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Symposium review: Lactococcus lactis from nondairy sources: Their genetic and metabolic diversity and potential applications in cheese[1]

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ABSTRACT

The widespread dissemination of species of the lactic acid bacteria (LAB) group in different environments testifies to their extraordinary niche adaptability. Members of the LAB are present on grass and other plant material, in dairy products, on human skin, and in the gastrointestinal and reproductive tracts. The selective pressure imparted by these specific environments is a key driver in the genomic diversity observed between strains of the same species deriving from distinct habitats. Strains that are exploited in the dairy industry for the production of fermented dairy products are often referred to as "domesticated" strains. These strains, which initially may have occupied a nondairy niche, have become specialized for growth in the milk environment. In fact, comparative genome analysis of multiple LAB species and strains has revealed a central trend in LAB evolution: the loss of ancestral genes and metabolic simplification toward adaptation to nutritionally rich environments. In contrast, "environmental" strains, or those from raw milk, plants, and animals, exhibit diverse metabolic capabilities and lifestyle characteristics compared with their domesticated counterparts. Because of the limited number of established dairy strains used in fermented food production today, demand is increasing for novel strains, with concerted efforts to mine the microbiota of natural environments for strains of technological interest. Many studies have concentrated on uncovering the genomic and metabolic potential of these organisms, facilitating comparative genome analysis of strains from diverse environments and providing insight into the natural diversity of the LAB, a group of organisms that is at the core of the dairy industry. The natural biodiversity that exists in these environments may be exploited in dairy fermentations to expand flavor profiles, to produce natural "clean label" ingredients, or to develop safer products.
Key words: *Lactococcus lactis*, niche adaptation, domesticated, environmental

INTRODUCTION

Consumption of fermented dairy foods is an age-old tradition that can be traced back thousands of years. From the spontaneous fermentations of the past to the industrial-scale manufacture of fermented dairy products of the present day, the starter cultures used for the production of fermented dairy foods are of great significance, driving the manufacture and development of the flavor and texture of these products. However, the pressures of the current global market and the desire for new products to meet customer demands can test the limits of microbial performance, resulting in the need for constant development of new starter blends with novel properties. As a result, today's starter cultures are developed mainly by design (Hansen, 2002). Strain discovery pipelines are now a common feature of the research and development units of many commercial culture suppliers and in research labs dedicated to rational strain discovery, non-GMO strain improvements, and their associated processes in industrial application. In addition, the application of state-of-the-art "-omics" technologies has provided sophisticated tools for a more knowledge-based approach to selection of desirable cultures (Mills et al., 2010; Kelleher et al., 2015).

Dairy consumers are willing to experiment with different flavors and ingredients and thus, dairy companies are striving to enhance the differentiation points of their products (Dairy Reporter, 2017). To facilitate this, many companies have looked at culture manipulation as a tool for flavor diversification. This has led to an increasing interest in "environmental" isolates, particularly isolates of *Lactococcus lactis* from plant material or other niches. Adaptation of such strains to the substrates encountered in these environments is expected to result in the development of unique traits and phenotypes that could be exploited in dairy applications. Until relatively recently, such isolates were poorly

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characterized. The aim of this review is to consider the most recent information emerging from studies on these so-called wild or environmental strains, isolated from raw milk, plants, animals, and other nondairy environments. We examine the genomic diversity within the *L. lactis* group, the domestication of well-known strains to the dairy environment, and the metabolic potential afforded to strains by traits known to confer an evolutionary advantage in nondairy environments. In addition, we review studies where environmental strains have been applied in dairy settings and comment on the future perspective of such strains being used commercially for the development of dairy products with novel product attributes.

THE ORIGINS OF MODERN CULTURE SYSTEMS

Modern industrial starter cultures originated mainly from natural contaminants of the raw materials used as substrates in fermentation. The contaminating microbiota was responsible for driving a process of spontaneous fermentation, long before the process of fermentation itself was even understood. The practice of "back-slopping," where a small amount of whey or cream from one day's fermentation was used to start the next day's fermentation, ensured maintenance of the starter (Mullan, 2014). However, a lack of quality and consistency in the resulting fermented products meant that such an approach was not satisfactory for today's industrial-scale production, and the concept of the defined strain starter culture was realized in the 1930s (Limsowtin et al., 1996). Traditional starter cultures were screened for individual strains with key properties, such as fast growth in milk, insensitivity to bacteriophages, and production of certain flavors and textures. Strains displaying these desired properties were combined as mixed-strain starters, with the strain combinations depending on the application. Although some artisanal fermented food producers still rely on their own undefined culture blends, most large-scale producers now rely on commercially produced, defined culture blends in which the strains have been specifically selected, blended, and cultivated under tightly controlled conditions to ensure an optimized fermentation each time. A recent review on general aspects of starter cultures is available and provides an overview of both traditional and modern culture systems (Parente et al., 2017). What these authors, and others who have discussed the topic, allude to is that the repeated isolation of single strains from undefined mixed starter blends, coupled with the sharing of these strains between laboratories in the early days of culture screening and analysis, has resulted in a relatively small pool of good starter cultures that forms the basis of the modern fermented dairy foods industry (Marshall, 1991; Kelly et al., 2010; Parente et al., 2017). This has reduced the biodiversity of strains from which to choose for novel applications and increased demand for unique strains from diverse sources, with some concerted efforts to mine the microbiota of natural environments, such as raw milk and plants, for strains of technological interest (Klijn et al., 1995; Kelly et al., 1998; Nomura et al., 2006; Alemayehu et al., 2014; Cavanagh et al., 2015).

LACTOCOCCUS LACTIS—THE QUINTESSENTIAL CHEESE STARTER

As primary components of the starter cultures used in fermented food production, members of the lactic acid bacteria (**LAB**) group are of key industrial importance. The major genera used in dairy fermentations include *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc*. Starter cultures used for the production of cheeses such as Cheddar, Edam, and Gouda typically consist primarily of mesophilic species, predominantly species from the *Lactococcus* genus. Twelve species of *Lactococcus* are currently recognized, along with 6 subspecies (Table 1). Although several species within the genus are derived from nondairy habitats, we are most familiar with the milk- and dairy-associated species. These include *Lactococcus lactis*, *Lactococcus raffinolactis*, and, more recently, *Lactococcus hircilactis* and *Lactococcus laudensis*. The latter 2 species are recently discovered members of the genus isolated from goat and cow milk, respectively (Meucci et al., 2015). The *L. lactis* and *L. raffinolactis* species are recorded in the International Dairy Federation's Inventory of Microbial Food Cultures (Bourdichon et al., 2012) and have long been associated with fermented dairy foods. Little is known about *L. raffinolactis*, but it has been found as a constituent of complex undefined mesophilic starter blends, and the genome sequence of one such strain was recently elucidated (Meslier et al., 2012). To date, 4 *L. lactis* subspecies have been defined: *lactis*; *cremoris*; *hordniae*, from the leafhopper *Hordnia circellata*; and *structae*, isolated from the intestinal mucus of a brown trout (Schleifer et al., 1985; Pérez et al., 2011).

Strains of the subspecies *lactis* and *cremoris* are central components of the defined strain culture blends used in the commercial production of cheese. The principal role of these strains is to produce lactic acid and contribute to the degradation of milk casein, thus influencing the flavor, texture, and quality of the final product. Although strains of *L. lactis* ssp. *lactis* are often considered fast acidifiers, *L. lactis* ssp. *cremoris* strains are often favored as defined starters as they tend to cause less bitterness and other defects. However, their heat sensitivity compared with *L. lactis* ssp. *lactis*

isolates often precludes their use in certain applications. Strains of *L. lactis* ssp. *lactis* biovar *diacetylactis* ferment citrate, which contributes to flavor and aroma through the production of diacetyl. The taxonomic classification of *L. lactis* ssp. *lactis* and ssp. *cremoris* is currently phenotype-based and distinguished on the basis of growth temperature, salt tolerance, and arginine utilization. However, with progress in molecular methods in the last decade, it has become clear that comparison of strains from a broad range of different environmental niches challenges these phenotypic distinctions and that a combination of genotype and phenotype is required to describe strains of this species. This will be discussed in more detail later.

A Plant-Based Origin for Dairy Lactococci

Initial comparative analysis of the first lactococcal strains to be sequenced at the genome level indicated that strains of dairy origin had undergone a reductive evolution process or a minimization of the chromosome toward specialized adaptation to growth in the nutrient-rich milk environment (Klaenhammer et al., 2002). This reductive evolution was shown to be linked to a reduction in genome size, loss of redundant genes, and acquisition of other genes that encoded abilities such as rapid growth in milk, certain stress responses, and host-defense systems relevant to its new habitat (for a review, see McAuliffe, 2017). These features were commonly plasmid-encoded and, indeed, it has been demonstrated the plasmid complement of the dairy specialist strains can contribute up to 200 kb to their overall genome size (Kelly et al., 2010).

Comparative analysis of the early lactococcal genome sequences also revealed numerous signs that the ancestor of certain lactococcal strains inhabited a plant niche (Wegmann et al., 2007). This was most clearly demonstrated by the retention of genes encoding enzymes involved in the metabolism of plant-derived sugars and cell-surface proteins associated with the breakdown of complex plant polysaccharides (Wegmann et al., 2007). Further sequencing and analysis of whole genomes and the plasmid complements of isolates from raw milk and some artisanal dairy products revealed the wider gene repertoire, and resultant metabolic potential, from which the well-characterized dairy strains originated. Analysis of the plasmids of *L. lactis* ssp. *lactis* biovar *diacetylactis* DPC3901 revealed plasmid-encoded markers that could theoretically trace the strain back to a plant origin (Fallico et al., 2011). The plasmids of this raw milk cheese isolate were found to encode genes with significant similarity to those responsible for the degradation of the plant cell wall in *Rhizobium* (Fallico et al., 2011). The authors suggested that the presence of such genes and others in dairy strains could be considered traceability markers, indicators of the previous habitats of the organism—habitats in which these gene functions conferred a real colonization advantage on the host. Following on from this and other similar studies, it is now a commonly held view that dairy strains used in modern cheese production originated from plant material (Kelly et al., 2010). Some authors have speculated that in nature, *L. lactis* stays dormant on plant surfaces; that is, alive but not actively growing, awaiting ingestion into the animal gastrointestinal tract along with the plant material, where it becomes active and multiplies intensively (Bolotin et al., 2001). However, the presence of plant niche-specific gene sets in plant-derived strains does suggest a more active role in the microbial community.

Table 1. Taxonomic classifications within the *Lactococcus* genus

Species	Subspecies	Type strain	Source of isolation	Reference
L. lactis	*lactis*	ATCC19435	Mesophilic dairy starter	Schleifer et al., 1985
	cremoris	NCDO607	Mesophilic dairy starter	Schleifer et al., 1985
	hordniae	NCDO2181	Leafhopper (*Hordnia circellata*)	Schleifer et al., 1985
	tructae	L105(T)	Brown trout	Pérez et al., 2011
L. raffinolactis		4877	Mesophilic dairy starter	Meslier et al., 2012
L. plantarum		NCDO1869 (52)	Frozen peas	Schleifer et al., 1985
L. piscium		NCFB2778	Salmonid fish	Williams et al., 1990
L. garvieae	*garvieae*	ATCC43921	Rainbow trout	Varsha and Nampoothiri, 2016
	bovis	BSN307(T)	Indian bison dung	
L taiwanensis		0905C15(T)	Fresh cummingcordia	Chen et al., 2013
L. chungangensis		CAU28T	Activated sludge foam	Cho et al., 2008
L. formosensis		516(T)	Fermented broccoli stems	Chen et al., 2014
L. fujiensis		NJ317(T)	Chinese cabbage leaves	Cai et al., 2011
L. laudensis		4195(T)	Cow milk	Meucci et al., 2015
L. hircilactis		117(T)	Goat milk	Meucci et al., 2015
L. nasutitermitis		M19T	Gut of wood-feeding termite	Yan Yang et al., 2016
L. reticulitermitis		Rs-Y01(T)	Gut of subterranean termite	Yuki et al., 2018

In contrast to their dairy counterparts, strains from nondairy environments exhibit diverse metabolic capabilities and lifestyle characteristics required for niche specialization. Examples include the nondairy *L. lactis* A12, which is capable of fermenting raffinose and arabinose, which reflects the adaptation of this strain to the sourdough environment, where it rapidly uses sugars that are more abundant due to their slow utilization by yeasts and lactobacilli (Passerini et al., 2013). Another nondairy isolate, *L. lactis* KF147, from mung bean sprouts, possesses numerous gene clusters that may enable it to survive in its environment, such as exopolysaccharide production and utilization of plant carbohydrates (Siezen et al., 2008). As more whole-genome sequences from strains of dairy and nondairy origin became available, it was possible to examine this niche adaptation at a global level. The pan-genome (or species genome) comprises the core genome (genes found in all strains of a species) and the dispensable genome (genes found in single strains or lineages; Lan and Reeves, 2000). A recent study investigated the pan-genome of *L. lactis* by analyzing sequences of a series of *L. lactis* strains of diverse origins with complete genome sequences (Kelleher et al., 2017). Niche adaptation appeared to play a significant role in governing the genetic content of the *L. lactis* strains examined, while genome decay and redundancy were evident in strains from a dairy niche (Kelleher et al., 2017).

The Concept of Domesticated Versus Environmental Strains

As more lactococcal strains from nondairy origins became available, it became possible to examine the natural variability and associated genomic diversity of *L. lactis* from both dairy and nondairy sources. Passerini et al. (2010) used multilocus sequence typing (**MLST**) and pulsed field gel electrophoresis (PFGE) to analyze a bank of *L. lactis* ssp. *lactis* isolates of various ecological sources and geographical areas at the level of both gene and genome. The findings of this study revealed an unexpectedly high level of variability within the subspecies. The variability did not necessarily correlate with division based on "dairy" and "nondairy" strains, but rather with division corresponding to "domesticated" and "environmental" strains. Two clonal complexes (CC1 and CC2) were uncovered by MLST analysis that contained exclusively domesticated strains or strains isolated from dairy starters, from fermented products, but also from milk processing activities. By contrast, strains isolated from raw milk, sourdough, plants, and animals were distributed into numerous unique sequence types (ST), referred to by the authors as environmental strains. Using the concatenated sequences from the MLST to build a phylogenetic tree further demonstrated this domesticated versus environmental division.

Genomic analysis points to an evolutionary trend toward the elimination of systems not required for growth in a complex nutritional environment such as milk. Passerini et al. (2010) speculated on the evolutionary steps that gave rise to domesticated dairy strains. Although it is difficult to predict with accuracy the evolutionary pathway of bacteria, their MLST analysis provided some evidence that the domestication of lactococci occurred by random genetic drift. Based on analysis of phylogenetic trees from theirs and another MLST study of lactococcal strains (Rademaker et al., 2007), they hypothesized that environmental strains appeared first, and domesticated strains emerged much later, quite recently in fact, following a single founder event (Passerini et al., 2010). Founder events are an important example of genetic drift, when a random event, such as the separation of a small group from the rest of the population (Figure 1A), results in the elimination of genes from a population, thus reducing adaptive potential (Dlugosch and Parker, 2008). This gene decay in *L. lactis*, coupled with the acquisition of plasmid-encoded genes for traits such proteolysis and lactose metabolism, could explain the dominance of this species in milk fermentations. However, the presence of such genes is not the only driving force toward domestication because strains in complex starter blends may lack these features or lose them due to general plasmid instability. Another explanation for the evolution of domesticated strains is that they emerged from a bottleneck event (Figure 1B). With the birth of industrial-scale cheese production, the sampling of complex traditional blends used for spontaneous fermentation probably led to the selection of strains from within the blend that exhibited technologically important features, such as fast acid production. This population bottleneck, or a drastic reduction in population size, would have resulted in the elimination of strains within the population that did not display these characteristics; thus, the natural genetic diversity observed in environmental strains would be lost (Passerini et al., 2010). Either way, the evidence emerging from MLST analysis performed by various groups suggests the recent emergence of the dairy genotypes (Rademaker et al., 2007; Passerini et al., 2010).

The adaptation of nondairy lactococci to the dairy environment was recently examined through a process of experimental evolution (Bachmann et al., 2012). The plant strain KF147, into which plasmid pNZ251 encoding the lactococcal extracellular protease (PrtP)

was introduced, was cultured for 1,000 generations in milk. Comparison of the mutant with the wild-type strains revealed several niche-specific adaptations similar to those occurring naturally through the evolutionary process. The downregulation of genes involved in branched-chain amino acid biosynthesis and plant polymer utilization observed, in addition to the loss of some mobile genetic elements, appears to be consistent with the genome decay that is reported to have occurred in dairy strains (Bachmann et al., 2012). Furthermore, mutations in the promoter region of the oligopeptide transport system, resulting in significant upregulation of these genes in milk-adapted strains, demonstrate the importance of this system for growth in milk (Bachmann et al., 2012). Thus, experimental evolution experiments mimic the evolution occurring in nature, whereby extensive adaptation of dairy lactococcal strains to the nutrient-rich milk environment occurs through a process of reductive evolution, resulting in a smaller genome size, a greater number of pseudogenes, and acquisition of a much more extensive plasmid complement (Bolotin et al., 2001; Kelly et al., 2010; Ainsworth et al., 2014).

Phenotype–Genotype Disparity in L. lactis

As previously mentioned, taxonomic classification of strains from subspecies *lactis* and *cremoris* is phenotype-based and distinguished on the basis of growth temperature, salt tolerance, and arginine utilization. Strains with a "*lactis* phenotype" demonstrate the capability to hydrolyze arginine and to grow at 4°C and in 4% NaCl, whereas strains with a "*cremoris* phenotype" do not. With the advent of molecular methods for genotyping, it became obvious that phenotypes and genotypes of strains often do not correlate, particularly when strains of environmental origin are examined. This phenotype–genotype disparity was reported by Cavanagh et al. (2015), in which several nondairy *L. lactis* strains were compared with a bank of dairy isolates through phenotypic and genotypic analysis; examples are given in Figure 2. Each of the strains was analyzed using 2 separate PCR-based genotyping methods. The first of these assays exploits the differences in the sequences of the 16S rRNA genes of each subspecies (Pu et al., 2002). In *L. lactis*, the 16S rRNA gene exhibits 0.07% variance between subspecies, and primer sets were designed

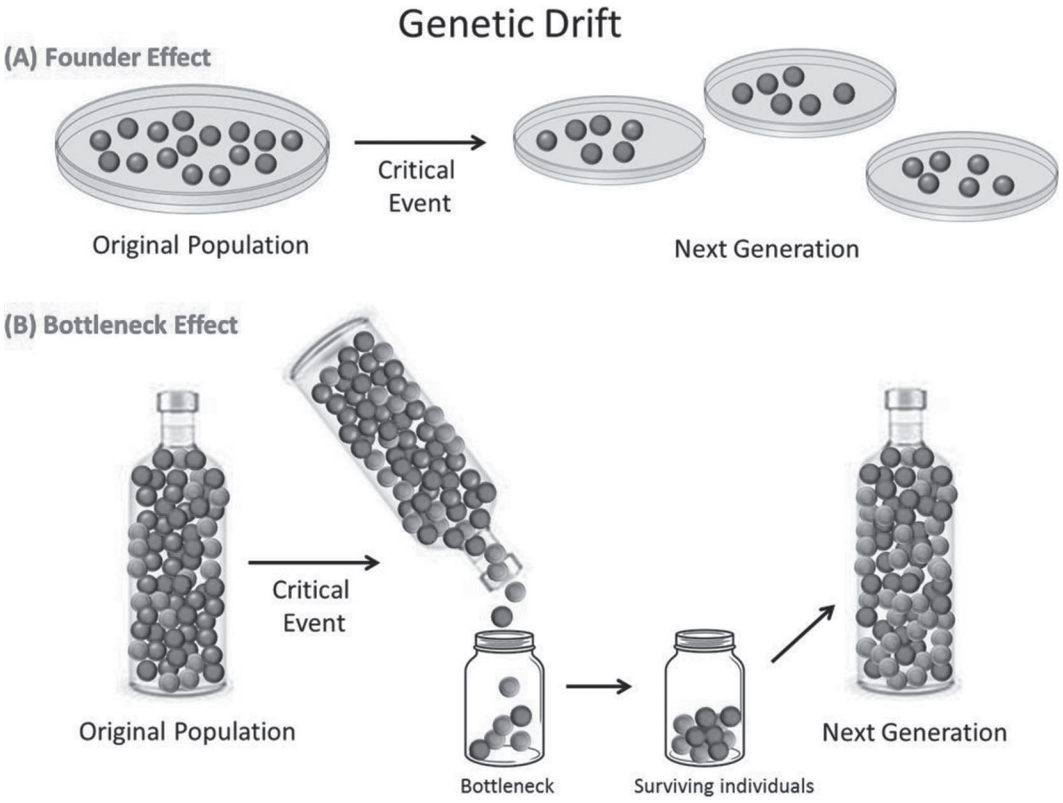

Figure 1. Examples of genetic drift, which can cause significant losses of genetic variation for small populations. (A) Founder effect: this occurs when a new colony is started by a few members of the original population. This small population size means that the colony may have reduced genetic variation relative to the original population or a nonrandom sample of the genes in the original population. (B) Bottleneck effect: this occurs when there is a sharp reduction in the size of a population due to environmental events. Color version available online.

with a common reverse primer and a subspecies-specific forward primer, LacF for subspecies *lactis* and CreF for subspecies *cremoris*. Amplicons are generated with one or other of the subspecies-specific forward primers but not both, depending on the strain (Pu et al., 2002). The dairy strain DPC4268 amplified with the LacF primer but not the CreF primer, indicating a "*lactis*" genotype. Conversely, the dairy strain AM1 amplified with the CreF primer, but not the LacF primer, indicating a "*cremoris*" genotype. These results were corroborated by a second assay, a PCR-based assay designed to detect sequence divergence in the histidine biosynthesis operon (Beimfohr et al., 1997). *Lactococcus lactis* ssp. *cremoris* possesses a 200-bp insertion in the *hisZ* gene, and primers designed to detect this insertion result in the amplification of a 1,149-bp product in subspecies *cremoris* strains and a 934-bp product in subspecies *lactis* strains. The phenotypic evidence confirmed that the phenotype and genotype matched in these strains. However, genotypic analysis of many of the nondairy strains, including DPC6860 from grass (Figure 2), indicated a "*cremoris*" genotype. This was unexpected, because the isolation of strains of the subspecies *cremoris* from environmental sources would be considered quite unusual (Klijn et al., 1995; Salama et al., 1995; Kelly and Ward, 2002). However, upon analysis of the phenotype, it became obvious that most of the nondairy strains tested, including DPC6860, exhibited a *lactis* phenotype, with the ability to hydrolyze arginine and to grow at 4°C and in 4% NaCl (Figure 2; Cavanagh et al., 2015). Other reports have identified subspecies genotypes with mismatching phenotypes (Fernández et al., 2011, Parapouli et al., 2013) and it is speculated that the "*lactis* phenotype," allowing for broader metabolic capabilities, is required for growth in more diverse environments.

Phenotype–genotype mismatching at the *L. lactis* subspecies level has raised the question of the classi-

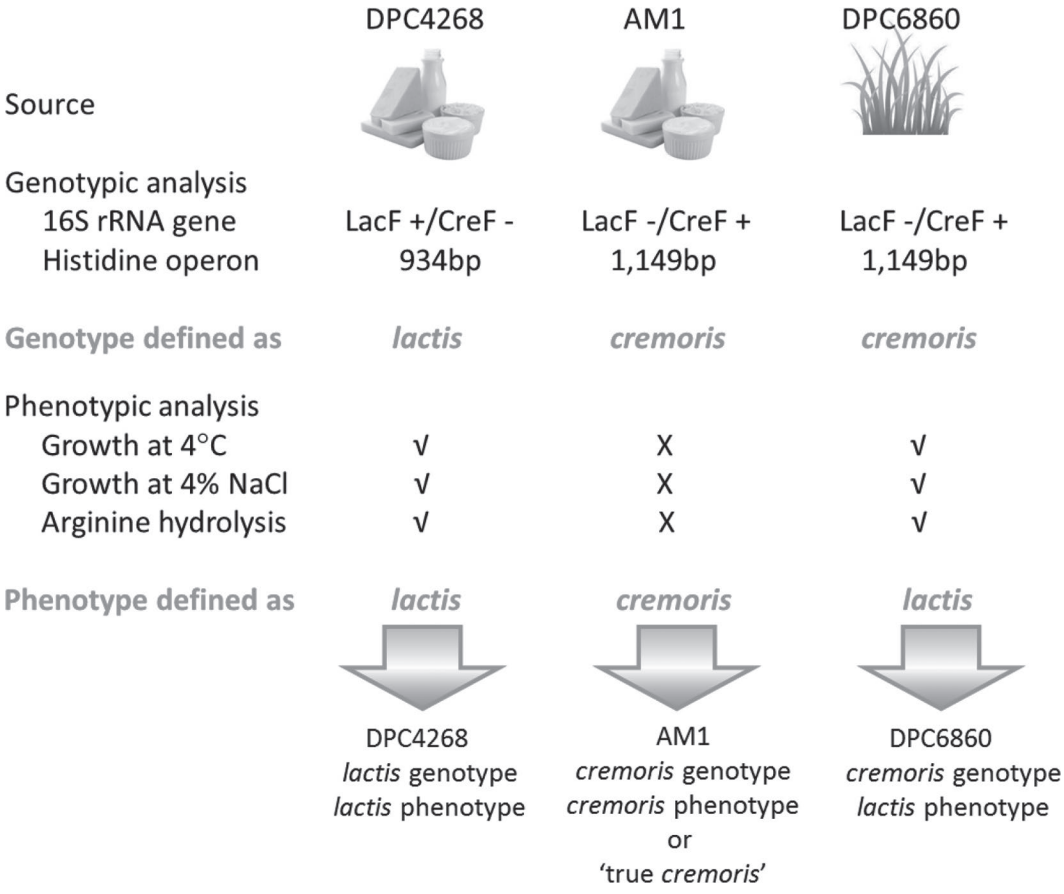

Figure 2. Phenotype–genotype mismatching in the nondairy strain *Lactococcus lactis* DPC6860, isolated from grass. Dairy and nondairy strains were subjected to genotypic tests as described by Beimfohr et al. (1997) and Pu et al. (2002) and assigned genotype on this basis. LacF and CreF are subspecies-specific forward primers for subspecies *lactis* and *cremoris*, respectively. Cultures were tested for the ability to grow at 4°C and in 4% (wt/vol) NaCl for 24 h in LM17 broth. Arginine utilization was assessed using the medium described by Beimfohr et al. (1997) with the addition of bromocresol purple (0.001% wt/vol) in place of phenol red. Information in the figure derived from Cavanagh et al. (2015). Color version available online.

fication of *lactis* and *cremoris* as separate subspecies (Cavanagh et al., 2015). Elucidation of the genome sequences of 3 nondairy strains—from grass (DPC6860), corn (DPC6953), and the bovine rumen (6856)—allowed further examination of the genomic diversity of the nondairy lactococci through comparisons with dairy strains and the estimation of average nucleotide identity (**ANI**) and tetranucleotide frequency correlation coefficients (**TETRA**; Richter and Rossello-Mora, 2009). These tools are utilized in prokaryotic species circumscription to define a species on the basis of genome sequence. Richter and Rossello-Mora (2009) proposed that if an ANI value of <95% is observed with a TETRA score of <0.99, the strains in question should be considered separate species. In the analysis by Cavanagh et al. (2015), strains from both dairy and nondairy origins belonging to subspecies *lactis* and *cremoris* demonstrated ANI values of 85.54 to 87.45%, below the cut-off for species circumspection (*L. lactis* ssp. *lactis* strains from wild environments, plus strain IL1403 interestingly, appear to form a new species). To further confound the issue, a TETRA value of <0.99 was only observed when certain strains were compared; for example, the dairy strain *L. lactis* ssp. *cremoris* TIFN3 and the corn isolate *L. lactis* ssp. *lactis* DPC6853. The low ANI values suggest that subspecies *lactis* and *cremoris* should be considered as different species, whereas the TETRA values only support this in certain cases. This information highlights the need for a revision of the way in which subspecies are designated in *L. lactis*. However, further analysis of both phenotypic and chemotaxonomic markers will be required to support a revision of *Lactococcus* subspecies as separate species.

ENVIRONMENTAL NICHE-SPECIFIC TRAITS

A key differentiation between dairy- and plant-derived strains is their capacity to metabolize a wide variety of sugars. Plant material presents a variety of different carbohydrates that are absent from the milk environment. Whole-genome sequencing of strains from plant-based habitats revealed that much of these genomes are dedicated to the metabolism of plant-based sugars. A diversity analysis of 39 *L. lactis* strains consisting of different subspecies (*cremoris*, *lactis*, and *hordniae*) from plant and dairy sources, assessed the presence or absence of genes involved in the breakdown of plant polymers using comparative genome hybridization (Siezen et al., 2011). The presence of genes for metabolism of the monosaccharide arabinose, a key component of cell wall polymers in plants, was found specifically in plant strains. Genes for the metabolism of α-galactosides, such as raffinose, melibiose, and stachyose, were also present in all plant strains examined. Degradation of complex sugars such as xylan, the main component of hemicelluloses found in both cereals and annual plants, requires multiple enzymes acting together. The gene cluster responsible, *aglA-pda-xynA-xynP-xynQ-xynS-siaA*, was present in some subspecies *lactis* strains of plant origin, but also in 2 dairy *lactis* strains (Siezen et al., 2011). A putative arabinogalactan endo-1,4-β-galactosidase was identified in strain DPC6860 derived from grass, which exhibited 57% amino acid identity to that of *Bacillus coagulans*, with no homologs in *L. lactis* (Cavanagh, 2015). This open reading frame was flanked by 2 transposases that may contribute to the mobilization of this gene. Sequence analysis revealed 2 glycosyl hydrolase family 53 protein domains (pfam07745) and 2 COG3867 domains from arabinogalactan endo-1,4-β-galactosidases. Similar to annual plants, grasses possess highly branched arabinogalactans involved in primary cell wall formation (Carpita, 1996) and it was suggested that the putative arabinogalactan endo-1,4-β-galactosidase protein may function in the degradation of plant carbohydrates by DPC6860 in the grass environment (Cavanagh, 2015).

Recently, functional genome distribution analysis was performed to compare the genome of a lactococcal strain derived from the bovine rumen with other nondairy lactococci in an attempt to understand the metabolism of carbohydrates by rumen-associated strains of *L. lactis* (Cavanagh, 2015). Functional genome distribution analysis enables the identification of nonconserved open reading frames between strain groups based on amino acid similarity, while taking into account genetic alterations associated with niche adaptation (Altermann, 2012). Little is known about *L. lactis* and its inhabitation of the bovine rumen. Due to the large consumption of grass by cattle, these organisms may be transient and simply pass through the bovine digestive system. On the other hand, *L. lactis* may actually colonize the rumen, where it can survive by fermenting plant material ingested by the animal. Comparative analysis of both the grass isolate DPC6860 and the rumen isolate DPC6856 identified several common gene clusters involved in carbohydrate utilization, supporting the hypothesis that the rumen isolate DPC6856 originated from a grass environment. These included a 4-gene cluster involved in xyloglucan metabolism, encoding a β-xylosidase, endoglucanase, permease, and transcriptional regulator, and a 3-gene cluster that encodes a β-glucosidase, a 6-phospho-β-glucosidase, and a sugar kinase, involved in cellulose degradation (Cavanagh, 2015). However, despite these similarities, evidence suggests that the rumen isolate DPC6856 may not be transient but a common rumen inhabitant. A putative lyxose ketol isomerase was identified that has only 2 significant matches in *L. lactis*,

strains IO-1 and KW2, isolated from drain water and fermented corn, respectively. This enzyme catalyzes the reversible conversion of D-lyxose to D-xylulose and has been shown to participate in the reversible conversion of L-ribose to L-ribulose and D-mannose to D-fructose in other species (Cho et al., 2007; Kwon et al., 2010). Other proximal genes were identified as phosphotransferase system (**PTS**) components with fructose specificity, and a putative fructose bisphosphate aldolase was also identified. Mannans such as galactomannans and glucomannans are composed of D-mannose and can be found in plant cell walls. Following the release of D-mannose monomers, these sugars are converted to D-fructose by the lyxose ketol isomerase and taken into the cell via a PTS system. Together, these genes may function in the utilization of D-mannose or other monosaccharides released into the rumen by the action of surrounding organisms. Furthermore, the absence of certain genes in the rumen isolate DPC6856 involved in complex carbohydrate degradation (i.e., arabinogalactan endo-1,4-β-galactosidase) and simple sugar utilization (i.e., xylose utilization) that are present in the grass isolate DPC6860 (Figure 3) hints that these functions may not be required in the rumen because of the contribution of the rumen microbiota to breakdown of grass-associated sugars (Cavanagh, 2015).

Phenotypic studies have confirmed that strains from nondairy niches exhibit a wider range of carbohydrate fermentation profiles than strains from dairy niches. Examples are the plant isolates KF147, from mung bean sprouts, and KF282, from mustard/cress, which grew on a much broader range of sugar substrates than either of the dairy strains IL1403 and SK11 (Siezen et al., 2008). A comparison of carbohydrate utilization profiles by a bank of dairy and nondairy lactococci revealed that most of the plant-derived strains were capable of fermenting D-mannitol, amygdalin, potassium gluconate, L-arabinose, D-xylose, sucrose and gentibiose, whereas none of the tested dairy strains were found to metabolize these sugars (Alemayehu et al., 2014). Consequently, nondairy lactococci have been found to ferment different plant-associated carbohydrates, with industrial dairy strains showing significantly reduced capacity in this regard (Siezen et al., 2008; Alemayehu et al., 2014). The most striking difference between the rumen isolate DPC6856 and the grass isolate DPC6860

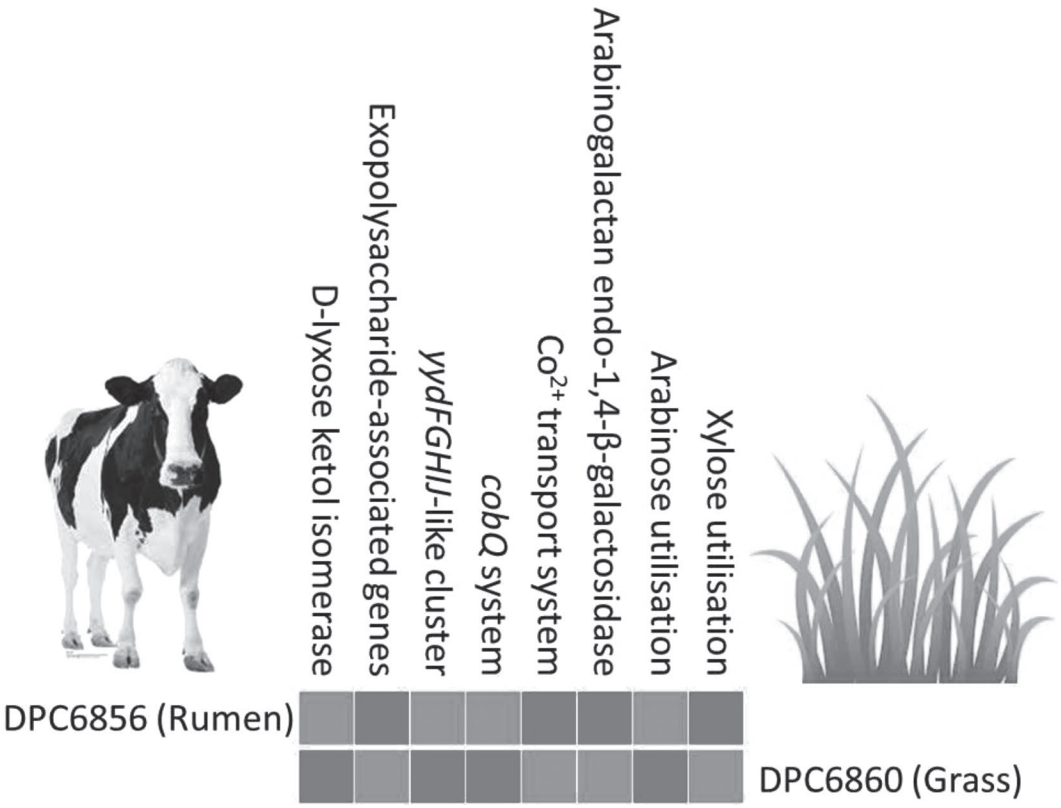

Figure 3. Comparison of the gene content of environmental lactococcal isolates from grass (*Lactococcus lactis* DPC6860) and the bovine rumen (*Lactococcus lactis* DPC6856) suggests that the rumen isolate may not be transient at this location but a specialized rumen inhabitant. The figure has been adapted and now includes EPS as exopolysaccharide. Information in the figure is derived from Cavanagh (2015). Red indicates the presence of genes in a particular strain; green indicates the absence of genes. Color version available online.

was the inability of the rumen isolate to grow on xylose as the sole carbon source (Cavanagh, 2015). However, DPC6856 did grow weakly in the presence of both L-arabinose and melibiose—carbohydrates that did not support the growth of the grass isolate DPC6860.

DAIRY-RELEVANT TRAITS IN ENVIRONMENTAL STRAINS

As previously mentioned, environmental isolates have been found to be more metabolically diverse and often more tolerant of environmental conditions than their domesticated counterparts. Adaptation to high temperatures, higher salt concentrations, and a wider range of pH values may lend these strains to certain dairy applications in which cook temperatures, salt concentrations, and pH ranges vary considerably. Nonetheless, key technological traits are of interest if such strains are to be applied in the industrial dairy setting.

Milk Acidification

Acidification rate is one of the most important technological considerations when choosing a culture for fermented food production. Strains that are to be used as the primary starter should display fast growth in milk and rapid production of lactic acid (Marshall, 1991). In many dairy lactococci, the genes for lactose utilization are located on large plasmids; in fact, strains using the plasmid-encoded phospho-β-galactosidase have been selected over time through intensive industrial use of lactococci (Mills et al., 2010). In nondairy strains, the paucity of plasmids could result in a lactose-negative phenotype in some isolates. However, other pathways for lactose utilization have been detected in *L. lactis*, which may be functional in strains of nondairy origin. These include the chromosomally encoded lactose permease-β-galactosidase, found in certain plasmid-free dairy strains (Aleksandrzak-Piekarczyk et al., 2005), and the chromosomally encoded cellobiose-specific PTS system, an alternate lactose uptake system in *L. lactis* MG1363, a strain described previously as being unable to utilize lactose (Aleksandrzak-Piekarczyk et al., 2011, 2015).

Upon examination of the acidification profiles of nondairy or wild lactococci, acid production in milk was shown to be variable but, in general, nondairy isolates were slower to coagulate milk than their dairy counterparts (Ayad et al., 2000; Fallico et al., 2011; Alemayehu et al., 2014; Cavanagh et al., 2015). This was shown, at least in the study by Alemayehu et al. (2014, to be most likely due to a compromised ability to metabolize lactose, because comparison of the growth of 2 *cremoris* strains in milk in the presence of 0.5% glucose revealed an apparently functioning proteolytic system (Alemayehu et al., 2014). Cavanagh et al. (2015) compared dairy and nondairy isolates from corn, grass, and the bovine rumen using the Pearce test, an activity test that simulates conditions during the Cheddar cheesemaking process (Pearce, 1969). The analysis showed that the nondairy strains, although capable of growing in milk, could not reach the desired pH under the conditions of Cheddar cheese manufacture (Cavanagh et al., 2015). Thus, these strains would not be suitable as primary starters for acid production. However, the fact that the strains were capable of growth in milk without the need for supplementation indicated a role for these strains as adjunct cultures (Ayad et al., 2000; Cavanagh et al., 2014b, 2015).

Activities of Key Flavor-Forming Enzymes

Starter cultures are heavily dependent on their proteolytic system for growth in milk, because the concentrations of both free amino acids and peptides in milk are relatively low (Smit et al., 2005). Cell wall–bound proteinases, peptide uptake systems, and peptidases are key enzymes for the establishment of growth in milk, whereas aminotransferases, decarboxylases, and others catalyze the conversion of the liberated amino acids to an array of key flavor and aroma compounds. Many environmental strains are capable of casein breakdown, as demonstrated by clearing zones surrounding colonies on skim-milk agar plates (Cavanagh et al., 2014b). The activities of aminotransferases for methionine and phenylalanine, a sulfur amino acid and aromatic amino acid, respectively, were tested in several nondairy strains (Cavanagh et al., 2015). The conversion products of phenylalanine (e.g., benzaldehyde, benzoic acid, phenylmethanol ethyl benzoate) had previously been detected in both hard and soft cheeses, whereas the sulfur compounds released from methionine afford onion and garlic flavor notes to cheeses such as Camembert and Cheddar (Marilley and Casey, 2004). Compared with dairy strains, all tested nondairy strains showed increased levels of activity for phenylalanine, with the grass isolate DPC6858 showing the highest activity for this amino acid among tested strains. Conversely, the dairy strains showed higher levels of activity for methionine (Cavanagh et al., 2015). Sequence analysis of several of the nondairy isolates uncovered several aspartate aminotransferases, in addition to the aromatic aminotransferase AraT. In other species, aspartate aminotransferases have been shown to harbor activity toward aromatic amino acids (Rijnen et al., 2003). The presence of these additional enzymes could result in the increased activity toward phenylalanine observed in this study. A recent study similarly observed that

methionine aminotransferase activity was high in many dairy-derived lactococcal isolates (Kelleher et al., 2017). However, aminotransferase activities for methionine and phenylalanine were measured for only one nondairy strain in that study, and activity levels for both enzymes were significantly lower than in the dairy strains.

The decarboxylation activity of nondairy strains, in monoculture and in coculture with an industrial dairy strain, was evaluated by Ayad et al. (2001). Strong decarboxylating activity toward α-ketoisocaproic acid (KICA) was observed for some wild strains in monoculture, as determined by high levels of the chocolate flavor compound 3-methyl butanal in the cell-free extracts. This activity was absent in the dairy control SK110. However, the authors found that by combining SK110, which possesses good proteolytic activity, with a nondairy strain that possesses strong decarboxylase activity, completion of the pathway to 3-methyl butanal formation occurred during fermentation (Ayad et al., 2001). Thus, an approach to strain blending based on knowledge of enzyme activities and flavor formation pathways can result in complementation of incomplete pathways, resulting in the production of certain desired flavor compounds.

Production of Volatile Flavor Compounds

As mentioned above, the development of flavor and aroma in dairy products is mainly an enzymatic process, with many of the enzymes coming from the starter or nonstarter microbiota of the product. The formation of volatile compounds is key to flavor enhancement, and production of these compounds is a result of the complex processes of glycolysis, lipolysis, citrate metabolism, and particularly proteolysis, where amino acids are precursors for their formation (Smit et al., 2005). In a study by Alemayehu et al. (2014), analysis of milk fermentates by solid-phase microextraction coupled to GC-MS analysis showed a clear separation of strains of dairy origin from isolates derived from peas, baby corn, and grass with respect to the diversity and concentration of compounds produced. The nondairy strains produced higher levels of branched-chain aldehydes and their corresponding alcohols from branched-chain amino acid metabolism, as well as higher levels of ethanol, acetoin, 2,3-butanediol, and diacetyl, generally produced in dairy strains from citrate fermentation and associated with desirable natural flavor in certain cheeses. In addition, levels of sulfur compounds were higher in the plant isolates. Similar observations were made in a study by Cavanagh et al. (2014b), where diverse aroma profiles were generated for plant-derived lactococci when grown in milk, compared with their dairy counterparts (Figure 4). In general, nondairy strains were observed to form relatively high abundances of a broad range of important volatile flavor compounds associated with positive flavor attributes in dairy products. It is anticipated that the ability of plant isolates to produce a more varied and diverse volatile profile could be beneficial in dairy applications, where these strains could be used to enhance the flavor profile of products, lead to flavor diversification and the creation of novel products by generating unique flavor profiles, or indeed to mask off-flavors created by dairy strains.

Insensitivity to Common Dairy Phages

The use of wild nondairy strains in commercial situations may depend on the inherent ability of the strains to resist infection by common dairy phages. In the study by Cavanagh et al. (2014a), the sensitivity of a series of nondairy isolates to common lactococcal phages was examined. Most of the dairy phages tested, including POO8, bIL170, HP, C2, and KSY1, were unable to infect the nondairy strains, whereas only one dairy phage, ML3, was found to infect the grass isolate DPC6855 and the rumen isolate DPC6856 (Cavanagh et al., 2014a). In addition, phage ebI was found to infect grass isolates DPC6854 and DPC6855, but not isolates from rumen or vegetable origin. In general, the nondairy isolates were insensitive to common dairy phages, but given the ability of phages to evolve and overcome bacterial phage defense mechanisms, it is expected that phages attacking these strains would eventually appear. Interestingly, phage L47, isolated from a nondairy environment against the grass isolate DPC6860 and found to infect several other grass isolates, is highly related to the dairy phage 949 but cannot infect the 949 dairy hosts (Cavanagh et al., 2014a). Phage L47 possesses an unusually long tail fiber not found in 949, which may be responsible for the specificity of this phage for nondairy isolates. Future work will investigate the diversity of nondairy lactococcal phages and their relationships to dairy phages.

POTENTIAL OF ENVIRONMENTAL STRAINS IN DAIRY APPLICATIONS

As certain environmental strains of lactococci have the potential to produce diverse volatile profiles in model systems compared with their dairy counterparts, there is interest in their behavior in the cheese environment and their potential to be applied to diversify flavor and develop new cheese varieties. A study by Ayad and colleagues (2000) assessed the potential of such strains in a pilot-plant cheese-making process.

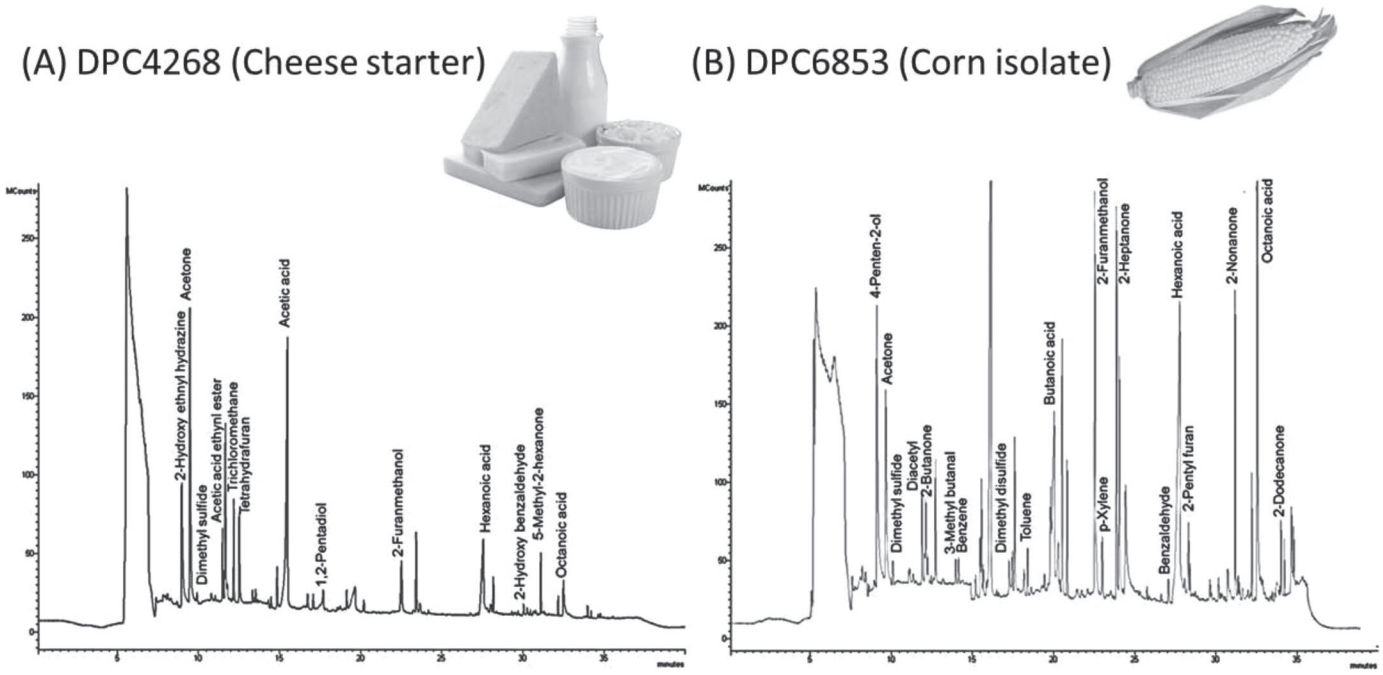

Figure 4. Comparison of the volatile profiles generated by a "domesticated" isolate of *Lactococcus lactis* (strain DPC4268, a cheese starter) and an "environmental" isolate of *L. lactis* (strain DPC6853, isolated from corn). Strains were grown in 10% reconstituted skim milk overnight and the volatile compounds in the fermentate were detected following solid-phase microextraction and subsequent gas-chromatography mass-spectrometry (SPME GC-MS). The strain isolated from corn shows a much more diverse profile of volatile compounds when compared with the dairy isolate, including a higher relative abundance of compounds present in both fermentates. Information in the figure is derived from Cavanagh (2015). Color version available online.

Direct vat inoculation (DVI) culture blends of the industrial dairy strain SK110 and combinations of nondairy wild strains were prepared and used to produce Gouda-type cheeses. The nondairy strains selected were derived from raw milk, milking machines, grass, and soil. Many of the wild nondairy strains grew well with the industrial starter, whereas others were shown to inhibit the growth of SK110, most likely due to production of antimicrobial peptides (Ayad et al., 2000). Assessment of sensory attributes of the resultant cheeses after 3 and 6 mo of ripening demonstrated consistent texture characteristics across all cheeses, and the wild nondairy strains produced flavors that were distinct from that of the SK110 control. Chocolate/cacao and malty flavors were described for cheeses produced from strains derived from raw milk niches, most likely resulting from methylaldehyes originating from leucine, valine, and isoleucine. Although these compounds are not normally found at high levels in Gouda cheese and have been recognized as off-flavors in fermented milk, they are recognized as important flavor compounds in certain cheese varieties, such as Parmesan and Proosdij cheeses (Bosset and Gauch, 1993; Barbieri et al., 1994). Farm cheese-like and Kernhem cheese-like flavor were described for other cheeses, flavors attributable to sulfur compounds. This was confirmed by volatile analysis, which showed high levels of H_2S, methanethiol, or dimethylsulfide in these cheeses, most likely originating from methionine breakdown. Certain sulfur compounds, such as dimethylsulfide, have a low odor threshold in cheese, and are recognized as being important in cheeses such as Cheddar, Gouda, and Limburger. Overall, Ayad et al. (2000) concluded that given the correct ratio of dairy and wild nondairy strains in the culture blend, new flavor combinations could be generated by using wild nondairy strains

A recent study by Cavanagh et al. (2014b) also assessed the potential for flavor diversification of a bank of nondairy lactococci in mini-Gouda cheeses. In this instance, the strains were derived from grass, baby corn, and the bovine rumen, and previous work had shown their technological potential for dairy applications. Mini-Gouda cheeses were produced with a commercial *L. lactis* starter and each of 5 nondairy strains as adjuncts. As a dairy comparison, a similar mini-cheese manufactured with a commercial adjunct, *Lactobacillus helveticus* Flav54, was selected. An additional cheese was produced using an attenuated culture of the corn isolate DPC6853 to determine the effect of attenuation by microfluidization of strains on cheese flavor. The strains DPC6854 (grass) and DPC6856 (bovine rumen) were found to delay acidification and, as a

result, the process time was lengthened. No significant differences were observed for the main compositional parameters of the cheeses; that is, moisture, protein, fat, nonfat substances, fat in DM, or salt in moisture. Strain-to-strain variation was noted in lactate dehydrogenase activity of cheese extracts, an indication of strain autolysis, which often has a positive correlation with flavor development. In addition, the levels of total free amino acids varied among strains but were highest in the cheese made with *Lb. helveticus* as the adjunct, a species known for its higher levels of intracellular peptidase activities. Sensory analysis demonstrated that cheeses made with nondairy strains as adjuncts were associated away from the control and *Lb. helveticus* cheeses and were linked with differences in nutty flavors and aromas and textural attributes, but also with bitterness and astringency (Cavanagh et al., 2014b). Attenuation of DPC6853 by microfluidization before addition of the adjunct to the cheese vat resulted in a less bitter cheese. Microfluidization involves the high-pressure disruption of the integrity of cells, resulting in specific cell populations that may be live, permeabilized, or lysed when added to the vat. This can enhance the enzymatic potential of the cultures during cheese ripening (Yarlagadda et al., 2014). The study by Cavanagh et al. (2014b) further suggests that microfluidization is a suitable method to fully exploit the enzymatic potential of these nondairy strains for flavor development. Overall, the study concluded that performance of the strains in the cheese environment in terms of their survival, lysis, enzymatic activity, and proteolysis was strain-dependent (Cavanagh et al., 2014b). Further work in terms of dosage, attenuation, and possible synergistic interactions between strains may maximize their potential in this or other cheese applications.

FUTURE PERSPECTIVES

The study of strains of dairy species from nondairy or environmental niches demonstrates the vast genetic and metabolic diversity that exists within these groups of organisms. Nondairy settings vary in their composition, and bacterial inhabitants of these environments are exposed to stresses and strains that dairy organisms are not. By selecting strains from environmental sources for dairy applications, we are, in effect, attempting to "turn back the clock" to a time before the domestication of these organisms and the loss of many of the physiological traits that enabled them to survive in the harsher nondairy environment. Exploitation of the diversity of nondairy isolates could have significant implications for new product development. In addition to examining dairy species from nondairy sources, there has been recent interest in examining species not normally associated with dairy products but that are close relatives of common dairy species. Some of the newly identified *Lactococcus* species possess some interesting genes of commercial significance, especially in cheese production. An example is *Lactococcus chungangensis*, an organism isolated from an activated sludge foam (Cho et al., 2008). This organism has been the subject of several reports, has amylase, lipase, and proteinase activities superior to those found in dairy isolates, and has been tested in dairy applications such as cream cheese and yogurt (Konkit et al., 2016; Konkit and Kim, 2016).

Broadening the search for strains for dairy applications to species outside those normally considered could give access to a vast repository of strains and a vast array of possibilities in terms of diversifying product attributes through culture manipulation. However, caution should also be advised. Although species from diverse sources may carry "generally regarded as safe" (GRAS) status, or in the absence of that, have a previous documented presence in foods (Bourdichon et al., 2012), they may nevertheless encode specific genes that, although considered niche adaptation factors, could contribute to these organisms being unsuitable for food applications. Traits such as antibiotic resistance or biogenic amine production, among others, could preclude the use of such strains. Therefore, strains isolated from diverse environments with potential for food applications should be carefully assessed before their introduction to the food production chain.

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