviewed the biosynthesis and functionality of the cell wall of LAB containing peptidoglycan decorated with a variety of cell surface components such as LTA, sugars, and proteins. The cell wall peptidoglycan is sufficiently robust to resist pressures of up to 20 atm with the ability to exclude molecules of ~55 kDa. Differences in cell surface properties between 15 parent starter lactococcal strains and their phage-resistant derivatives were shown by Crow et al. (1995b). Phage resistance was reflected in surface hydrophobicity with some resistant strains having either greater (3 strains), lesser (5 strains), or similar (7 strains) hydrophobicity than parent strains.

However, although no clear relationship could be found between variation in cell surface components such as LTA and phage resistance, it was clear that major differences exist in morphological features of lactococcal strains. These anomalies point to gaps in knowledge that could benefit from research attention in the future. Our viewpoint on the most likely mechanism of starter autolysis has emerged from our recent study where strain-related viability, permeabilization, intracellular enzyme accessibility, and autolytic properties were primarily induced by Cheddar cheese cooking temperature (Yanachkina et al., 2020). The strain-dependent salt tolerance response can be considered a secondary effect on the above properties. The exact molecular mechanisms thereafter are possibly the combined results of the disruption of autolysin regulatory control at the genomic level either with, or without,

induction of prophage in certain strains such as AM2, cell survival strategies, along with nutritional starvation over prolonged storage.

INFLUENCE OF CHEESE PROCESSING ON AUTOLYSIS

Clearly, exposure of starter lactococci to processing conditions during cheese manufacture affects viability and autolysis. The influence of Cheddar cheese cooking temperature on viability and autolysis is strain related (Lowrie et al., 1974; Feirtag and McKay, 1987a; Wilkinson et al., 1994; O'Donovan et al., 1996).

In the manufacture of Cheddar-Swiss hybrid cheeses, salting method (dry or brine salting) had a greater effect on viability and autolysis than cook temperature (40 to 50°C), as reduced viability, higher levels of autolysis, and proteolysis were found for *Lb. helveticus* in dry salted cheese compared with brine salted cheeses irrespective of cooking temperature (Hickey et al., 2017). No such effects for salting method or cooking temperature were noted for the *St. thermophilus* strain used. Interestingly, the highest LDH levels were noted in the dry salted cheese cooked to 40°C, which had the lowest salt in moisture levels. No clear explanation was offered for this finding, but the authors suggest that a combination of factors (other than salt in moisture level only) including the interactive effects of cook temperature, salting method, pH, moisture, and salt in moisture levels may also influence LDH release. Overall, lower viability and elevated LDH levels noted in the dry salted cheeses (at both cook temperatures) was attributed to the formation of supersaturated pools of brine at the surface of the curd chips before pressing of the blocks in comparison to a slower migration of salt inward from brine-salted cheese with a delayed onset of salt equilibrium within the latter cheeses. However, this study did not measure salt levels or starter viability and LDH activities within inner or outer layers of the dry or brine salted cheeses.

In contrast, Husson-Kao et al. (1999) using an autolytic strain of *St. thermophilus* showed that exposure to environmental conditions such as 2% NaCl, lactose limitation, heat, and cold shock triggered autolysis in buffered milk systems with extensive PepX release. Hickey et al. (2018) examined viability and autolysis of *Lb. helveticus* and *St. thermophilus* strains within the outer and inner layers of a cheese salted in 18 or 20% brine with a cooking temperature of 50°C. Three cheeses were manufactured using either a single strain *Lb. helveticus* starter, a single strain *St. thermophilus* starter, or a blend of both starters. Viable plate counts of *Lb. helveticus* either as a single strain or in combination with *St. thermophilus* declined significantly in the

outer layer of cheese with higher salt content (~2.5%) compared with the inner layer having lower salt content (~0.5%) and was affected by brine concentration. Viability of *St. thermophilus* was unaffected by salt concentration or location within cheese layers. In contrast, autolysis, as determined by LDH release, was higher for *Lb. helveticus* starter only, within the *Lb. helveticus* and *St. thermophilus* combination and for the inner layer.

Flow cytometry enabled both strains to be distinguished from each other and indicated a greater decline in viability of individual or combined starters within the inner layer of the cheese, supporting LDH data. Forward light scatter in flow cytometry profiles of individual or combined starter strains in the outer layer with highest salt concentration indicated potential shrinkage of cells, not noted within the inner layer, pointing to osmotic effects due to salt. Cells in the outer layer appeared to undergo greater oxidative stress with a greater level of reactive oxygen species generated along with lower autolysis and viability, suggesting reactive oxygen species may be the major factor contributing to the lower viable cell counts and lower proteolysis noted in the outer layer. Although viability of *Lb. helveticus* was lower in the outer layer, released LDH levels were not higher. Hickey et al. (2018) suggest that cell membrane functionality of starters in the outer layer of cheese with high salt levels may have been altered by a rearrangement of fatty acids designed to ensure cell survival in a nonculturable state by inhibition of the release of intracellular contents including enzymes such as LDH. This possible new cellular response to salt and osmotic stress by *Lb. helveticus* may constitute one of the factors other than salt in moisture level noted in viability and LDH release trends discussed in the earlier study by Hickey et al. (2017) for dry and brine salted cheeses.

A study of the growth of 34 lactococcal strains including strains Z8, HP, SK11, and AM2 in a modified medium having high osmolarity to which was added either 1, 2, 4, or 6.5% salt was undertaken by Obis et al. (2001). Following addition of 2% salt, strains Z8 and HP were unable to grow, whereas strain AM2 appeared to be tolerant at 2% salt addition but not at 4%. Higher osmotolerance of *L. lactis* subsp. *lactis* strains appeared related to accumulation of the osmoprotectant betaine within the cell. *L. lactis* subsp. *cremoris* strains Z8 and HP did not accumulate betaine at 1% salt, whereas strain AM2 accumulated betaine at 1% salt but not to the same extent at 2% salt. Indeed, strains Z8 and HP did not possess the *busA* operon coding for the betaine transport system.

Both a common and strain-related transcriptional response to cooking for several bitter and nonbitter starters was noted by Taïbi et al. (2011) who showed that exposure to stressors such as acid and salt dur-

ing simulated cheesemaking conditions influenced gene expression. These workers found SK11 had a higher level of expression of the oligopeptide transport genes (*optCDF*) after cooking, suggesting increased peptidase activity for subsequent debittering in cheese, which may affect PepX release and accessibility. Strains SK11 and E8S responded to cooking temperature by an early induction of genes coding for osmoprotectant/thermoprotectant glycine betaine transporters (*busAA* and *busBB*) (Taïbi et al., 2011).

In summary, salt diffusion from outer to inner layers affects the viability of cheese bacteria in a strain-dependent manner, whereas the autolytic response to salting can be more (*Lc. lactis*, *Lb. helveticus*) or less (*Lc. lactis*, *St. thermophilus*) pronounced among species.

INDUCTION OF STARTER PERMEABILIZATION AND AUTOLYSIS

Chemical and Mechanical Methods

Induction of early and extensive permeabilization and autolysis of starter lactococci for intracellular enzyme release is often part of the strategy for accelerated ripening (Wilkinson and Kilcawley, 2005). This frequently involves attenuation of starter bacteria such that acidification is impaired, and cultures are then suitable to be added as adjuncts to the cheese milk.

The choice of attenuation process may render these cells more susceptible to autolysis during ripening and hence enhance intracellular enzyme release (Doolan and Wilkinson, 2009). As mentioned previously, induced autolysis of lysozyme-treated cells following salting caused a 3-fold increase in FAA concentration in experimental cheeses (Law et al., 1976).

A lactococcal starter strain was exposed to differing *n*-butanol concentrations to permeabilize cells and enhance intracellular PepN and PepX accessibility (Exterkate 2006). In addition, the effects on cell envelope proteinase PrtP (CEP I-type activity) was examined. An optimal concentration of 5% *n*-butanol was determined for access of PepN substrate into the cell, whereas 6.5 to 7.0% *n*-butanol was required for optimal PepX substrate accessibility. In contrast, concentrations of up to 10% *n*-butanol did not enhance substrate access for the PrtP activity, indicating it was unaffected by the permeabilization process. Hydrolysis of chymosin-generated peptides was evident from permeabilized cells at 5% *n*-butanol, which may have resulted from enhanced PepN substrate accessibility. Concentrations of FAA also increased with increasing *n*-butanol concentration. This study ascribed

the effects of *n*-butanol to increased accessibility of substrate into the cell rather than increased intracellular release.

Autolysis can be induced by various simulation methods, and a range of treatments on the permeabilization and accessibility of *Lc. lactis* subsp. *cremoris* AM2, HP, and *Lactococcus lactis* subsp. *lactis* 303 has been evaluated (Doolan and Wilkinson, 2009). The cationic surfactant cetyl-trimethylammonium bromide (CTAB) caused high levels of permeabilization of all strains with enhanced accessibility to PepN and especially PepX. All strains showed increased permeability and enzyme accessibility when exposed to SDS or 70% isopropyl alcohol; flow cytometry also indicated the most extensive permeabilization and enzyme accessibility was due to CTAB treatment. Strains grown in 10% RSM had higher levels of both enzymes compared with cells grown on L-M17.

Methods to induce autolysis and permeabilization in 2 commercial starter strains of *St. thermophilus* were examined by Peralta et al. (2019) by monitoring release of both β -galactosidase and LDH. Enzymatic and mechanical methods (mutanolysin, bead mill, or ultrasonic bath) induced lysis, whereas chemical treatments (SDS, 40% ethanol) caused cell permeabilization. Heat or freeze shock were unsuitable for inducing either lysis or permeabilization. However, methods such as high pressure and microfluidization, a type of high-pressure homogenization, have been used to release intracellular enzymes.

Two slow-lysing lactococcal strains were exposed to 200 MPa for 20 min at 20°C and added as adjuncts to Cheddar cheese milk by Upadhyay et al. (2007). Acid production from these attenuated strains was impaired, whereas some increase in FAA levels released during cheese ripening was noted. From this study, the extent of cell lysis and permeabilization resulting from the treatment was unclear.

When 4 lactococcal starter strains were treated to a range of pressure regimens from 300 to 400 MPa, LDH activity was not increased, suggesting that autolysis had not occurred and no significant effect on cheese proteolysis was noted (O'Reilly et al., 2002). The use of microfluidization at 172,369 kPa for generation of attenuated starter lactococci as adjuncts in Gouda cheese manufacture was reported by Yarlagaadda et al. (2014). Strain-related effects on cheese ripening were noted, and in some cases, the rapidly lysing commercial control starter generated higher levels of LDH and FAA than experimental cheeses. Overall, more work is required at the cellular level to understand the changes in morphology and the mechanisms of permeability and autolysis provoked by such treatments.

Natural Antimicrobials, Bacteriocins, and Nisin

Natural antimicrobials such as bacteriocins have been used to lyse or permeabilize starter lactococci. The addition of a citrate-positive *Lc. lactis* subsp. *lactis* strain producing lactococcins A, B, and M as adjunct culture together with a slow-lysing, acid-producing strain *Lc. lactis* subsp. *cremoris* HP with a view to enhancing LDH release and secondary proteolysis in Cheddar cheese was described by Morgan et al. (1997).

An earlier release of LDH was noted during Cheddar cheese manufacture compared with the control, and LDH activity at salting was 8 times higher for the cheese containing bacteriocin-producing culture. During ripening, elevated LDH activity was found for cheeses with adjunct culture included, whereas FAA levels also increased.

A *Lc. lactis* subsp. *lactis* strain producing the lantibiotic lacticin 481 was isolated by O'Sullivan et al. (2002). This strain induced significantly elevated levels of LDH and PepX release from sensitive lactococcal strains without impairing growth, as measured by optical density and viable plate counts. Laboratory-scale cheesemaking trials using the lantibiotic-producing strain as an adjunct induced elevated enzyme release from *Lc. lactis* subsp. *cremoris* HP. O'Sullivan et al. (2002) suggested that lacticin 481 may be inducing transient pores in cells, thereby allowing enzyme leakage without affecting overall growth of the culture, a scenario similar to strain-related permeabilization profiles during cheese ripening noted by Sheehan et al. (2005).

The effects of several bacteriocins including lacticin 3147; lactococcins A, B, and M; and enterocin AS-48 on permeability and autolysis of a *Lc. lactis* strain was examined using flow cytometry and PI staining to evaluate membrane damage (Martínez-Cuesta et al., 2006). Nisin was included for comparison purposes and in buffer at pH 5.4 with 4.5% added salt; its addition caused ~95% of cells to become permeable to PI after 15 min exposure. All experimental bacteriocins induced >80% permeabilization to PI after exposure times ranging from 1 to 5 h. The extent of permeabilization did not appear to be directly related to viability as determined by plate counts. Indeed, nisin-treated cells retained a higher viability compared with the greater reduction in viability caused by lactococcins A, B, and M, and lacticin 3147.

The effect of bacteriocin treatments on aldehyde formation in cell supernatants and in cell suspensions indicated that highest formation of these compounds in supernatants for the lactococcins and lacticin 3147. In the case of cell suspensions, the same treatments also resulted in higher aldehyde formation compared with

controls and appeared to be related to both the extent and nature of the cell membrane damage caused by these treatments. The authors suggest that lactococcins A, B, and M along with lacticin 3147 may have initially induced permeabilization, which was then followed by a complete cell autolysis enabling conversion of amino acids to aldehydes.

The addition of bacteriocin-producing adjuncts is a promising approach toward enhancing autolysis and intracellular enzyme release. However, it is first necessary to ensure that adjunct addition rates are optimized such that the acidifying sacrificial strain is not excessively affected to avoid altering or prolonging the cheese manufacturing process.

Gene-Based Technologies

Genetic manipulation of both intracellular peptidase and autolysin gene expression has shown that autolysis and enzyme release are not necessarily directly related. A novel expression cassette containing the prophage holin and lysin genes with a transcriptional activator on a high copy number plasmid was developed by Tuler et al. (2002) to investigate externalization of cloned intracellular peptidases (PepX, PepN, PepC, PepA, and PepO). Despite increased activity of PepN, PepC, and PepX in transconjugant cells, only 6% of the total enzyme activity was detected externally.

Additional promoters used to express PepO and PepA showed that while intracellular activity increased, externalization of these activities did not occur. The cloned peptidases had molecular masses of up to 100 kDa and were within the size range used in a cassette system to produce leaky cells able to externalize β -galactosidase (117 kDa), but without host cell autolysis (Walker and Klaenhammer, 2001). Hence, it would appear that the size of the proteins or degree of autolysis were not the key issues determining externalization.

The nisin-controlled gene expression system (NICE) was used by Hickey et al. (2004) to express enterolysin A from *Enterococcus faecalis* in a *Lc. lactis* strain, which autolyzed on contact with nisin added to the medium. When added along with the poorly autolyzing strain *Lc. lactis* subsp. *cremoris* HP in laboratory-scale cheese, a 27-fold increase in LDH release was found compared with controls. However, strain HP was not extensively lysed because viable cell populations were similar for this strain in both cheeses. Thus, LDH activity originated from autolysis of the transconjugant strain. These studies highlight the complexity of molecular processes leading to release of intracellular enzymes in lactococci and suggest that mechanisms other than autolysis are also involved.

STARTER PERMEABILIZATION DURING CHEESE RIPENING

The existence of permeabilized or damaged starter cells during ripening has been suggested from microscopy studies (Ohmiya and Sato, 1970). Evidence for a discrete permeabilization event was reported by Niven and Mulholland (1998) who developed an assay using CTAB as a permeabilizing agent along with PI fluorescence to measure permeabilization. The presence of CTAB increased fluorescence 20-fold compared with nonpermeabilized cells, and this value was considered as being the maximum fluorescence of the total cell population. Fluorescence data from PI entering cells in the absence of CTAB treatment therefore represented the percentage of cells having damaged or compromised membranes.

A “permeability index” was calculated by expressing the net value of fluorescence due to PI in the absence of CTAB as a percentage of the value for CTAB-treated cells. An increase in extracellular PepX activity with an increase in permeability index indicated a contribution to released enzymes from cells with permeable membranes. This fluorescent staining assay, although not performed on cheese extracts or by using flow cytometry, provided a very good basis from which to determine cell permeability.

A SYTO9/PI staining combination along with flow cytometry was used by Bunthof et al. (2001b) to

monitor the release of LDH, PepN and confocal laser scanning electron microscopy to demonstrate the effects of mutanolysin on *Lc. lactis* subsp. *lactis* cells. Mutanolysin at varying concentrations caused the extensive release of LDH when cells were suspended in buffer at pH 6.5 with or without a stabilizing agent (5% BSA). Release of LDH was much more extensive in buffer without added BSA with or without added mutanolysin. The flow cytometry analysis also agreed with microscopy and indicated 70% cell permeabilization at 22 h after exposure to 100 U of mutanolysin. Stabilization of the buffer resulted in a marked difference in enzyme distribution, with 75% of PepN activity being cell associated and 25% released extracellularly, whereas for the control buffer this distribution in activity was almost reversed.

Bunthof et al. (2001b) noted that conditions in a cheese may enable cells to persist as protoplasts and maintain cell integrity despite autolysis. Over a 42-d ripening period of Cheddar cheese, a significant percentage of permeabilized cells was present on d 1 of ripening for both *Lc. lactis* subsp. *cremoris* AM2 and HP (Sheehan et al., 2005). As ripening progressed, the percentage of dead and permeabilized cells increased for both strains but were higher for strain AM2 and by d 42 permeabilized cells comprised 95% of the flow cytometry profile for AM2 compared with 49% for HP. Increasing permeability of AM2 cells was accompanied by increased accessibility to LDH, PepX, and PepN.

Figure 3. Flow cytometric profiling of starter lactic acid bacteria strains during cheese ripening, comparing the proportion of live, permeabilized, and dead cells according to temperature sensitivity, salt tolerance, and phage induction. Green cells are viable (1), and red cells are permeable to the membrane integrity dye, representing both permeable (2) and dead (3) cell populations. Viable plate counts decrease in proportion to the sensitivity of each strain to cooking temperature, salt, and prophage induction. *Lc.* = *Lactococcus*.

In contrast, for strain HP, increasing permeability of cells was not accompanied by increased accessibility to these enzymes, suggesting enzyme accessibility and permeabilization may not be directly related. Flow cytometry thus demonstrates the relationship between strain-related permeabilization, autolytic, and intracellular enzyme release properties for starters in Cheddar cheese, as summarized in Figure 3.

Recently, Yanachkina et al. (2020) screened a range of lactococcal starters to identify cheesemaking strains suitable for low-salt cheese production based on high salt sensitivity and optimal intracellular enzyme release at reduced sodium concentrations. Data indicated strain-related effects of both salt level and cooking temperature on autolysis and permeabilization, typical for “bitter” and “nonbitter” strains. Most importantly, the strain-related response to cooking temperature directly influenced accessibility or entry of substrates into the cells that were not undergoing autolysis. Differences noted in enzyme accessibility were also related to the phenotypic profiles. Overall, strains such as *Lc. lactis* subsp. *cremoris* AM2 and *Lc. lactis* subsp. *cremoris* SK11 were considered as having optimal performance in low-salt model systems (Figure 3).

The salt tolerance of lactococcal isolates from a commercial DL-culture used for Danbo cheese manufacture was examined at 3.4%, 8.0%, and 15% salt by Kristensen et al. (2020). Viability generally decreased with increasing salt level and was reflected by flow cytometry profiles of viable cells. No indication of effects on permeabilized subpopulations was given by the authors, who recognized the need for more in-depth analysis of salt sensitivity of starter strains.

STARTER ENZYME RELEASE DURING CHEESE RIPENING

Taïbi et al. (2011) noted that despite induction of gene expression for holin and a cell wall-associated hydrolase, LDH was not released. The chaperone gene *htrA* in SK11 was highly expressed at 4% salt addition at the end of the Pearce test. However, Desfossés-Foucault et al. (2014) found that *htrA* was not expressed at 2% salt addition for strain SK11. As the HtrA protein slows autolysis by degradation of the secreted autolysin, further investigation into autolysis as affected by salt level is required. Activity of AcmA is limited through degradation by the CEP I and HtrA proteinase; however, its role during ripening is unclear.

Pillidge et al. (1998) suggested that AcmA contributed to autolysis in milk but not cheese, indicating a possible lack of expression of the gene or protein non-functionality under Cheddar cheese conditions. In the study by Yanachkina et al. (2020), assays were calcium

free so CEP I activity should have been unaffected and therefore would degrade AcmA unhindered, suggesting a lesser role for autolysin in permeabilization and autolytic responses to salt levels. Clearly, additional work is required to clarify the mechanisms surrounding enzyme function during cheese ripening.

NEW EVIDENCE FOR STARTER PERSISTENCE IN CHEESE

In general, it was assumed that starter bacteria viability became less important beyond early ripening contributing thereafter primarily through released intracellular enzymes. However, a subpopulation of the starter microbiota may remain viable until the later stages of ripening based on their ability to grow on selective media.

Studies of PR cheese ripening has indicated the ability of a natural whey starter to persist up to 20 mo of ripening (De Dea Lindner et al., 2008). In this study, a range of selective agar media, under various incubation conditions, enumerated mesophilic and thermophilic lactobacilli and lactococci in cheese from internal and external areas of the cheese. Generally, the total population reached $\sim 10^7$ cfu/g, which declined slowly from 6 mo to reach $\sim 10^5$ cfu/g by 20 mo of ripening. Lactobacilli enumerated on MRS (pH 5.4) agar at 25 or 42°C were higher than lactococci toward the latter stages of ripening. Staining by SYTO9 and PI enumerated live and nonviable populations in curd samples, and it appeared that high levels of both cell types were present throughout ripening with a general decrease from $\sim 10^9$ to $\sim 10^7$ cfu/g noted. Total cell numbers determined by epifluorescent staining (live plus nonviable cells) were consistently lower than the total populations as determined by plate counts, indicating the ability of staining to enumerate damaged and permeabilized cells.

This study illustrated the persistence of a significant population of starter lactobacilli in the viable, reproductive state in a long-ripened cheese. As a mixed strain whey-based starter was used, it was not possible to definitively state which strain/strains were the predominant survivors and further colony examination of isolates on MRS and M17 agar would have been useful in estimating whether “crossover” growth was occurring on these media during the advanced ripening stages. Use of culture-independent methods has provided new data on survival of LAB starter strains during advanced stages of ripening.

A qPCR method was used for monitoring *Lc. lactis* subsp. *lactis* in cheese by Ruggirello et al. (2014) who compared it to data obtained from culture-dependent techniques. A range of ripened commercial cheese varieties was purchased ($n = 33$) and analyzed for the

presence of *Lc. lactis* cells. Populations of cells detected by qPCR ranged from 10^7 cfu/g for ripened Toma, Asiago, and Pecorino cheese varieties to 10^3 cfu/g for Fontina cheese. Data from plate counting on M17 agar indicated viable populations of 10^5 to 10^9 cfu/g. Genetic analysis of 396 random isolates found that only 24 were definitively *Lc. lactis* strains, indicating a lack of selectivity of this medium for starter enumeration in late ripening. The differences noted in data from media and qPCR may be indicative of a high population of VBNC starter cells in cheese.

This study provided useful data but was carried out on commercial cheeses, which did not enable a controlled investigation into the fate of a particular starter culture. However, a further study examined the viability of 8 commercial starter strains in a Tome-like cheese model system up to 180 d of ripening using culture-independent PCR-based methods and traditional plate counting on M17 agar (Ruggirello et al., 2016). The cultures studied were 4 *Lc. lactis* subsp. *lactis* strains, 2 *Lc. lactis* subsp. *cremoris* strains, and a blend of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*. The origin, descriptors, or other technical characteristics of these strains were coded to protect confidentiality.

Starter populations on M17 agar reached $\sim 10^9$ cfu/g after manufacture with a strain-dependent decline during ripening. Three *Lc. lactis* subsp. *lactis* starters maintained high viability ($\sim 10^8$ cfu/g) until the end of ripening, whereas 4 starters, 2 *Lc. lactis* subsp. *lactis*, 1 *Lc. lactis* subsp. *cremoris*, and the blend of both subspecies had a reduction to 10^5 cfu/g after 180 d. Colonies detected on M17 medium at the end of ripening were composed of the original added starter for strains with lowest viability. However, not all colonies detected on M17 were confirmed as the original added starter, again suggesting lack of selectivity of M17 especially late in ripening or perhaps the presence of VBNC cells. Overall, 4 strains persisted in the viable state throughout ripening with populations $\sim 10^3$ cfu/g noted for 3 strains on M17.

Comparative RNA-based detection generally indicated somewhat lower populations were present, which may have arisen due to difficulties in using direct RNA analysis. Three *Lc. lactis* subsp. *lactis* strains and 1 *Lc. lactis* subsp. *cremoris* strain that lost viability over ripening and were unable to grow on L-M17 agar by d 180 of ripening were examined for their ability to resuscitate from the VBNC state. At 180 d of ripening, enrichments were carried out in M17 broth with either added lactose (2% wt/vol) or glucose at 0.5, 1, or 2.5% (wt/vol), incubated at 37°C for 24 h, then plated onto L-M17 agar (48 h at 30°C). Resultant colonies were then examined using species-specific qPCR to verify their identity. A glucose concentration of 2.5% (wt/vol)

was sufficient to recover the *Lc. lactis* subsp. *cremoris* strain but only one of the *Lc. lactis* subsp. *lactis* strains. The other 2 strains could not be recovered at any concentrations of added glucose or lactose. These data indicated the complexity of the nutritional requirements that may be necessary for strains to recover from potentially differing degrees of cell damage during extended ripening.

Desfossés-Foucault et al. (2013) compared the microbiota of Cheddar cheese produced using either thermized milk (63°C for 16 s) or pasteurized milk (72°C for 16 s) and inoculated with an undefined mixed strain starter containing *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* strains. Both DNA- and RNA-based approaches were combined with plate counting to monitor the viability of the starter lactococci in cheese ripened for 6 mo at 4, 7, or 12°C. Plate counts decreased from $\sim 10^6$ to 10^7 to 10^4 to 10^6 cfu/g over ripening. Genetic analyses indicated a reduction in genome copy number and cDNA copies, which reflected a decrease in culturable lactococcal cells from 10^8 to 10^9 to 10^6 to 10^7 after 6 mo. The higher numbers determined by culture-independent genetic methodologies may indicate the presence of dead or nonculturable cells that were intact. Lactococci represented the dominant population present throughout, accounting for 98% of total LAB detected.

The effect of milk heat treatment on microbiota showed that lactobacilli in pasteurized milk cheeses initially decreased by 1 log but maintained a stable population from 1 mo of ripening onward. In contrast, thermized milk cheeses appeared to support the growth of lactobacilli to higher population densities. The effect of starter autolysis on growth of lactobacilli was noted toward the end of ripening when a decrease in genome copy number for lactococci coincided with increased growth of lactobacilli. This study provides strong evidence for the persistence of viable starter lactococci in significant populations from the outset to the conclusion of a 6-mo ripening period. These data highlighted the important influence of milk heat treatment on the dynamics of the cheese microbiota.

PERSISTENT CELL CHARACTERISTICS

The ability of bacteria to exist in dormant and VBNC states has been recognized in the areas of medical microbiology where persister cells have been frequently detected following antibiotic therapy (Wood et al., 2013). A distinguishing feature of VBNC cells is their inability to resuscitate following restoration of optimal growth conditions or removal of antibiotics while persister cells can revert to normal type under these conditions. In persister cells, a downregulation

of biosynthetic pathways (including absence of protein synthesis) is evident along with a low or absent growth rate, leading to a state of dormancy (Wu et al., 2017). Within a population, a certain percentage of cells become persisters before exposure to antibiotics (Balaban et al., 2004).

Persister cells are considered to arise as a noninherited phenotype resulting in a switch to a slow growth rate. Hence, the incidence of persistence increases under nutrient starvation, thereby reducing competition for scarce resources, and also when cells originate from a single clonal lineage (Gardner et al., 2007).

Kim et al. (2018) examined whether VBNC and persister cells are actually the same. Kim et al. (2018) induced a VBNC state in an *Escherichia coli* population following starvation and the persister state in a separate population following pretreatment with rifampicin. Cells were stained using a SYTO9/PI viability combination and enumerated by plate counting with antibiotic to enumerate persister cells. A reduction in viability from 10^8 to 10^3 cfu/mL was found over a 7-wk period. However, PI-positive cells only amounted to 4% of the population.

Persister cells increased from 10^3 to 10^5 within 2 wk. Culturable cells from the VBNC-inducing starvation conditions became persister cells and remained in this condition for the remainder of the 7-wk incubation period. Therefore, starvation induced the formation of persister cells and culturable cells from the VBNC state appeared identical to persister cells in this case. Using differences in cytosol density (dense in viable control cells, low or absent in nonviable cells), 99.5% of the VBNC population consisted of dead cells based on the cytosol criteria.

The metabolic activity of persister and VBNC cells was determined by flow cytometry using a RedoxSensor Green and PI stain combination and both groups appeared similar to dead cell controls. As before, PI staining did not reflect viability as only ~15% of the VBNC population were PI positive. Although microscopy indicated a loss of cytosolic material, the low level of PI suggests a high degree of intact membranes, and the authors propose loss of material through “blebbing” while retaining membrane integrity.

In the area of food safety, *Listeria monocytogenes* persister cells have been demonstrated in response to nisin treatment (Wu et al., 2017). For cheese, recent data suggesting long-term survival of starter LAB raise the question as to whether persister cells are present in cheese. If we consider that persistence is a population-level response to stress, then we can understand that the cheesemaking process, incorporating temperature increases and salt addition followed by low storage temperature with nutrient depletion, constitutes a series

of adverse events for the starter cell. Hence, viability profiles during ripening may represent the strain-dependent proportion of persister cells present.

Genotypically, it is recognized that persister cells are identical to other cells but with a differing phenotype enabling slow growth under adverse conditions. The significance of potential persister cells during ripening is unclear and raises questions such as whether it confers an advantage on these cells in cheese. In addition, although persister cells may be genetically similar to the parent cell, are these cells more active enzymatically in terms of proteolysis and other biochemical transformations?

To date we have made significant progress toward understanding permeabilization and autolysis of nonviable cells; however, we may now need a holistic population-based approach toward understanding the interactions between various subpopulations in cheese during ripening, including persister cells.

A stronger research focus into the question of prolonged starter survival is now becoming an integral feature of recent studies on cheese ripening. Future perspectives toward a holistic population approach include methods targeting in situ activity as well as analysis of single cells, combining microscopy with advanced technologies of cell sorting and genome sequencing.

CONCLUSIONS

Starter lactic bacteria are essential to cheese manufacture and ripening to ensure typical texture and flavor development. The study of starter bacteria has proceeded through a series of advances in our understanding of the characteristics of starters beginning from the early reports on classification of responses of strains to cooking temperature, salt sensitivity, and phage through to the current highly sophisticated genomic and proteomic studies of control of cell regulatory mechanisms. However, gaps in our knowledge still remain, especially regarding the area of starter nonviability responses during prolonged ripening.

To gain a more complete picture as to the phenotypic, genotypic, and ultimately flavor production pathways from these cells, a more integrated research approach is needed. A key area for additional research clearly identified in this review is a more detailed microstructural study of the morphological changes undergone by starter cells during their transition from intact and fully functional units right through to completely autolyzed cell remnants. Recent reports of cell “ballooning” or possible membrane “blebbing” during this transition require further attention to understand the functionality of these structures.

Current studies on genomics and proteomics also require a more coherent integration into an overall study of the cell and the interplay of vitality and survival strategies. Therefore, future starter research should involve a combination of technologies such as flow cytometry, cell sorting together with whole-genome sequencing, enzymology, and flavor chemistry to understand the evolution of the various subpopulations of cells and their relative contributions during ripening. The links between autolysis, proteolysis, and nitrogen metabolism are also becoming stronger and need to be examined within a wider cell survival strategy in the cheese environment.

Our current thinking regarding a continuous “conveyor belt” and “forward-only” process approach to starters going from viable to permeabilized to completely autolyzed cells needs careful examination to uncover whether reversible reactions or transitions between cell states or subpopulations occur. Is it possible that permeabilized cells can repair, recover functionality, and resume growth during ripening?

Work undertaken in the 1970s and 1980s on starter phenotypic classification, the contribution and characterization of key proteolytic enzymes, and elucidation of the main flavor generation pathways and associated flavor chemistry advances can rightly be regarded as a “golden age” in starter research, yielding many practical and academic outcomes for the dairy industry.

However, we are now entering an exciting era for starter research that will open up new vistas and insights into a group of microorganisms which sometimes have been taken for granted but which continue to offer fresh possibilities for academic and industrial study enabling commercial exploitation and ultimately additional practical benefits for the consumer.

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