Symposium review: Interaction of starter cultures and nonstarter lactic acid bacteria in the cheese environment¹

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ABSTRACT

The microbiota of ripening cheese is dominated by lactic acid bacteria, which are either added as starters and adjunct cultures or originate from the production and processing environments (nonstarter or NSLAB). After curd formation and pressing, starters reach high numbers, but their viability then decreases due to lactose depletion, salt addition, and low pH and temperature. Starter autolysis releases cellular contents, including nutrients and enzymes, into the cheese matrix. During ripening, NSLAB may attain cell densities up to 8 log cfu per g after 3 to 9 mo. Depending on the species and strain, their metabolic activity may contribute to defects or inconsistency in cheese quality and to the development of typical cheese flavor. The availability of gene and genome sequences has enabled targeted detection of specific cheese microbes and their gene expression over the ripening period. Integrated systems biology is needed to combine the multiple perspectives of post-genomics technologies to elucidate the metabolic interactions among microorganisms. Future research should delve into the variation in cell physiology within the microbial populations, because spatial distribution within the cheese matrix will lead to microenvironments that could affect localized interactions of starters and NSLAB. Microbial community modeling can contribute to improving the efficiency and reduce the cost of food processes such as cheese ripening.

Key words: cheese, interaction, starter culture, nonstarter lactic acid bacteria, metabolism

THE PLACE OF MICROBES IN CHEESE-MAKING

The potential for controlling the course of fermentation and ripening is a strong driver for developing defined cultures. Concurrently, there is consumer demand

for traditional products. Both perspectives can benefit from a better understanding of microbial community interactions. According to their roles, the microbiota involved in the process of cheese manufacture and ripening can generally be divided into 2 groups: starter lactic acid bacteria (starter **LAB** or **SLAB**), such as mesophilic lactococci for Cheddar cheese, and adventitious nonstarter LAB (**NSLAB**), such as lactobacilli. The SLAB are mainly responsible for acid development during cheese production and contribute to the initial ripening process (Beresford et al., 2001). The NSLAB have been shown to play a somewhat contradictory role during ripening by enhancing flavor development or deteriorating cheese quality (Martley and Crow, 1993). The microbial succession during cheese ripening is related to the ability of the microbial populations to adapt to specific environmental conditions, influencing the features of cheeses. The microbiota of long-ripened cheeses has been widely studied (Neviani et al., 2013; Santarelli et al., 2013; Gatti et al., 2014).

Flavor develops in cheese by the combined metabolic activity of the microbial community on milk fat, proteins, and carbohydrates, accompanied by further enzymatic and chemical conversions in the cheese matrix. The major LAB metabolic pathways involved in cheese flavor formation are metabolism of lactose (or glycolysis) and citrate, as well as proteolysis and the subsequent catabolism of AA (Figures 1 and 2). The release of free fatty acids (lipolysis) and their metabolism can be also involved but to a lesser extent (Lazzi et al., 2016). Specifically, in the characterized aroma profile of Cheddar, one half of the potent odorants originate from lactose fermentation or citrate degradation (and a few from lipolysis), and the other half result from leucine and methionine degradation (Yvon and Rijnen, 2001). Methional and methanethiol, which are the major aroma compounds produced from methionine, contribute to the Cheddar aroma and the desirable garlic note, respectively (Yvon and Rijnen, 2001). This review will focus mainly on semihard and hard cheeses, and will not attempt to cover the advances made in understanding microbial interactions in smear-ripened and mold-ripened cheeses.

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Figure 1. Factors influencing the ecological interactions among microorganisms in cheese. Schematic representation of possible interactions among starter lactic acid bacteria (SLAB) and nonstarter lactic acid bacteria (NSLAB) during cheese manufacture and ripening. Competition for residual lactose between SLAB and NSLAB depends on the salt-in-moisture (S/M) content. At low S/M, residual lactose is mainly converted to L-lactate by salt-sensitive SLAB in the absence of NSLAB. At high S/M, SLAB activity is reduced, so the greater residual lactose may be converted to D-lactate by NSLAB, particularly at higher ripening temperatures (top). The ability of SLAB and NSLAB strains to produce bacteriocins confers a competitive advantage. Cellular lysis induced by bacteriocins or autolysis leads to release of enzymes such as peptidases and esterases (center). The SLAB degrade large and intermediate-sized peptides, providing smaller peptides for the proteolytic system of NSLAB. Intracellular bacterial peptidases released after autolysis may degrade small peptides to free AA. Microbial and chemical conversion of metabolites and AA subsequently contributes to flavor formation (bottom). Color version available online.

Role of Starter Cultures

The LAB are gram-positive bacteria that produce lactic acid as a primary fermentation end product, so they are useful as starter cultures to aid in coagulation of milk proteins during the process of cheese making. For artisanal cheese products, the back-slopping technique has traditionally been used, which requires the inoculation of milk with whey or fermentate. Starter culture mixes consisting of unknown strains are termed “undefined starters.” Further efforts in controlling starter quality have led to defined starter cultures, consisting of a specific number of strains of bacteria that may have been isolated from undefined starter cultures (Kelleher et al., 2015). The application of defined starter culture blends and rotation aims to ensure consistency of the fermentation process (Kelleher et

al., 2015). However, when strain diversity is reduced, blends may become susceptible to several factors such as carbon and nitrogen constraints, salt stress, temperature and pH changes, as well as phage predation during milk fermentation (Gatti et al., 2014). Mixed-strain and undefined cultures may be more resilient and display a more robust performance (Erkus et al., 2013), which is enhanced by the presence of a rich consortium of microbes. In spite of increasing efforts to identify the microbial composition of mixed cultures, little is known about diversity at the strain level, mainly due to the need to develop molecular tools to investigate the presence of specific strains (Johansen et al., 2014). Metagenomics has been applied recently to study the structure of microbial communities in undefined and defined starter cultures; however, resolution at the strain level is still difficult to achieve (Erkus et al.,

2013; Johansen et al., 2014). The development of stable starter cultures will depend on population dynamics resulting from interactions among the strains during milk fermentation (Smit et al., 2005).

Starter cultures can also be divided into mesophilic starters, with an optimal growth temperature of 25 to 30°C, and thermophilic starters, which grow best between 40°C and 45°C. Mesophilic starters may contain only acidifying strains or a mix of acidifying and citrate-fermenting LAB. The most frequently used LAB in mesophilic starter cultures are *Lactococcus lactis*, including the subspecies *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*, but undefined starters can also contain strains of *Leuconostoc* spp. for citrate fermentation. Thermophilic starter cultures commonly include strains of *Streptococcus thermophilus*, which are typically used for the production of semihard and hard cheeses (e.g., Italian and Swiss varieties). Strains of *Lactobacillus* spp. (e.g., *Lactobacillus helveticus* and *Lactobacillus*

delbrueckii) are widely used as adjunct cultures for flavor development.

The LAB use lactose as a carbon source, resulting in the production of high concentrations of lactic acid (Figure 1) and the growth of LAB in milk during the early stages of cheese production (Gatti et al., 2014). Lactic acid results in a decrease in pH, which assists in curd formation and prevents the growth of pathogens (Beresford et al., 2001; Crow et al., 2002). The citrate present in milk constitutes a secondary energy source for some LAB (Drici et al., 2010). Three types of dairy starters are known to be able to uptake and metabolize citrate to additional flavor compounds: *L. lactis* ssp. *lactis* biovar *diacetylactis*, *Leuconostoc* spp., and *Weissella paramesenteroides* (Drici et al., 2010). *Lactobacillus casei* and *Lactobacillus plantarum* are also able to metabolize citrate. The co-metabolism of citrate and lactose leads to production of CO₂ and C4 aroma compounds such as diacetyl (butter flavor; Drici et al.,

Figure 2. Biochemical pathways leading to the formation of flavor compounds because of starter lactic acid bacteria (SLAB) and nonstarter lactic acid bacteria (NSLAB) interactions during cheese manufacturing and ripening. S/M = salt-in-moisture; LDH = lactose dehydrogenase; α-KG = α-ketoglutarate; AT = aminotransferase; FFA = free fatty acids; BcAA = branched-chain AA; ArAA = aromatic AA; GDH = glutamate dehydrogenase; *L. lactis* = *Lactococcus lactis*; *L. mesenteroides* = *Leuconostoc mesenteroides*; *L. casei* = *Lactobacillus casei*; *L. plantarum* = *Lactobacillus plantarum*. Color version available online.

2010). Starter cell densities in most cheese varieties are approximately 10^8 to 10^9 cfu/g during cheese manufacture (Parente et al., 2017). During the early steps of ripening, a substantial decrease in the SLAB population can occur, accompanied by cell autolysis and release of intracellular enzymes, depending on the characteristics of the strains used (Steele et al., 2013). However, NSLAB can also remain subdominant to starter lactococci throughout the ripening of Cheddar cheese (Desfossés-Foucault et al., 2013). Bacteriocin producers have been used as adjuncts in starters to increase the rate of starter cell lysis. Bacteriocins inhibit the growth of pathogenic and spoilage bacteria, but they can also inhibit some species of LAB. The ability of lactococcal and NSLAB strains to produce bacteriocins confers a competitive advantage (Settanni and Moschetti, 2010).

Once the cheese curd is formed and pressed, bacterial mobility is minimal. This leads to the formation of colonies within curds, although multispecies communities have been found at the borders of curds in Serra cheese (Parker et al., 1998). The microscopy techniques used for studying bacterial colonies within cheese have been recently reviewed (Hickey et al., 2015). Bacteria are found mainly in the whey pockets and at the fat-protein interface, so they are not homogeneously distributed (Hickey et al., 2015). Within 3 mo of manufacture, the selective conditions of ripening cheese (low moisture, pH and temperature, high salt environment) usually decrease starter viability, leading to a decline in survival and to increased autolysis in some cases (Wilkinson et al., 1994). High starter counts in Cheddar cheese were reported as the result of elevated ripening temperatures (15, 17.5, and 20°C) compared with those at 8°C (Cromie et al., 1987).

Role of NSLAB

In artisanal cheese production without direct inoculation of starter cultures, the microbial agents involved in these fermentations are unquestionably enriched from the starting material and environmental sources (Bokulich and Mills, 2013). Furthermore, the inherent and unique flavors known in raw milk cheese are likely a product of native microbiota rather than starter cultures, and have been described (Marilley and Casey, 2004).

The NSLAB are adventitious bacteria that play an important role during ripening by enhancing flavor development (Settanni and Moschetti, 2010). Although NSLAB may be present at low concentrations after curd manufacture (10^2 to 10^3 cfu/g), their populations slowly increase about 4 to 6 orders of magnitude (log cfu/g) within the first months of ripening (Settanni and Moschetti, 2010). Depending on the rate of death of

the starter bacteria, NSLAB can dominate the viable microbiota of cheeses throughout most of the ripening period (Sousa et al., 2001). This is common in hard and semihard cheeses, especially in Cheddar, Emmental, and Gruyere (Irlinger et al., 2017). Although NSLAB cell density may decrease if cheese aging is prolonged, NSLAB autolysis has not been well described (Gobbetti et al., 2015).

The ranges of temperature, pH (around 5.2), and salt in moisture (4–6%) that prevail in Cheddar cheese have little effect on NSLAB final cell numbers attained after ripening (Lazzi et al., 2014). Higher moisture content (45%) reduced the lag time in growth of NSLAB. Higher temperatures can be used to accelerate growth, but the final cell count attained at 9 mo was similar for growth at 8, 12, or 16°C (Folkertsma et al., 1996). Starter activity also had little effect on the final population of NSLAB attained (Folkertsma et al., 1996), and the limit to growth of NSLAB is thought to be independent of the lactose content of the curd (Fox et al., 1998). Until 17 wk of ripening, there appears to be little variation occurring in the overall spatial distribution of NSLAB strains within Cheddar cheese, although temporal changes in the species and strains were observed (Fitzsimons et al., 2001). Some isolates were specific to certain samples, whereas others were more widely distributed.

Development of NSLAB during cheese ripening can be attributed partially to their ability to use the alternate nutrient sources available (Lazzi et al., 2014). Because lactose in cheese is metabolized in the first weeks of ripening, NSLAB can derive energy from compounds such as lactic acid, citric acid, ribose, fatty acids, glycerol, and AA (Broadbent et al., 2003). Redox potential affects the production of flavor compounds through microbial metabolism and chemical reactions. Several equilibria influence the changes in oxidation-reduction potential (McSweeney et al., 2010); namely lactate-pyruvate, ascorbate-riboflavin, and thiol-sulfhydryl compounds. Reducing conditions are necessary for AA conversion by *Lactococcus* to produce carboxylic and hydroxy acids from Phe, Leu, and Met, for example. Oxidizing conditions lead to aldehyde production from Leu and Met. Under low redox conditions in cheese, dimethyl disulfide and dimethyl trisulfide are produced from methanethiol. The general decline in redox potential due to microbial growth is strain-specific, and NSLAB have strain-specific growth responses to the redox potential of the medium (Boucher et al., 2006). Boucher et al. (2006) proposed starter-adjunct blends to control adventitious NSLAB.

Although the origin of NSLAB in cheese may differ, the main sources of NSLAB are raw milk and, to a lesser extent, natural whey culture (Gobbetti et al., 2015).

The factory environment can be a potential source of NSLAB, especially for those mesophilic bacteria with capacity to form biofilms and survive in the equipment and plant environment after cleaning and disinfecting treatments (Somers et al., 2001). Bokulich and Mills (2013) reported the existence of facility-specific “house” microbiota and suggested their potential role in shaping site-specific product characteristics. The dominant NSLAB in Cheddar-type cheeses typically consists of mesophilic, facultative heterofermentative lactobacilli (FHL, including *Lb. casei*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. plantarum*, and *Lb. curvatus*; Broadbent et al., 2003). *Lactobacillus* species are more diverse early in Cheddar ripening. Although 8-wk-old Irish Cheddar cheese contained *Lb. paracasei*, *Lb. plantarum*, and *Lb. curvatus* (Jordan and Cogan, 1999), 9- to 24-mo-old cheese was dominated by *Lb. paracasei* (96.4%) (Fitzsimons et al., 2001). In Cheddar cheese, the role of NSLAB has been related to flavor development due to an increased level of proteolysis and enhanced flavor intensity (Gobbetti et al., 2015). Some strains appear to produce high-quality Cheddar, whereas others result in cheese with acid and bitter flavor defects (Beresford et al., 2001).

Heterofermentative LAB are commonly found in milk and dairy products. If they grow to high levels, the acid and CO₂ can cause defects such as slits. Although minor components of the NSLAB population, obligate heterofermentative lactobacilli (OHL), including *Lb. brevis*, *Lb. fermentum*, *Lb. wasatchii* sp. nov., and *Lb. parabuchneri*, for example, have several metabolic pathways leading to CO₂ production from sugars, lactate, and AA (Fröhlich-Wyder et al., 2015; Ortakci et al., 2015). The FHL produce gas only under certain conditions or from specific substrates such as lactose, galactose, lactate, citrate, and urea (O’Sullivan et al., 2016). O’Sullivan et al. (2016) showed that presence of excess carbohydrates (lactose and galactose) and citrate due to absence or failure of starter bacteria during the initial ripening stages will likely provide *Lb. casei* (FHL) with sufficient substrates for excessive CO₂ and eye formation defect in Swiss-type cheese. Thus, in the presence of other gas-forming bacteria, such as propionic acid bacteria, adjunct FHL, such as *Lb. casei*, can be used to control excess gas formation in artisanal Swiss-type cheeses by inhibiting propionic acid bacteria activity (O’Sullivan et al., 2016). During the manufacture of Dutch-type cheeses, the inoculation of NSLAB results in the dominance of the adjunct, *Lb. paracasei* and *Lb. rhamnosus* after 4 wk of ripening (Porcellato et al., 2013). *Lactobacillus rhamnosus*, which can also arise from raw milk and natural whey culture, persist in long-ripened cheeses throughout ripening. This is mainly due to their metabolic activities, which allow them

to tolerate the environment of cheese through the use of nonconventional energy sources (Bove et al., 2012). The addition of *Lb. paracasei* ssp. *paracasei* and *Lb. plantarum* to Cheddar cheese influences cheese flavor, leading to acceleration of ripening (Lynch et al., 1999).

METABOLIC PATHWAYS OF SLAB AND NSLAB DURING CHEESE MANUFACTURE AND RIPENING

The dynamic interactions of microbiota, from either the starter culture or the environment, are crucial for the development of the unique sensory characteristics of each cheese variety (Figure 1). Although the relevance of microbial communities for flavor development in cheese is clear, studies are reducing the gap in our knowledge concerning the interactions between the components of the cheese microbiota. With these interactions defined, cheese producers can better control the ripening process and, therefore, cheese quality. However, the inability to simulate the exact natural conditions of microbial communities under laboratory conditions, especially during cheese ripening, makes these objectives difficult to define. Thus, studies on the interactions between SLAB and NSLAB during cheese production that produce unique cheese flavors are still needed (Kieronczyk et al., 2003; Desfossés-Foucault et al., 2013).

Competition and Cooperation in Carbohydrate Metabolism

Carbohydrates are the primary energy and carbon sources for LAB during growth in milk. The lactose used by LAB as a carbon source is depleted within a few days of ripening, as it is converted into lactic acid (Beresford et al., 2001). The depletion of lactose in cheese curd is essential to avoid the development of undesirable secondary microorganisms, which will negatively affect cheese quality (McSweeney and Fox, 2017). The rate of lactose fermentation depends on the salt-in-moisture (S/M) content of the curd. Lactococci are more salt-sensitive than NSLAB. Thus, at low S/M concentrations and low populations of NSLAB, residual lactose is converted mainly to L-lactate by SLAB. Conversely, D-lactate is produced when NSLAB attain high populations (mainly pediococci and mesophilic lactobacilli) by fermentation of residual lactose or by isomerization of L-lactate (McSweeney and Fox, 2017). Lactate can also be oxidized to acetate and CO₂ by NSLAB activity through the phosphoketolase pathway (pentose phosphate; Murtaza et al., 2014).

Citrate, which is present at relatively low concentrations in milk, can be metabolized by some strains of lactococci, such as *L. lactis* ssp. *lactis* biovar *diacetyl-*

lactis. Strains containing a citrate plasmid coding for citrate uptake are citrate-positive or **Cit+** (McSweeney and Fox, 2017). In the presence of a fermentable carbohydrate, citrate is metabolized with the production of diacetyl, acetate, acetoin, and CO₂ (McSweeney and Fox, 2017). During cheese ripening, residual citrate can be metabolized by nonstarter lactobacilli (*Lb. plantarum*, *Lb. helveticus*), which leads to succinate production (Drici et al., 2010) using a similar pathway to that of Cit+ lactococci, only in the presence of a fermentable sugar. It has been shown that the presence of *L. plantarum* accelerated the depletion of citrate (Budinich et al., 2011). Thus, both SLAB and adventitious NSLAB may compete for citrate in milk (Figure 2). In the absence of L-lactate, citrate is taken up by the citrate transport system in LAB (**CitP**) with the citrate metabolic pathway intermediates or end products pyruvate, α -acetolactate, and acetate (Pudlik and Lolkema, 2013). Pudlik and Lolkema (2013) demonstrated the involvement of CitP in flavor-producing pathways driven by α -ketoglutarate, which is considered the limiting factor for the conversion of AA into aroma compounds by LAB (Figures 2 and 3).

Citrate metabolism, important in certain varieties (e.g., Dutch cheeses), is affected by the interaction between *Leu. mesenteroides* and *L. lactis* in cheese starter cultures. *Leuconostoc mesenteroides* can only produce diacetyl and acetoin from citrate at acidic pH, and acidification is predominantly driven by lac-

tose fermentation by the lactococci (McSweeney and Sousa, 2000). Pure cultures of *Leuconostoc* spp. do not produce acetoin and diacetyl because pyruvate is an intermediate of both lactose and citrate metabolism, and the pyruvate produced from citrate metabolism is converted to lactate and acetylphosphate (McSweeney and Sousa, 2000). Conversely, under acidic conditions (below pH 5.5), *Leuconostoc* spp. produce diacetyl and acetoin, perhaps due to a reduction in their ability to take up lactose (Smid and Kleerebezem, 2014).

During cheese ripening, the growth of NSLAB can also be stimulated by oligosaccharides synthesized by SLAB under limiting conditions (Crow et al., 2002). One of the mechanisms of adaptation of NSLAB that allows them to persist in the cheese matrix during long-term ripening is the production of acetic acid coupled with generation of ATP. Specifically, Lazzi et al. (2014) reported that when *L. rhamnosus* PR1019 was grown in a cheese-like medium, genes linked to the conversion of pyruvate to acetate and to the pathway of ribose degradation were overexpressed, suggesting the activation of alternate pathways for ATP generation. Pyruvate is an intracellular metabolite that could be released in the cheese matrix with starter autolysis. Similarly, ribonucleosides that are released when the starter undergoes lysis could be a source of ribose that represents a fermentable carbohydrate under carbon starvation during cheese ripening (Lazzi et al., 2016). Both pyruvate degradation and ribose catabolism induce a

Figure 3. Cooperation between the starter lactic acid bacteria (SLAB) *Lactococcus lactis* ssp. *cremoris* NCDO763 and glutamate dehydrogenase (GDH)-positive *Lactobacillus* strains in the formation of aroma compounds from branched-chain AA. The GDH-positive lactobacilli convert branched-chain AA to α -keto acids by transamination and partially reduce α -keto acids to hydroxy acids. *Lactococcus lactis* ssp. *cremoris* NCDO763 completes by converting α -keto acids and hydroxy acids to carboxylic acids. NSLAB = nonstarter lactic acid bacteria. Color version available online.

metabolite flux toward acetate, which is then coupled with ATP production via acetate kinase, replacing the lack of ATP generation from glycolysis. In addition to lactococci, which use the phosphoenolpyruvate:phosphotransferase system for lactose transport, *Lb. casei* and *Lb. plantarum* also ferment lactose into phosphoenol pyruvate, which is further hydrolyzed to glucose and galactose-6-phosphate. Both sugars are then fermented by glycolysis, yielding 2 mol of lactic acid/mol of hexose used. Budinich et al. (2011) suggested that substrate availability does not limit the growth of *Lb. paracasei* in cheese up to 8 mo. In that study, it was also reported that *Lb. paracasei* growth was supported by energy sources other than lactose, galactose, and citrate. Milk-derived complex carbohydrates and starter-derived components were suggested as substrates. Starter cell autolysis leads to the release of substrates that are available for the subsequent catabolism by the remaining intact cells in the cheese (Smid and Kleerebezem, 2014).

Desfossés-Foucault et al. (2014) reported an up-regulation of lactate dehydrogenase (*ldh*) and alcohol dehydrogenase (*adh*) in *L. lactis* ssp. *cremoris* SK11 in mixed culture with *Lb. paracasei* ATCC 334, genes normally induced in response to carbon starvation. The *ldh* gene is upregulated at the end of ripening under energy starvation conditions, which indicates competition for substrates (e.g., lactose, citrate). Conversely, *ldh* was downregulated in ATCC 334 in mixed culture, which suggests a switch to mixed acid fermentation. They also showed that deoxyribose-phosphate aldolase (*deoC*) and phosphofructokinase 1 (*pfk*) were upregulated in ATCC 334 in the presence of SK11 during cheese manufacture, as a result of carbohydrate competition under heat and salt stresses (Desfossés-Foucault et al., 2014). In contrast, the galactokinase gene (*galK*) was upregulated throughout ripening in single culture, indicating carbon limitation, whereas expression remained stable in mixed culture, indicating the ability of *Lb. casei* to use other sources of energy when in competition with SK11 (Desfossés-Foucault et al., 2014).

Scavenging Nucleotides and Other Cell Components

Nucleotides are required by all organisms for the synthesis of DNA, RNA, and several coenzymes, and *L. lactis* requires nucleotides to grow in milk (Kilstrup et al., 2005). Lactic acid bacteria are able to produce nucleotides de novo, but these pathways are usually silenced under conditions of excess purines, pyrimidines, and their precursors (Kilstrup et al., 2005). Conversely, many lactobacilli are auxotrophic for both purines and pyrimidines, and the purine requirement cannot be satisfied in milk (Kilstrup et al., 2005). However,

the secretion of nucleases is widely distributed among milk-related lactobacilli species (Péant and LaPointe, 2004). Péant and LaPointe (2004) tested 14 *Lactobacillus* strains, showing that each strain constitutively secreted nucleases and that the levels of production and secretion were strain-dependent. They concluded that the nucleotides released due to the action of nucleases might be utilized by active bacteria present in the medium for synthesis of DNA, RNA, and coenzymes (Péant and LaPointe, 2004).

Gatti et al. (2008) also suggested the potential use of DNA as a source of carbon, nitrogen, and nucleotide bases by NSLAB during ripening. Low levels of SLAB, whole and lysed cells, were found after 24 mo of ripening using the culture-dependent length heterogeneity (LH)-PCR technique (Gatti et al., 2008). During earlier stages of ripening (6 mo), those authors reported a decrease in the peak areas of SLAB whole cells and an increase in the peak areas of SLAB lysed cells. Thus, the presence of dead SLAB during ripening, together with the ability of lactobacilli to release nucleotides in the medium and to further assimilate them, might confer a growth advantage on NSLAB during cheese ripening (Péant and LaPointe, 2004; Gatti et al., 2014). Also, the ability of some species among NSLAB (e.g., *Lb. rhamnosus* and *Lb. paracasei*) to use nonconventional energy sources such as ribose may allow them to persist throughout long ripening times (Lazzi et al., 2014). Lazzi et al. (2014) reported that when *Lb. rhamnosus* PR1019 was grown in a cheese-like medium, genes linked to the pathway of ribose degradation were overexpressed, probably due the presence of ribonucleosides in the cheese matrix being released after starter autolysis. Ribose catabolism, together with pyruvate degradation, was also overexpressed in PR1019, redirecting metabolite flux toward acetate, coupled with ATP production via acetate kinase.

During milk fermentation, the DNA replication genes (*dnaG*, *purD*) in *Lb. paracasei* ATCC 334 were upregulated in the presence of *L. lactis* ssp. *cremoris* SK11, which indicates the need for ATCC 334 to synthesize more purines during fermentation (Desfossés-Foucault et al., 2014). However, SK11 viable populations (10^9 cfu/mL) did not decrease, which was indicated by the absence of cell lysis (Desfossés-Foucault et al., 2014). Moreover, Taïbi et al. (2011) showed that salting caused a downregulation of genes responsible for biosynthesis of purines in *L. lactis* ssp. *cremoris*, which was correlated with a decrease in growth and cell division of *L. lactis* ssp. *cremoris* during milk fermentation. Concomitantly, after 6 d of ripening, a rapid decline in *L. lactis* ssp. *cremoris* populations (10^7 cfu/mL) was also reported by Desfossés-Foucault et al. (2014). These results may indicate that the endonucleases of lactobacilli

and subsequent release of nucleotides may favor their growth during advanced ripening, after the SLAB cell lysis (Gatti et al., 2008).

Cooperation in Protein, Peptide, and Amino Acid Degradation

Proteolysis is one of the chief biochemical events for flavor formation during cheese ripening, releasing AA that are subsequently catabolized, leading to many volatile compounds (McSweeney, 2017). Proteolysis in cheese is catalyzed by enzymes from the coagulant, milk, and enzymes from the starter, nonstarters, or secondary cultures (McSweeney and Sousa, 2000). Caseins are hydrolyzed mainly by the coagulant and to a lesser extent by plasmin (Gobbetti et al., 2015), resulting in the formation of large and intermediate-sized peptides (Figure 1), which are degraded subsequently by the coagulant and enzymes from LAB and NSLAB (Figure 2; Gobbetti et al., 2015). Starters, together with residual chymosin and plasmin, have a greater role than NSLAB in protein breakdown (Gobbetti et al., 2015).

The optimal growth of lactococci in milk depends heavily on their proteolytic systems, as milk has very low concentrations of free AA and peptides (Smit et al., 2005). Thus, high peptidase expression provides a selective advantage in response to the proteolytic activity of *L. lactis* ssp. *cremoris* in the high protein environment of milk. Peptide degradation indicates potential debittering activity that could positively contribute to flavor development (Smeianov et al., 2007). The extracellular cell envelope-associated proteinase (**CEP**) of *Lactococcus* (lactocepain, **PrtP**) contributes to the formation of small peptides in cheese (probably by hydrolyzing the large peptides produced from α_{S1} -casein by chymosin or from β -casein by plasmin; McSweeney, 2017). Similar to the lactococcal lactocepins, other CEP have been isolated from *Lactobacillus* strains. A limited number of the resulting peptides are internalized by the oligopeptide transport system and degraded to AA by a pool of cytoplasmic peptides (McSweeney, 2017). Intracellular SLAB peptidases can be released after cell lysis and are responsible for the degradation of short peptides and production of free AA (McSweeney and Sousa, 2000). The intracellular peptidases of *Lb. helveticus* are thought to play an effective role in the production of free AA in Swiss cheese (Valence et al., 1998). The extracellular proteinase is not present in all LAB strains, even within the same population, as it is often encoded by a plasmid that may be lost, so the resulting cells become Prt[−] (Smit et al., 2005). The Prt[−] cells depend on the Prt⁺ activity of other cells in the starter culture for the production of peptides and AA (Smit et al., 2005). For instance, the *Leu. mesenteroides* popula-

tion (1%) present in the starter culture used in Gouda cheese depends on the caseinolytic *L. lactis* strains for the supply of essential free AA or small peptides (Smid and Kleerebezem, 2014). In the presence of the proteolytic activity of *L. lactis* SK11, the peptidase genes of *Lb. casei* are induced (Desfossés-Foucault et al., 2014).

The branched-chain (Val, Leu, and Ile), aromatic (Tyr, Trp, and Phe), and sulfur-containing (Met and Cys) AA are the major AA sources for flavor production by LAB (Smit et al., 2005). Amino acid catabolism can be initiated by transamination, which requires an α -keto acid as an amino group acceptor for the aminotransferases (Yvon and Rijnen, 2001; Figures 2 and 3). Aminotransferase activity is widespread among NSLAB and lactococci, although higher levels were shown in the SLAB *L. lactis* ssp. *cremoris* NCDO763 than in 2 lactobacilli (*Lb. paracasei*; Kieronczyk et al., 2003). The limiting factor of this reaction is the α -keto acid α -ketoglutarate, which has been shown to improve keto acid formation from AA and is produced from glutamate (Glu) by the activity of glutamate dehydrogenase (**GDH**). Only a few strains of *L. lactis* are capable of producing α -ketoglutarate by deamination of Glu (Pudlik and Lolkema, 2013), whereas some NSLAB genomes code for GDH and this enzyme activity has been reported in *Lb. plantarum* and *Lb. fermentum* (Amarita et al., 2001). Kieronczyk et al. (2003) reported that under cheese-like conditions and in the presence of α -ketoglutarate, *Lactobacillus* strains degraded less AA from Leu, Phe, and Met compared with *L. lactis* ssp. *cremoris* NCDO763. The lactobacilli strains produced high levels of keto acids and hydroxy acids (Figure 3), whereas *L. lactis* ssp. *cremoris* NCDO763 produced mainly carboxylic acids (Kieronczyk et al., 2003). In that study, cooperation between GDH-positive lactobacilli and *L. lactis* ssp. *cremoris* NCDO763 was thought to stimulate flavor formation in cheese (Figure 3). Lactobacilli convert AA to α -keto and hydroxy acids, which can be converted to carboxylic acids by *Lactococcus*. In contrast, some strains of *L. casei* can divert keto acids to components without flavor (e.g., α -hydroxy acids) via the action of D-hydroxyisocaproic acid dehydrogenase, thus reducing the concentration of flavor compounds (Broadbent et al., 2004). Oxidative deamination of Glu to α -ketoglutarate may change the intracellular oxido-reduction potential, which may facilitate the higher production of carboxy acids in the presence of GDH activity (Kieronczyk et al., 2003).

The production of AA by NSLAB is affected by ripening temperature, which also affects their growth. Higher levels of peptides and total free AA were seen in cheeses ripened at 8°C than in those ripened at 1°C (Rehman et al., 2000). Cooperation among microbes during cheese manufacture and ripening also stimulates

flavor formation from AA, as was indicated by the interaction between starter *L. lactis* and GDH-positive *Lactobacillus* (Kieronczyk et al., 2003). Ayad et al. (2001) also reported the production of high levels of branched-chain aldehydes, which are responsible for the development of a malty or chocolate flavor (3-methyl butanal) through cooperative completion of a metabolic pathway between 2 strains of *L. lactis* in milk. *Lactococcus* starters were combined with peptidases from *Lactobacillus* to increase the peptidolytic activity during cheese ripening, thus accelerating and diversifying proteolysis in cheese (Courtin et al., 2002).

Desfossés-Foucault et al. (2014) reported downregulation of *cysK* gene (encoding cystathionine- β -synthetase) expression in single culture of *L. lactis* SK11, whereas this gene was upregulated in mixed culture with *Lb. paracasei* ATCC 334. This effect could lead to the use of Met and Cys in mixed culture by ATCC 334 during ripening. Methionine is the major sulfur-containing amino acid in milk proteins and, among other S-containing compounds, contributes to the cheesy, cabbage, and garlic flavors of cheese. Methanethiol is formed in 2 ways from methionine: directly via a methionine- γ -lyase or indirectly through a methionine transferase (**MetAT**; Figure 2). Amarita et al. (2001) reported that among 22 strains of *Lb. plantarum* and 7 of *Lb. casei*, only 5 and 2, respectively, showed MetAT activity, which indicated that this activity is highly strain-dependent. The same authors reported that *L. lactis* NCDO 763 transaminated more Met than any other strain when α -ketoglutarate was used as the amino group acceptor. None of the 29 lactobacilli strains showed Met lyase activity (Amarita et al., 2001). The relative proportions of individual AA have been found to be similar in many varieties of cheese, which indicates that the subsequent enzymatic or chemical modification of AA could be the critical factor rather than the production of AA itself (Wallace and Fox, 1997).

Lipolysis and Fatty Acid Conversion

Lipolytic degradation of triglycerides of milk fat during cheese ripening results in the release of free fatty acids (**FFA**) affecting the final flavor of cheese (Collins et al., 2003b). Although considered essential in varieties such as blue, surface-ripened (smear), and hard Italian types, extensive lipolysis is considered undesirable in many varieties such as Cheddar, Gouda, and Swiss cheeses (McSweeney and Sousa, 2000). Free fatty acids contribute to desirable flavors in Cheddar cheese at low levels, although at high levels, butyric acid and other short-chain fatty acids lead to undesirable or rancid off-flavors (Collins et al., 2003b). The lipolytic activity observed in Cheddar cheese may be influenced by the

release of intracellular enzymes from the starter bacteria in cheese caused by autolysis (Collins et al., 2003a). Lipolytic enzymes from LAB are mainly intracellular; thus, they must be released upon autolysis to be exposed to their substrates (Figure 1). Several approaches have been proposed to induce cell autolysis and thus accelerate cheese ripening (Thierry et al., 2017).

Lipolytic enzymes in cheese can originate from milk, rennet paste, SLAB, microbial secondary cultures, NSLAB, and exogenous lipase preparations. Milk contains a potent endogenous lipoprotein lipase (**LPL**), which is relatively unstable to heat (complete inactivation of LPL occurs when heating at 78°C for 10 s). Thus, LPL activity would be more significant in raw milk cheese than in cheeses made from pasteurized milk. Short- and medium-chain fatty acids are preferentially released by LPL (Thierry et al., 2017). In cheeses made of pasteurized milk, LAB would contribute more to lipolysis than endogenous enzymes.

Native milk lipids are composed mainly of emulsified triglycerides that can only be hydrolyzed by lipases. However, mono- and diglycerides produced by triglyceride hydrolysis could be hydrolyzed by some esterases, also known as carboxyl ester hydrolases (EC 3.1.1.-). Esterases carry out the hydrolysis and synthesis of ester bonds, and can be divided into lipases (lipolytic enzymes) and nonlipolytic esterases (Thierry et al., 2017). As reviewed by Collins et al. (2003b), LAB can possess esterases and lipolytic activity that are due mainly to intracellular enzymes capable of hydrolyzing fat in milk and cheese (Figure 2). Such activity has been shown for 9 strains of *L. lactis* ssp. *cremoris* using β -naphthyl dodecanoic acid (C12:0) as substrate (Piatkiewicz, 1987) as well as for *Lb. casei* and *Lb. plantarum* using milk fat as substrate (cited in Collins et al., 2003b). In spite of the weak lipolytic system of lactococci and lactobacilli compared with other lipolytic microbes, their enzymes can be responsible for the liberation of significant FFA levels in cheese because cheese made without starter or with low fat leads to lower levels of FFA (Collins et al., 2003b). Lipolytic enzymes from LAB are optimally active at pH 7.0 to 8.5 and temperatures around 35 to 40°C. The *estA* gene in *L. lactis* showed activity on short-chain *para*-nitrophenol (p-NP) esters of fatty acids from C2:0 to C12:0 (Collins et al., 2003b). Only one esterase gene is present in *L. lactis* but it is responsible for the main capacity of *L. lactis* to synthesize short-chain fatty acid esters in vitro (Nardi et al., 2002). On the other hand, the esterase genes of *Lb. helveticus* (*estA*) and *Lb. casei* (*estB* and *estC*) selectively hydrolyzed p-NP esters of fatty acids of C2:0–C4:0 and C3:0–C4:0, respectively, decreasing their selectivity with fatty acid chain lengths longer than 4 carbons (C4). Desfossés-Foucault et al. (2014)

reported that the *estA* gene of SK11 showed the highest expression after incubation at 38°C and after salting (2%). The presence of *Lb. paracasei* ATCC 334 did not alter the expression of *estA* during cheese manufacture, whereas during ripening, this gene was upregulated in SK11 in mixed culture with *Lb. paracasei* compared with single culture, which may indicate competition for the substrates. The genes *estA*, *estB*, and *estC* were active in *Lb. casei* LILA under cheese ripening conditions (Fenster et al., 2000). No differences were observed in ATCC 334, where the genes were downregulated in all conditions, suggesting that this strain of *Lb. paracasei* did not contribute to lipolysis or fatty acid and ester flavor formation in Cheddar cheese (Desfossés-Foucault et al., 2014).

Stress Responses

The cheese environment changes throughout the manufacturing and ripening process. Lactic acid bacteria are subject to various stresses due to modifications in temperature, pH, water activity, redox potential, and carbon availability (Ganesan et al., 2007). These changes affect the expression of technologically important enzymes, which affects the texture and flavor of cheese. The stress response of LAB is strain-specific in spite of the presence of a core gene expression response, which includes regulation of metabolic pathways to maintain metabolism and energy production (Xie et al., 2004; Taïbi et al., 2011). More specifically, molecular chaperones (*dnaK*, *dnaJ*, *GroESL*) and proteinase-chaperone complexes (*ClpP*, *ClpX*, *FstH*) have been reported to be responsible for heat shock and acid stress responses (Parente et al., 2017). Cretenet et al. (2011) reported induction of all these genes, accompanied by a growth arrest of *L. lactis* after 24 h in cheese made from UF milk. Desfossés-Foucault et al. (2014) showed upregulation of stress response genes (*clpC*, *dnaJ*, and *groES*) of *Lb. paracasei* ATCC 334 during growth in mixed culture with SK11. However, during ripening, *Lb. paracasei* ATCC 334 showed lower expression of stress response genes, which could be a consequence of the decrease of viable *L. lactis* ssp. *cremoris* SK11 over time. The authors suggested that lysing starter cells might release substrates that could be further catabolized by NSLAB. In the same study (Desfossés-Foucault et al., 2014), a potential cumulative response to stress at the end of the cheese making was linked to the highest expression of *groEL* in SK11. The presence of both strains SK11 and ATCC 334 amplified their response to cumulative heat and osmotic stresses.

In the case of carbohydrate starvation stress, lactococci respond with several strategies, including becom-

ing nonculturable but remaining active for up to a few years (Ganesan et al., 2007). During this state, cell division, as well as sugar metabolism, is repressed, whereas cells are able to maintain transcription, metabolic activity, and energy production, and to switch from sugar to amino acid metabolism. The ability of lactococci to survive in this state is related to their ability to use energy for protein and biomolecule synthesis, which will be further degraded to generate peptides and AA (Ganesan et al., 2007). Two AA, Met and its precursor Ser, are produced during carbon starvation and cheese ripening (Wallace and Fox, 1997). Although genes involved in autolysis are not expressed during the nonculturable state (Ganesan et al., 2007), bacterial autolysis has been reported after carbon depletion. Temperature shifts such as those encountered during Cheddar cheese manufacturing (from 38 to 40°C) induced lysis of thermolytic *L. lactis* strains (Meijer et al., 1998). Induction of autolysis of *Strep. thermophilus* strains was also reported in response to NaCl concentration, the presence of low levels of ethanol in the medium, or heat shock. Taïbi et al. (2011) observed an increase in the expression of the chaperone protease gene *htrA* for SK11 after a 4% salting, whereas no differences in *htrA* gene expression were observed by Desfossés-Foucault et al. (2014) in SK11 when only 2% salt was used. This gene was most highly expressed after heating during cheese making in both single (SK11) and mixed cultures (with ATCC 334). A substantial reduction of the overall community size of starter culture triggered by the effect of salt addition during brining has been reported (Erkus et al., 2013). A faster decline in viable population was observed in protease-negative *L. lactis* ssp. *cremoris* during the first 2 wk of ripening.

During later stages of cheese ripening, Bachmann et al. (2010) suggested that the expression of the *ldh* gene might be upregulated as the result of energy starvation stress. The pyruvate obtained from lactate by the action of lactate dehydrogenase enzymes might in turn be used for the production of acetate, thereby generating additional energy in the form of ATP (Goffin et al., 2006). Desfossés-Foucault et al. (2014) observed a positive effect on *L. lactis* *ldh* expression in mixed culture, whereas a negative effect was observed on the expression of *ldh* in *Lb. paracasei*. This could indicate that *L. lactis* was more highly affected by carbon starvation than *Lb. paracasei*. Low values of pH (~4.8–5.3) during ripening cause intracellular acidification, decreasing the activity of cytoplasmic enzymes. At pH ~5.0, the F-ATPase system has to pump protons out of the cell to protect intracellular enzymes. It has been reported that the F-ATPase activity of *Lb. casei* and *Lb. plantarum* is optimal at pH 5.0–5.5, whereas that of *L. lactis* is much

higher (pH 7.0–7.5). In contrast to most lactococci, NSLAB are able to produce GDH, which catalyzes the reversible deamination of glutamate to α -ketoglutarate and NH_3 (Figure 2). Although NH_3 production confers protection against acid stress, α -keto acids are catabolized into ketones, aldehydes, alcohols, or acids to improve the cellular redox potential or synthesize ATP. These mechanisms present in NSLAB clearly indicate an adaptation to the cheese environment during ripening in comparison to SLAB.

Autolysis of Starters

After autolysis of SLAB cells, intracellular enzymes (peptidases, esterases, lipases, and enzymes of amino acid catabolism) are released into the medium and reach substrates present in the cheese matrix (Figure 1). Released intracellular bacterial peptidases can decrease bitterness resulting from the hydrolysis of casein-derived hydrophobic peptides. Autolysis of adjunct cultures (*Lb. helveticus* and propionic acid bacteria) used for Swiss-type cheeses also influences the ripening process (Valence et al., 1998). Cell lysis is caused by the activity of endogenous peptidoglycan hydrolases, which are capable of hydrolyzing bonds in their own protective cell wall peptidoglycan (Lortal and Chapot-Chartier, 2005). The ability of LAB to lyse is highly strain dependent, as was extensively demonstrated in Cheddar and Saint-Paulin cheeses (Lortal and Chapot-Chartier, 2005). This strain-dependent character was also reported in dairy *Leuconostoc* strains (Cibik and Chapot-Chartier, 2000). The lysis of some *L. lactis* strains can be induced after a shift in cooking temperature from 38 to 40°C or after one carbon source is depleted in the media (Feirtag and McKay, 1987; Riepe et al., 1997). The differences in autolysis observed in 2 strains of *L. lactis* ssp. *cremoris* were used by Collins et al. (2003a) to suggest the relationship between starter autolysis and lipolysis in Cheddar cheese during ripening. In that study, cheese prepared using the highly autolytic *L. lactis* ssp. *cremoris* strain AM2 had higher levels of lipolysis, probably due to a more efficient and extensive release of lipolytic and esterolytic enzymes. This is in agreement with the fact the most esterases from LAB seem to be located intracellularly (Collins et al., 2003b). Because of lysis, the products of peptidolytic and lipolytic activity can serve as substrates for intact cells for the formation of volatile aroma compounds by increasing the pool of AA (Smid and Kleerebezem, 2014). Thus, the release of intracellular enzymes upon cellular lysis and the metabolic activity of intact cells determine the formation of the typical cheese aroma compounds (Smid and Kleerebezem, 2014). Rapposch

et al. (1999) suggested the ability of *Lb. helveticus* to provide sufficient carbon sources to support the growth of FHL to $>10^7$ cfu/mL. After induction of *L. helveticus* cell lysis, the levels of ribose increased after a week. Later, the decrease of free ribose was correlated with the exponential growth of the ribose-fermenting *Lactobacillus* strains. This hypothesis was recently confirmed by Sgarbi et al. (2014). Those authors used the cell sonicated substrate membrane system, a device consisting of a polycarbonate membrane, which serves as a growth support for NSLAB and a broken SLAB-based (BSM) medium as the culture media. The SLAB *Lb. helveticus* cell lysate was used as the only nutrient source for NSLAB. This methodology confirmed that NSLAB were able to grow on the BSM medium, thus it is possible that NSLAB populations present during ripening use the products of *Lb. helveticus* lysed cells, as previously indicated (Rapposch et al., 1999).

Aroma formation in cheese is influenced by the timing and degree of autolysis (Lazzi et al., 2016). In the case of Grana Padano cheese, the 2-mo time point was shown to be a crucial moment for microbial characterization, which affected the aromatic definition of ripened cheese (Santarelli et al., 2013; Lazzi et al., 2016). Recently, Lazzi et al. (2016) studied the influence of autolysis of LAB on the volatile fraction of Grana Padano cheese. The levels of bacterial lysis were quantified by measuring the activity of aminopeptidases located intracellularly and by LH-PCR of the lysed cell fraction. Length heterogeneity-PCR allows the study of the dynamics of whole and lysed LAB cells, by analyzing the cheese extracts after sterilizing by filtration (filter pore size of 0.22 μm). The authors showed that cheese with an anticipated SLAB cell lysis after brining was characterized by a higher content of FFA, particularly those deriving from milk fat lipolysis, benzaldehyde, and organic acids such as pGlu and citric acid. Early autolysis implies that more enzymes have more time to act in the cheese, but also that cytoplasmic enzymes released after lysis allow easier contact of the enzyme with the precursor molecules, advancing cheese ripening. Another cheese made at a different dairy processing plant showed a greater complexity of volatile compounds, which was associated with a more complex microbial composition following SLAB lysis and NSLAB growth during ripening.

For all these reasons, lytic behavior is one of the phenotypic factors to consider when selecting starters (Parente et al., 2017). Genetic engineering studies have focused on the construction of strains in which lytic behavior can be controlled by environmental conditions, striving to accelerate cheese ripening (cited in Smid and Kleerebezem, 2014).

SYSTEMS INTEGRATION THROUGH GENOMICS AND POST-GENOMIC APPROACHES

Genomic studies have greatly contributed to advancing our knowledge about bacterial evolution, physiology, and metabolic pathways, which have key roles in cheese flavor development (Makarova et al., 2006; Smeianov et al., 2007; Almeida et al., 2014). Culture-independent methods based on the analysis of microbial nucleic acids have provided access to the genetic information contained in a large number of LAB. DNA extracted from cells separated from the cheese matrix was used to study the dynamics of lactococci and lactobacilli during Cheddar cheese ripening (Desfossés-Foucault et al., 2013).

Metagenomics allows access to the genetic information within food environments, specifically in fermented dairy products. Microbial communities from cheese have recently been studied by amplicon-based high-throughput sequencing (HTS; De Filippis et al., 2017), revealing spatial and temporal variations as well as the influence of abiotic factors (O'Sullivan et al., 2013; Dugat-Bony et al., 2016). The data obtained from a sequencing-based approach of 137 cheese rinds were used to reconstruct and manipulate the cheese rind populations under in vitro conditions (culture-based approach; Wolfe et al., 2014). This in situ to in vitro approach could be used to define the mechanisms that influence community structure and function in other cheese ecosystems (Wolfe et al., 2014). With the aim of collecting results from the increasing number of amplicon-targeted HTS analyses performed and using them for meta-studies, a new tool has been developed, called FoodMicrobionet (http://www2.unibas.it/parente/fmbn1_0web/), which will help in the construction of networks of food microbial communities (Parente et al., 2016). As more sequence data are made available, this tool can be used to improve the visualization of microbial interactions in more complex systems (De Filippis et al., 2017).

Amplicon-based HTS is useful in many situations but the poor discriminatory power of most of the phylogenetic markers used in these studies, mainly 16S rRNA gene and internal transcribed spacer (ITS) genes, can be resolved by sequencing the total DNA present in samples. In this case, whole-metagenome sequencing helps us understand the taxonomic and functional composition of the microbial communities (Johansen et al., 2014). It has also served to identify, for the first time, the involvement of the genus *Pseudoalteromonas* in the production of flavor compounds in cheese (Wolfe et al., 2014). Similarly, whole-metagenome sequencing was recently used to correlate the presence of *Thermus thermophilus* with pinking defects in cheese

(Quigley et al., 2016). By targeting DNA, it is possible to characterize the metabolic potential of the cheese microbial communities. Notably, the level of metabolic complementation between *Lactococcus* and *Leuconostoc* strains was evaluated by superimposing the metabolic pathways maps obtained by projecting the clusters of orthologous group categories of *L. lactis* TIFN1-7 pan-genome and the *Leu. mesenteroides* TIFN8 genome separately (Erkus et al., 2013). In cheese, genome-scale metabolic models can predict formation of desired and undesired flavors by each strain and conditions that affect that flavor formation (Flahaut et al., 2013) and possible complementary interactions at the species or strain level. These DNA-based approaches reveal only the potential activity of the microbial communities because of the presence of metabolically inactive and dead cells as well as the variations in transcription activity of different genes under certain conditions (De Filippis et al., 2017). Thus, the combination of approaches, such as transcriptomics, proteomics, and metabolomics, will provide detailed understanding of the functions of microbes in cheese and its relationship with cheese flavor development (Macori and Cotter, 2017). Most of these approaches have been applied independently to study the response of LAB during cheese manufacture and ripening (Table 1).

Transcriptomics

Analysis of DNA extracted from cheese reveals information on both live and dead microbial populations, resulting in overestimations of the active portion of the microbiota. Activity of a species, however, can be measured by its rRNA transcription, which shows the ability of bacteria to make ribosomal RNA and eventually synthesize protein. Investigating bacterial populations using DNA-based methods gives cumulative information, whereas the RNA-based methods give transient snapshots. Furthermore, as rRNA transcripts are often more abundant per cell than genome copies, RNA-based molecular techniques may have enough sensitivity to quantify subdominant species (Desfossés-Foucault et al., 2013). Desfossés-Foucault et al. (2013) studied abundance and rRNA transcriptional activity of lactococci and lactobacilli during Cheddar cheese ripening by targeting the 16S rRNA gene using quantitative PCR (qPCR) and reverse-transcription quantitative PCR (RT-qPCR). Although lactococcal DNA was still dominant after 6 mo of ripening, the rRNA synthesis (cDNA) never exceeded the 16S rRNA genome copy numbers until the 6-mo time point.

Community composition profiling using DNA is not the same as when RNA is analyzed through qPCR and RT-qPCR (Desfossés-Foucault et al., 2013). Lactococ-

Table 1. Post-genomics studies on lactic acid bacteria behavior during cheese manufacturing and ripening

Approach	Method	Medium	Focus	Reference
Transcriptomics	Microarray designed for <i>Staphylococcus aureus</i> and <i>Lactococcus lactis</i> IL1403	Chemically defined medium	Effect of <i>L. lactis</i> on <i>Staph. aureus</i> gene expression	Even et al. (2009)
	Recombinant in vivo expression technology (R-IVET) assay in combination with a high-throughput cheese-manufacturing protocol	Gouda-type cheese	Identification of <i>L. lactis</i> genes specifically induced during specific moments of cheese manufacturing and ripening	Bachmann et al. (2010)
	Real-time quantitative PCR (RT-qPCR)	UF-cheese	In situ global gene expression profile of <i>L. lactis</i> in response to environmental stress	Cretenet et al. (2011)
	RT-qPCR	Cheddar cheese slurry	Interactions between <i>L. lactis</i> ssp. <i>cremoris</i> SK11 and <i>Lactobacillus paracasei</i> ATCC 334 during Cheddar cheese simulation	Desfossés-Foucalt et al. (2014)
	cDNA-amplified fragment length polymorphism and RT-qPCR	Parmigiano Reggiano cheese	Identification of genes potentially responsible for adaptation of <i>Lactobacillus rhamnosus</i> to cheese environment during ripening	Lazzi et al. (2014)
Proteomics	RNA sequencing	Caciocavallo Silano cheese	Exploration of bacterial pathways highly activated during cheese manufacture and ripening	De Filippis et al. (2016)
	Glutamate dehydrogenase activity	Gouda and Cheddar cheese	Cooperation between <i>L. lactis</i> and glutamate dehydrogenase (GDH)-positive nonstarter lactobacilli in the formation of cheese aroma from AA	Kieronczyk et al. (2003)
	Size exclusion chromatography and MS [matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/nanoscale liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS)]	Emmental cheese	Analysis of proteins released in cheese after cell lysis	Gagnaire et al. (2009)
	MALDI-TOF-MS/MS and LC-nano-ESI-MS/MS	Cheese broth	Metabolic and proteomic adaptation of <i>Lb. rhamnosus</i> strains cultivation under cheese-like conditions	Bove et al. (2012)
	Isobaric tagging for relative and absolute quantification (iTRAQ)	Experimental Swiss-type cheeses	Quantitative proteomic analysis of <i>Streptococcus thermophilus</i> and <i>Lactobacillus helveticus</i> enzymes released in cheese during ripening	Jardin et al. (2012)
Metabolomics	Integration of metabolomics (HPLC, solid-phase microextraction-GC/MS) to a genomics-scale metabolic network	Chemically defined medium	Development and validation of a genome-scale model of <i>L. lactis</i> MG1363 to predict flavor formation	Flahaut et al. (2013)
	GC/time-of-flight-MS	Cheddar, Gouda and Parmigiano-Reggiano cheese	Prediction of the sensory characteristics related to hydrophilic low-molecular-weight compounds during ripening	Ochi et al. (2012)
	Headspace GC	Milk	Investigation of the complementary interactions of selected lactococci strains in flavor formation	Ayad et al. (2001)
	Solid-phase microextraction-GC/MS	Caciocavallo Silano cheese	Study of the effect of increasing the ripening temperature on microbial metabolism and cheese maturation rate	De Filippis et al. (2016)

cal rRNA synthesis decreased over the ripening period but remained dominant in the microbial population. Five species were monitored during Cheddar cheese ripening for 6 mo: *Lb. paracasei*, *Lb. buchneri/parabuchneri*, *Lb. rhamnosus*, *Lb. brevis*, and *Lb. coryniformis*. In pasteurized milk cheese, *Lb. brevis* dominated in terms of genome copy abundance, but not in rRNA transcription, whereas *Lb. buchneri/parabuchneri* and *Lb. paracasei* dominated thermized milk cheese. *Lactobacillus paracasei* was the most transcriptionally active of the lactobacilli, especially in cheese made with thermized milk. *Lactobacillus paracasei* and *Lb. buchneri/parabuchneri* showed rRNA transcription in cheese made from pasteurized milk, whereas other *Lactobacillus* species showed low levels of rRNA. *Lactococcus* spp. remained dominant in cDNA (rRNA transcription) throughout ripening. Transcriptional activity of both lactococci and lactobacilli was higher in thermized milk cheeses than those made from pasteurized milk. The cDNA copy number of *L. paracasei* reached 7 logs at 4 to 7°C, and 10 logs at 12°C, demonstrating the effect of ripening temperature on growth and activity of this species.

The adaptive responses of LAB to different stress conditions common to cheese making and ripening have been tested using transcriptomics with the objective to explore the activation of pathways and regulatory systems along with detection of the expression of the main genes involved (Steele et al., 2013). Identification of specific genes expressed under stress conditions helps to select strains that can survive and function under the selective conditions of cheese production. Cheese manufacturing and ripening conditions induce several stress-related genes in *L. lactis* (Bachmann et al., 2010). The global gene expression model of *L. lactis in situ* in UF cheese reveals several adaptive strategies developed by *L. lactis* in response to cheese matrix conditions, especially induction of acidic and oxidative stress response genes (Cretenet et al., 2011). In simulated Cheddar cheese, the effect of consecutive heat, acid, and osmotic stresses on the transcriptional profiles of 4 closely related strains of *L. lactis* ssp. *cremoris* was evaluated. Although all strains showed a core genome expression (stress response, carbohydrate, and amino acid metabolism) to heat stress, strain-specific responses were also observed in most strains (Taïbi et al., 2011).

In addition to bacterial metabolism and their response to certain stresses, interactions between starter culture strains (e.g., *Lactococcus* spp.) and between starter and NSLAB strains (mainly lactobacilli) are proposed to affect cheese flavor by modifying the profile of flavor compounds (Desfossés-Foucault et al., 2014). Interactions between *L. lactis* SK11 and *Lb. paracasei* ATCC 334 were studied using milk fermentation (Pearce activ-

ity test) and a cheese slurry model (Desfossés-Foucault et al., 2014). The transcriptional profiles of *L. lactis* ssp. *cremoris* SK11 and *Lb. paracasei* ATCC 334 in mixed culture in simulated Cheddar cheese showed the overexpression of several genes coding for stress, protein and peptide degradation, and carbohydrate metabolism compared with pure culture, which indicates interactions of starter bacteria and NSLAB toward flavor formation. Gene expression in *L. lactis* ssp. *cremoris* SK11 was not influenced by coculture, but was affected by milk versus cheese slurry, where genes coding for AA metabolism were more highly expressed. On the interaction of strains, a microarray was used to investigate the effect of *L. lactis* on gene expression of *Staph. aureus* in mixed culture (Even et al., 2009). Those authors found that several virulence factors and regulators (such as the *agr* locus, *sarA*, and some enterotoxins) were strongly induced in mixed compared with pure cultures, which emphasizes bacterial interactions at the transcriptome level.

In the initial transcriptomic studies, gene expression was studied in culture media or milk and with laboratory strains. Subsequent studies attempted to apply conditions more closely related to industrial conditions of actual products to predict their effects on flavor formation (Ndoye et al., 2011; Desfossés-Foucault et al., 2014). The transcriptome of lactococci in the cheese matrix was studied during manufacturing and ripening using advanced recombinant *in vivo* expression technology (R-IVET) combined with high-throughput cheese manufacturing (Bachmann et al., 2010). Real-time study of gene expression was carried out (for up to 8 d of manufacturing) using bacterial luciferase as reporter. This method enables *in situ* identification and validation of induced genes, such as *cysD*, *bcaP*, *dppA*, *hisC*, *gltA*, *rpsE*, *purL*, *amtB*, their corresponding promoters, and potential genes involved in the interactive responses between the different strains present in cheese at specific times of cheese making and ripening (Bachmann et al., 2010).

Proteomics

Although transcriptomics reveals valuable information on bacterial activities, the proteome indicates the physiological state of the microbiota, because not all mRNA are translated to proteins (Steele et al., 2013). Identification of bacterial stress proteins and metabolic enzymes shows that microorganisms adjust their metabolism for adaptation to those conditions by moving toward alternate metabolic responses (Steele et al., 2013). Upregulation of genes related to pyruvate degradation, ribose catabolism (Lazzi et al., 2014), lactate dehydrogenation (Desfossés-Foucault et al., 2014),

and a shift to AA metabolism (Ganesan et al., 2007), among others, are examples of adaptive metabolic responses to carbohydrate depletion during ripening. Heat stress in cheese whey was shown to induce expression of several proteins related to heat adaptation such as stress proteins, glycolysis-related machinery, and regulatory factors such as DNA-binding protein II and ATP-dependent protease by *Lb. helveticus* (Gagnaire et al., 2009). In Emmental cheese, *Propionibacterium freudenreichii*, *Lb. helveticus*, and *Strep. thermophilus* were shown to overexpress stress proteins, indicating their adaptation to stress conditions (Gagnaire et al., 2009). The proteomes of dairy products have been well studied, resulting in databases that are helpful in detection of in vitro and in vivo adaptation mechanisms of different strains. Identification of strain-dependent active metabolic pathways in cheese is even more reliable with development of quantitative proteomics (Gagnaire et al., 2009). Thirty proteins from SLAB *Lb. helveticus* and *Strep. thermophilus* (enzymes from central carbon metabolism as well as stress proteins) and bovine origin were identified and quantified using a nano-liquid chromatography (LC) coupled online with electrospray ionization quadrupole time-of-flight (ESI-QTOF) MS (Jardin et al., 2012). The samples were labeled with isobaric tagging reagent for quantitative proteomic analysis (iTRAQ), which allows the identification and quantification of the proteins released in cheese during ripening in a single LC-MS/MS run. In situ bacterial protein quantification helped us understand the response of SLAB metabolism (increase of concentration of most of the glycolytic enzymes identified throughout ripening) to a specific cheese composition (*P. freudenreichii* ssp. *freudenreichii* represented the main ripening bacteria; Jardin et al., 2012).

Metabolomics

Study of the unique chemical fingerprints produced by specific cellular processes—metabolomics—is the final step in omics analysis of cheese flavor. Small changes in fluxes through pathways result in significant changes in the concentration of metabolites, showing tiny but crucial changes that might not be perceived as quickly by other analytical approaches. This makes metabolomics a vital element in systems biology (Teusink et al., 2011).

Advances in metabolomics approaches such as application of extraction and concentration methods, including solid-phase microextraction, have resulted in huge progress in identification of flavor-forming compounds (volatiles) with lower rates of degradation, fragmentation, and generation of chemical artifacts, and loss of

highly volatile components, which are often in trace amounts (Frank et al., 2004).

As with proteomics and transcriptomics, metabolomics has revealed interactions of different strains and their influence on cheese flavor via complementary action of enzymes on substrates (Hassan et al., 2013). Completion of metabolic pathways is a common theme for interactions between SLAB and NSLAB leading to aroma compounds (Kieronczyk et al., 2003). For example, as previously discussed, the combination of *L. lactis* B1157 and SK110 strains resulted in an increase of 3-methylbutanal in milk (Figure 3). Study of the enzyme activities involved in related pathways showed that when SK110, a highly proteolytic strain lacking decarboxylating activity, was cultivated together with B1157, which expresses the decarboxylase, formation of more 3-methylbutanal was observed (Ayad et al., 2001).

Identification of active flavor compounds using metabolomics has made it possible to create a library that can be used to associate desirable cheese flavor or defects to specific molecules (Steele et al., 2013). By fingerprinting hydrophilic low-molecular-weight compounds, which provides data to build sensory prediction models, Ochi et al. (2012) showed that metabolomics has the potential to predict some sensory properties in cheese. They studied different sensory attributes of cheese using GC/time-of-flight MS and a partial least squares model and successfully predicted 2 attributes: rich and sour flavor in ripened cheese. Gan et al. (2016) investigated the volatile compounds of Cheddar cheeses (headspace volatile profiles) using atmospheric pressure chemical ionization (APCI)-MS, coupled with GC-MS, and partial least squares-linear discriminant analysis. Cheese age was successfully predicted at a rate of 70%, based on their key headspace volatile profiles (Gan et al., 2016).

The cheese metabolome is influenced by the spatial distribution of colonies. Spatial distribution of starter lactococci was manipulated in a cheese model (UF cheese) using UF milk by varying the starter inoculum (allowing 8 h of growth) and time of rennet addition (Le Boucher et al., 2015). A high starter inoculum (>7 logs cfu/mL) was used to obtain cheeses with small colonies, whereas a low inoculum (5 logs) gave cheeses with large colonies. Regardless of the spatial distribution, lactococcal counts were 9 logs up to 27 d of ripening. A total of 26 metabolites were affected by spatial distribution, including 4 organic acids and AA from *L. lactis* metabolism. A total of 10 volatiles were higher in small-colony cheeses than in large-colony cheeses. The spatial distribution of colonies affects the ripening process through 2 mechanisms: (1) the surface area

of exchange between colonies and the matrix, and (2) the diffusion rates of small molecules within the matrix and colonies. Diffusion within colonies depends on the physicochemical properties of each metabolite, bacterial surface properties, and physicochemical properties of the surrounding cheese matrix. Some metabolites such as dextran (155 kDa; flexible and neutral charge) do diffuse, whereas proteins such as α_{S1} -casein, lactoferrin, and BSA do not, due to rigidity and charge (Floury et al., 2010). Higher metabolic activity was noted for small colonies, where higher concentrations of some metabolites accumulated due to proteolysis or carbohydrate hydrolysis than in large colony cheeses.

Multi-omics Approaches

A multi-omics approach, combining genomics and post-genomics as an integrated system of genes, environmental conditions, biochemical pathways, proteins, and metabolites, will lead to a detailed picture of the cheese ecosystem and the potential microbial functions and interactions taking place during cheese manufacture and ripening. To date, just a few studies have combined different omics approaches in fermented food (De Filippis et al., 2017; Macori and Cotter, 2017). In cheese, the combined data obtained from metabolomics, metatranscriptomics, and metagenomics were used to facilitate better understanding of the contribution of NSLAB to the maturation process and revealed a direct effect of increasing the ripening temperature in the expression of genes related to proteolysis and lipolysis (De Filippis et al., 2016). These studies provide an in-depth analysis of the microbial community dynamics and help to shed light on the potential interactions among the components of the cheese system and its relationship with flavor formation (Gagnaire et al., 2009).

CONCLUSIONS AND PERSPECTIVES

The interaction between NSLAB and SLAB allows the completion of metabolic pathways favoring aroma formation, but metabolic pathways are often strain-dependent. Recent advances in the genetics of LAB and a better understanding of the interaction between SLAB and NSLAB will facilitate the selection of strains to accelerate cheese ripening, provide consistency in the quality of flavor production during ripening, especially in long-ripened cheeses, and open up possibilities to enhance specific or novel flavor development.

With the help of new knowledge on systems biology and metabolomics, innovative methods can be developed to achieve enhanced and diversified cheese flavor properties and to accelerate flavor development in cheese. However, our knowledge of the metabolic path-

ways of each strain and inter-strain interactions needs to advance to include the intra-population diversity through single cell physiology approaches. Understanding metabolic networks will be crucial to developing functional consortia of LAB for fermentations (de Vos, 2011).

Beyond integrating multi-omics technological approaches, there is the potential application of complex system science, which was first applied in environmental ecosystem modeling (Perrot et al., 2011). More complex predictive models for cheese ripening are thus on the horizon for cheese ripening (Sicard et al., 2012). Multiple species community modeling is overcoming many challenges, especially as it is applied to cheese. Rapid innovation in analytical methods will allow microbial community engineering to reach its full potential in contributing to cheese quality.

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REFERENCES

- Almeida, M., A. Hebert, A. L. Abraham, S. Rasmussen, C. Monnet, N. Pons, C. Delbes, V. Loux, J. M. Batto, P. Leonard, S. Kennedy, S. D. Ehrlich, M. Pop, M. C. Montel, F. Irlinger, and P. Renault. 2014. Construction of a dairy microbial genome catalog opens new perspectives for the metagenomic analysis of dairy fermented products. *BMC Genomics* 15:1101.
- Amarita, F., T. Requena, G. Taborda, and L. Amigo. 2001. *Lactobacillus casei* and *Lactobacillus plantarum* initiate catabolism of methionine by transamination. *J. Appl. Microbiol.* 90:971–978.
- Ayad, E. H., A. Verheul, W. Engels, J. Wouters, and J. Smit. 2001. Enhanced flavour formation by combination of selected lactococci from industrial and artisanal origin with focus on completion of a metabolic pathway. *J. Appl. Microbiol.* 90:59–67.
- Bachmann, H., L. De Wilt, M. Kleerebezem, and J. E. T. van Hylckama Vlieg. 2010. Time-resolved genetic responses of *Lactococcus lactis* to a dairy environment. *Environ. Microbiol.* 12:1260–1270.
- Beresford, T., N. Fitzsimons, N. Brennan, and T. Cogan. 2001. Recent advances in cheese microbiology. *Int. Dairy J.* 11:259–274.
- Bokulich, N. A., and D. Mills. 2013. Facility-specific “house” microbiome drives microbial landscapes of artisan cheesemaking plants. *Appl. Environ. Microbiol.* 79:5214–5223.
- Boucher, B., C. Brotherson, and J. Broadbent. 2006. Influence of starter and nonstarter lactic acid bacteria on medium redox. *Aust. J. Dairy Technol.* 61:116–118.
- Bove, C. G., M. D. Angelis, M. Gatti, M. Calasso, E. Neviani, and M. Gobetti. 2012. Metabolic and proteomic adaptation of *Lactobacillus rhamnosus* strains during growth under cheese-like environmental conditions compared to de Man, Rogosa, and Sharpe medium. *Proteomics* 12:3206–3218.

- Broadbent, J. R., K. Houck, M. Johnson, and C. Oberg. 2003. Influence of adjunct use and cheese microenvironment on nonstarter bacteria in reduced-fat Cheddar-type cheese. *J. Dairy Sci.* 86:2773–2782.
- Broadbent, J. R., S. Gummalla, J. E. Hughes, M. E. Johnson, S. A. Rankin, and M. A. Drake. 2004. Overexpression of *Lactobacillus casei* D-hydroxyisocaproic acid dehydrogenase in cheddar cheese. *Appl. Environ. Microbiol.* 70:4814–4820.
- Budinich, M. F., I. Perez-Diaz, H. Cai, S. Rankin, J. Broadbent, and J. Steele. 2011. Growth of *Lactobacillus paracasei* ATCC 334 in a cheese model system: A biochemical approach. *J. Dairy Sci.* 94:5263–5277.
- Cibik, R., and M. P. Chapot-Chartier. 2000. Autolysis of dairy leucostoc and detection of peptidoglycan hydrolases by renaturing SDS-PAGE. *J. Appl. Microbiol.* 89:862–869.
- Collins, Y. F., P. L. McSweeney, and M. G. Wilkinson. 2003a. Evidence of a relationship between autolysis of starter bacteria and lipolysis in cheddar cheese during ripening. *J. Dairy Res.* 70:105–113.
- Collins, Y. F., P. L. H. McSweeney, and M. G. Wilkinson. 2003b. Lipolysis and free fatty acid catabolism in cheese: A review of current knowledge. *Int. Dairy J.* 13:841–866.
- Courtin, P., M. Nardi, U. Wegmann, V. Joutsjoki, J. C. Ogier, J. C. Gripon, A. Palva, B. Henrich, and V. Monnet. 2002. Accelerating cheese proteolysis by enriching *Lactococcus lactis* proteolytic system with lactobacilli peptidases. *Int. Dairy J.* 12:447–454.
- Cretenet, M., V. Laroute, V. Ulve, S. Jeanson, S. Nouaille, S. Even, M. Piot, L. Girbal, Y. Le Loir, P. Loubiere, S. Lortal, and M. Coccagn-Bousquet. 2011. Dynamic analysis of the *Lactococcus lactis* transcriptome in cheeses made from milk concentrated by ultrafiltration reveals multiple strategies of adaptation to stresses. *Appl. Environ. Microbiol.* 77:247–257.
- Cromie, S. J., J. E. Giles, and J. R. Duley. 1987. Effect of elevated ripening temperatures on the microflora of Cheddar cheese. *J. Dairy Res.* 54:69–76.
- Crow, V., B. Curry, M. Christison, K. Hellier, R. Holland, and S. Liu. 2002. Raw milk flora and NSLAB as adjuncts. *Aust. J. Dairy Technol.* 57:99–105.
- De Filippis, F., A. Genovese, P. Ferranti, J. A. Gilbert, and D. Ercolini. 2016. Metatranscriptomics reveals temperature-driven functional changes in microbiome impacting cheese maturation rate. *Sci. Rep.* 6:21871.
- De Filippis, F., E. Parente, and D. Ercolini. 2017. Metagenomics insights into food fermentations. *Microb. Biotechnol.* 10:91–102.
- de Vos, W. M. 2011. Systems solutions by lactic acid bacteria: From paradigms to practice. *Microb. Cell Fact.* 10:S2.
- Desfossés-Foucault, E., G. LaPointe, and D. Roy. 2013. Dynamics and rRNA transcriptional activity of lactococci and lactobacilli during Cheddar cheese ripening. *Int. J. Food Microbiol.* 166:117–124.
- Desfossés-Foucault, E., G. LaPointe, and D. Roy. 2014. Transcription profiling of interactions between *Lactococcus lactis* ssp. *cremoris* SK11 and *Lactobacillus paracasei* ATCC 334 during Cheddar cheese simulation. *Int. J. Food Microbiol.* 178:76–86.
- Drici, H., C. Gilbert, M. Kihal, and D. Atlan. 2010. Atypical citrate-fermenting *Lactococcus lactis* strains isolated from dromedary's milk. *J. Appl. Microbiol.* 108:647–657.
- Dugat-Bony, E., L. Garnier, J. Denonfoux, S. Ferreira, A. S. Sarthou, P. Bonnamme, and F. Irlinger. 2016. Highlighting the microbial diversity of 12 French cheese varieties. *Int. J. Food Microbiol.* 238:265–273.
- Erkus, O., V. C. de Jager, M. Spus, I. J. van Alen-Boerrigter, I. M. van Rijswijk, L. Hazelwood, P. W. Janssen, S. A. van Hijum, M. Kleerebezem, and E. J. Smid. 2013. Multifactorial diversity sustains microbial community stability. *ISME J.* 7:2126–2136.
- Even, S., C. Charlier, S. Nouaille, N. L. Ben Zakour, M. Cretenet, F. J. Cousin, M. Gautier, M. Coccagn-Bousquet, P. Loubiere, and Y. Le Loir. 2009. *Staphylococcus aureus* virulence expression is impaired by *Lactococcus lactis* in mixed cultures. *Appl. Environ. Microbiol.* 75:4459–4472.
- Feirtag, J. M., and L. L. McKay. 1987. Isolation of *Streptococcus lactis* C2 mutants selected for temperature sensitivity and potential use in cheese manufacture. *J. Dairy Sci.* 70:1773–1778.
- Fenster, K. M., K. L. Parkin, and J. L. Steele. 2000. Characterization of an arylesterase from *Lactobacillus helveticus* CNRZ32. *J. Appl. Microbiol.* 88:572–583.
- Fitzsimons, N. A., T. M. Cogan, S. Condon, and T. Beresford. 2001. Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. *J. Appl. Microbiol.* 90:600–608.
- Flahaut, N. A. L., A. Wiersma, B. van de Bunt, D. E. Martens, P. J. Schaap, L. Sijtsma, V. A. M. dos Santos, and W. M. de Vos. 2013. Genome-scale metabolic model for *Lactococcus lactis* MG1363 and its application to the analysis of flavor formation. *Appl. Microbiol. Biotechnol.* 97:8729–8739.
- Floury, J., S. Jeanson, S. Aly, and S. Lortal. 2010. Determination of the diffusion coefficients of small solutes in cheese: A review. *Dairy Sci. Technol.* 90:477–508.
- Folkertsma, B., P. F. Fox, and P. L. H. McSweeney. 1996. Accelerated ripening of Cheddar cheese at elevated temperatures. *Int. Dairy J.* 6:1117–1134.
- Fox, P. F., P. L. H. McSweeney, and C. M. Lynch. 1998. Significance of non-starter lactic acid bacteria in cheddar cheese. *Aust. J. Dairy Technol.* 53:83–89.
- Frank, D., C. Owen, and J. Patterson. 2004. Solid phase microextraction (SPME) combined with gas-chromatography and olfactometry-mass spectrometry for characterization of cheese aroma compounds. *Food Sci. Technol.* 37:139–154.
- Fröhlich-Wyder, M.-T., W. Bisig, D. Guggisberg, S. Irmeler, E. Jakob, and D. Wechsler. 2015. Influence of low pH on the metabolic activity of *Lactobacillus buchneri* and *Lactobacillus parabuchneri* strains in Tilsit-type model cheese. *Dairy Sci. Technol.* 95:569–585.
- Gagnaire, V., J. Jardin, G. Jan, and S. Lortal. 2009. Invited review: Proteomics of milk and bacteria used in fermented dairy products: From qualitative to quantitative advances. *J. Dairy Sci.* 92:811–825.
- Gan, H. H., B. Yan, R. S. Linforth, and I. D. Fisk. 2016. Development and validation of an APCI-MS/GC-MS approach for the classification and prediction of Cheddar cheese maturity. *Food Chem.* 190:442–447.
- Ganesan, B., M. R. Stuart, and B. C. Weimer. 2007. Carbohydrate starvation causes a metabolically active but nonculturable state in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 73:2498–2512.
- Gatti, M., B. Bottari, C. Lazzi, E. Neviani, and G. Mucchetti. 2014. Invited review: Microbial evolution in raw-milk, long-ripened cheeses produced using undefined natural whey starters. *J. Dairy Sci.* 97:573–591.
- Gatti, M., J. D. D. Lindner, A. De Lorentiis, B. Bottari, M. Santarelli, V. Bernini, and E. Neviani. 2008. Dynamics of whole and lysed bacterial cells during Parmigiano-Reggiano cheese production and ripening. *Appl. Environ. Microbiol.* 74:6161–6167.
- Gobbetti, M., M. De Angelis, R. Di Cagno, L. Mancini, and P. F. Fox. 2015. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. *Trends Food Sci. Technol.* 45:167–178.
- Goffin, P., L. Muscarello, F. Lorquet, A. Stukkens, D. Prozzi, M. Sacco, M. Kleerebezem, and P. Hols. 2006. Involvement of pyruvate oxidase activity and acetate production in the survival of *Lactobacillus plantarum* during the stationary phase of aerobic growth. *Appl. Environ. Microbiol.* 72:7933–7940.
- Hassan, F., M. El-Gawad, and A. Enab. 2013. Flavour compounds in cheese (review). *Res. Precision Instrument Machinery* 2:15–29.
- Hickey, C. D., J. J. Sheehan, M. G. Wilkinson, and M. A. Auty. 2015. Growth and location of bacterial colonies within dairy foods using microscopy techniques: A review. *Front. Microbiol.* 6:99.
- Irlinger, F., S. Helinck, and J. Jany. 2017. Secondary and adjunct cultures. Pages 273–300 in *Cheese: Chemistry, Physics and Microbiology*. 4th ed. P. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Academic Press, London, UK.
- Jardin, J., D. Mollé, M. Piot, S. Lortal, and V. Gagnaire. 2012. Quantitative proteomic analysis of bacterial enzymes released in cheese during ripening. *Int. J. Food Microbiol.* 155:19–28.
- Johansen, P., J. Vindelov, N. Arneborg, and E. Brockmann. 2014. Development of quantitative PCR and metagenomics-based ap-

- proaches for strain quantification of a defined mixed-strain starter culture. *Syst. Appl. Microbiol.* 37:186–193.
- Jordan, K. N., and T. M. Cogan. 1999. Heat resistance of *Lactobacillus* spp. isolated from Cheddar cheese. *Lett. Appl. Microbiol.* 29:136–140.
- Kelleher, P., J. Murphy, J. Mahony, and D. van Sinderen. 2015. Next-generation sequencing as an approach to dairy starter selection. *Dairy Sci. Technol.* 95:545–568.
- Kieronczyk, A., S. Skeie, T. Langsrud, and M. Yvon. 2003. Cooperation between *Lactococcus lactis* and nonstarter lactobacilli in the formation of cheese aroma from amino acids. *Appl. Environ. Microbiol.* 69:734–739.
- Kilstrup, M., K. Hammer, P. R. Jensen, and J. Martinussen. 2005. Nucleotide metabolism and its control in lactic acid bacteria. *FEMS Microbiol. Rev.* 29:555–590.
- Lazzi, C., M. Povolo, F. Locci, V. Bernini, E. Neviani, and M. Gatti. 2016. Can the development and autolysis of lactic acid bacteria influence the cheese volatile fraction? The case of Grana Padano. *Int. J. Food Microbiol.* 233:20–28.
- Lazzi, C., S. Turrone, A. Mancini, E. Sgarbi, E. Neviani, P. Brigidi, and M. Gatti. 2014. Transcriptomic clues to understand the growth of *Lactobacillus rhamnosus* in cheese. *BMC Microbiol.* 14:28.
- Le Boucher, C., V. Gagnaire, V. Briard-Bion, J. Jardin, M. B. Mailard, G. Dervilly-Pinel, B. Le Bizec, S. Lortal, S. Jeanson, and A. Thierry. 2015. Spatial distribution of *Lactococcus lactis* colonies modulates the production of major metabolites during the ripening of a model cheese. *Appl. Environ. Microbiol.* 82:202–210.
- Lortal, S., and M. P. Chapot-Chartier. 2005. Role, mechanisms and control of lactic acid bacteria lysis in cheese. *Int. Dairy J.* 15:857–871.
- Lynch, C. M., C. C. Muir, J. M. Banks, P. L. H. McSweeney, and P. F. Fox. 1999. Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *J. Dairy Sci.* 82:1618–1628.
- Macori, G., and P. D. Cotter. 2017. Novel insights into the microbiology of fermented dairy foods. *Curr. Opin. Biotechnol.* 49:172–178.
- Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA* 103:15611–15616.
- Marilley, L., and M. G. Casey. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *Int. J. Food Microbiol.* 90:139–159.
- Martley, F. G., and V. L. Crow. 1993. Interaction between non-starter microorganisms during cheese manufacture and ripening. *Int. Dairy J.* 3:461–483.
- McSweeney, P. L. H. 2017. Biochemistry of cheese ripening: Introduction and overview. Pages 379–387 in *Cheese: Chemistry, Physics and Microbiology*. P. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Academic Press, London, UK.
- McSweeney, P. L. H., V. Caldeo, A. Topcu, and D. R. Cooke. 2010. Ripening of cheese: Oxidation-reduction potential and calcium phosphate. *Aust. J. Dairy Technol.* 65:178–184.
- McSweeney, P. L. H., and P. F. Fox. 2017. Metabolism of residual lactose and of lactate and citrate. Pages 411–421 in *Cheese: Chemistry, Physics and Microbiology*. 4th ed. P. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Academic Press, London, UK.
- McSweeney, P. L. H., and M. Sousa. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. *Lait* 80:293–324.
- Meijer, W., C. Dobbelaar, and J. Hugenholtz. 1998. Thermoinducible lysis in *Lactococcus lactis* ssp. *cremoris* SK110: Implications for cheese ripening. *Int. Dairy J.* 8:275–280.
- Murtaza, M. A., S. Ur-Rehman, F. M. Anjum, N. Huma, and I. Hafiz. 2014. Cheddar cheese ripening and flavor characterization: A review. *Crit. Rev. Food Sci. Nutr.* 54:1309–1321.
- Nardi, M., C. Fiez-Vandal, P. Tailliez, and V. Monnet. 2002. The EstA esterase is responsible for the main capacity of *Lactococcus lactis* to synthesize short chain fatty acid esters in vitro. *J. Appl. Microbiol.* 93:994–1002.
- Ndoye, B., E. A. Rasolofo, G. LaPointe, and D. Roy. 2011. A review of the molecular approaches to investigate the diversity and activity of cheese microbiota. *Dairy Sci. Technol.* 91:495–524.
- Neviani, E., B. Bottari, C. Lazzi, and M. Gatti. 2013. New developments in the study of the microbiota of raw-milk, long-ripened cheeses by molecular methods: The case of Grana Padano and Parmigiano Reggiano. *Front. Microbiol.* 4:36.
- O'Sullivan, D. J., L. Giblin, P. L. McSweeney, J. J. Sheehan, and P. D. Cotter. 2013. Nucleic acid-based approaches to investigate microbial-related cheese quality defects. *Front. Microbiol.* 4:1.
- O'Sullivan, D. J., P. L. H. McSweeney, P. D. Cotter, L. Giblin, and J. J. Sheehan. 2016. Compromised *Lactobacillus helveticus* starter activity in the presence of facultative heterofermentative *Lactobacillus casei* DPC6987 results in atypical eye formation in Swiss-type cheese. *J. Dairy Sci.* 99:2625–2640.
- Ochi, H., H. Naito, K. Iwatsuki, T. Bamba, and E. Fukusaki. 2012. Metabolomics-based component profiling of hard and semi-hard natural cheeses with gas chromatography/time-of-flight-mass spectrometry, and its application to sensory predictive modeling. *J. Biosci. Bioeng.* 113:751–758.
- Ortakci, F., J. R. Broadbent, C. J. Oberg, and D. J. McMahon. 2015. Growth and gas production of a novel obligatory heterofermentative Cheddar cheese nonstarter lactobacilli species on ribose and galactose. *J. Dairy Sci.* 98:3645–3654.
- Parente, E., L. Coccolin, F. De Filippis, T. Zotta, I. Ferrocino, O. O'Sullivan, E. Neviani, M. De Angelis, P. D. Cotter, and D. Ercolini. 2016. FoodMicrobionet: A database for the visualisation and exploration of food bacterial communities based on network analysis. *Int. J. Food Microbiol.* 219:28–37.
- Parente, E., T. Cogan, and I. Powell. 2017. Starter cultures: general aspects. Pages 201–226 in *Cheese: Chemistry, Physics and Microbiology*. P. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Academic Press, London, UK.
- Parker, M. L., P. A. Gunning, A. C. Macedo, F. X. Malcata, and T. F. Brocklehurst. 1998. The microstructure and distribution of micro-organisms within mature Serra cheese. *J. Appl. Microbiol.* 84:523–530.
- Péant, B., and G. LaPointe. 2004. Identification and characterization of a conserved nuclease secreted by strains of the *Lactobacillus casei* group. *J. Appl. Microbiol.* 96:367–374.
- Perrot, N., I. C. Trelea, C. Baudrit, G. Trystram, and P. Bourguine. 2011. Modelling and analysis of complex food systems: State of the art and new trends. *Trends Food Sci. Technol.* 22:304–314.
- Piatkiewicz, A. 1987. Lipase and esterase formation by mutants of lactic acid streptococci and lactobacilli. *Milchwissenschaft* 42:561–564.
- Porcellato, D., H. M. Østlie, M. E. Brede, A. Martinovic, and S. B. Skeie. 2013. Dynamics of starter, adjunct non-starter lactic acid bacteria and propionic acid bacteria in low-fat and full-fat Dutch-type cheese. *Int. Dairy J.* 33:104–111.
- Pudlik, A. M., and J. S. Lolkema. 2013. Uptake of alpha-ketoglutarate by citrate transporter CitP drives transamination in *Lactococcus lactis*. *Appl. Environ. Microbiol.* 79:1095–1101.
- Quigley, L., D. J. O'Sullivan, D. Daly, O. O'Sullivan, Z. Burdikova, R. Vana, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, P. L. McSweeney, L. Giblin, J. J. Sheehan, and P. D. Cotter. 2016. *Thermus* and the pink discoloration defect in cheese. *mSystems* 1:00023–16.
- Rapposch, S., F. Eliskases-Lechner, and W. Ginzinger. 1999. Growth of facultatively heterofermentative lactobacilli on starter cell suspensions. *Appl. Environ. Microbiol.* 65:5597–5599.
- Rehman, S.-U., J. M. Banks, P. L. H. McSweeney, and P. F. Fox. 2000. Effect of ripening temperature on the growth and significance of non-starter lactic acid bacteria in Cheddar cheese made from raw or pasteurised milk. *Int. Dairy J.* 10:45–53.

- Riepe, H. R., C. J. Pillidge, P. K. Gopal, and L. L. McKay. 1997. Characterization of the highly autolytic *Lactococcus lactis* ssp. *cremoris* strains CO and 2250. *Appl. Environ. Microbiol.* 63:3757–3763.
- Santarelli, M., B. Bottari, C. Lazzi, E. Neviani, and M. Gatti. 2013. Survey on the community and dynamics of lactic acid bacteria in Grana Padano cheese. *Syst. Appl. Microbiol.* 36:593–600.
- Settanni, L., and G. Moschetti. 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. *Food Microbiol.* 27:691–697.
- Sgarbi, E., B. Bottari, M. Gatti, and E. Neviani. 2014. Investigation of the ability of dairy nonstarter lactic acid bacteria to grow using cell lysates of other lactic acid bacteria as the exclusive source of nutrients. *Int. J. Dairy Technol.* 67:342–347.
- Sicard, M., N. Perrot, R. Reuillon, S. Mesmoudi, I. Alvarez, and S. Martin. 2012. A viability approach to control food processes: Application to a Camembert cheese ripening process. *Food Control* 23:312–319.
- Smeianov, V. V., P. Wechter, J. R. Broadbent, J. E. Hughes, B. T. Rodriguez, T. K. Christensen, Y. Ardo, and J. L. Steele. 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Appl. Environ. Microbiol.* 73:2661–2672.
- Smid, E. J., and M. Kleerebezem. 2014. Production of aroma compounds in lactic fermentations. *Annu. Rev. Food Sci. Technol.* 5:313–326.
- Smit, G., B. A. Smit, and W. J. Engels. 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. *FEMS Microbiol. Rev.* 29:591–610.
- Somers, E. B., M. E. Johnson, and A. C. Wong. 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. *J. Dairy Sci.* 84:1926–1936.
- Sousa, M., Y. Ardö, and P. L. H. McSweeney. 2001. Advances in the study of proteolysis during cheese ripening. *Int. Dairy J.* 11:327–345.
- Steele, J., J. Broadbent, and J. Kok. 2013. Perspectives on the contribution of lactic acid bacteria to cheese flavor development. *Curr. Opin. Biotechnol.* 24:135–141.
- Taïbi, A., N. Dabour, M. Lamoureux, D. Roy, and G. LaPointe. 2011. Comparative transcriptome analysis of *Lactococcus lactis* ssp. *cremoris* strains under conditions simulating Cheddar cheese manufacture. *Int. J. Food Microbiol.* 146:263–275.
- Teusink, B., H. Bachmann, and D. Molenaar. 2011. Systems biology of lactic acid bacteria: a critical review. *Microb. Cell Fact.* 10(Suppl. 1):S11.
- Thierry, A., Y. Collins, M. Abeijón Mukdsi, P. McSweeney, M. Wilkinson, and E. Spinnler. 2017. Lipolysis and metabolism of fatty acids in cheese. Pages 423–444 in *Cheese: Chemistry, Physics and Microbiology*. 4th ed. P. McSweeney, P. F. Fox, P. Cotter, and D. Everett, ed. Academic Press, London, UK.
- Valence, F., R. Richoux, A. Thierry, A. Palva, and S. Lortal. 1998. Autolysis of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* in Swiss cheeses: first evidence by using species-specific lysis markers. *J. Dairy Res.* 65:609–620.
- Wallace, J. M., and P. F. Fox. 1997. Effect of adding free amino acids to cheddar cheese curd on proteolysis, flavour and texture development. *Int. Dairy J.* 7:157–167.
- Wilkinson, M. G., T. P. Guinee, and P. F. Fox. 1994. Factors which may influence the determination of autolysis of starter bacteria during cheddar cheese ripening. *Int. Dairy J.* 4:141–160.
- Wolfe, B. E., J. E. Button, M. Santarelli, and R. J. Dutton. 2014. Cheese rind communities provide tractable systems for in situ and in vitro studies of microbial diversity. *Cell* 158:422–433.
- Xie, Y., L. S. Chou, A. Cutler, and B. Weimer. 2004. DNA Microarray profiling of *Lactococcus lactis* ssp. *lactis* IL1403 gene expression during environmental stresses. *Appl. Environ. Microbiol.* 70:6738–6747.
- Yvon, M., and L. Rijnen. 2001. Cheese flavour formation by amino acid catabolism. *Int. Dairy J.* 11:185–201.