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Minireview

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Growth and adaptation of microorganisms on the cheese surface

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One-sentence summary: This review summarizes the evolutionary processes in the cheese habitat and the main factors and functions involved in the growth of microorganisms on the cheese surface.

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ABSTRACT

Microbial communities living on cheese surfaces are composed of various bacteria, yeasts and molds that interact together, thus generating the typical sensory properties of a cheese. Physiological and genomic investigations have revealed important functions involved in the ability of microorganisms to establish themselves at the cheese surface. These functions include the ability to use the cheese's main energy sources, to acquire iron, to tolerate low pH at the beginning of ripening and to adapt to high salt concentrations and moisture levels. Horizontal gene transfer events involved in the adaptation to the cheese habitat have been described, both for bacteria and fungi. In the future, *in situ* microbial gene expression profiling and identification of genes that contribute to strain fitness by massive sequencing of transposon libraries will help us to better understand how cheese surface communities function.

Key words: cheese rind; smear-ripened cheese; ripening; Brevibacterium; Arthrobacter; Geotrichum candidum; Debaryomyces hansenii

INTRODUCTION

Surface-ripened cheeses are characterized by a complex surface microflora composed of various types of bacteria, yeasts and molds. Until 10 years ago, very few investigation strategies were available for studying typical microorganisms that live on cheese surfaces. Most studies concerned the monitoring of the growth of selected species in model cheeses and the assay of enzymatic activities or substrates and metabolic products. However, the rapid development of microbial genome sequencing has offered new investigation opportunities. Comparative genomic analyses help to identify genetic determinants specific to the cheese habitat and to understand the emergence of species adapted to the cheese surface. Cheese-originating strains whose genome sequences are available or for which there is a genome sequencing project are listed in Table 1. Functional metagenomics provides avenues for a deeper understanding of microbial communities living on cheese surfaces (Wolfe *et al.*, 2014). In addition, over the last years, considerable progress has been achieved for the *in situ* quantification of mRNA transcripts by reverse transcription-quantitative PCR (Duquenne *et al.*, 2010; Falentin *et al.*, 2012; Monnet *et al.*, 2013; Desfossés-Foucault,

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Table 1. List of strains isolated from cheeses with the available genome sequence or with a genome sequencing project*

Strain	NCBI Bioproject ID.	Genome status	Source
FUNGI			
Ascomycota			
Penicillium camemberti FM013	PRJEB4962	In progress	Cheese
Penicillium roqueforti FM164	PRJNA239656	Permanent draft	Cheese
Geotrichum candidum CILB 918	PRJEB5752	In progress	Milk from Pont l'Evêque cheese
BACTERIA			
Actinobacteria			
Agrococcus casei LMG_22410	PRJEB311	Permanent draft	Soft smear-ripened cheese
Arthrobacter arilaitensis Re117	PRJNA53509	Complete	Smeared slightly pressed cheese (Reblochon)
Arthrobacter arilaitensis 3M03	PRJEB261	Permanent draft	Soft smear-ripened cheese
Arthrobacter arilaitensis GMPA29	PRJEB354	Permanent draft	Soft smear-ripened cheese
Arthrobacter bergerei Ca106	PRJEB277	Permanent draft	Mould-ripened soft cheese
Brachybacterium alimentarium CNRZ925	PRJEB293	Permanent draft	Cooked hard cheese
Brevibacterium antiquum CNRZ918	PRJEB292	Permanent draft	Hard cheese
Brevibacterium aurantiacum ATCC 9174†	PRJNA54109	Permanent draft	Smear-ripened cheese (Romadur)
Brevibacterium casei CIP 102111	PRJEB281	Permanent draft	Cheddar Cheese
Brevibacterium linens ATCC 9172	PRJEB273	Permanent draft	Cheese (Harzerkase)
Corynebacterium ammoniagenes ws_2211	PRJEB360	Permanent draft	Cheese surface
Corynebacterium casei LMG S-19264	PRJNA186910	Complete	Surface of a smear-ripened cheese
Corynebacterium casei UCMA 3821	PRJNA78139	Permanent draft	Soft smear-ripened cheese (Livarot)
Corynebacterium flavescens Mu128	PRJEB324	Permanent draft	Soft smear-ripened cheese with raw milk
Corynebacterium flavescens OJ8	PRJNA242338	In progress	Cheese
Corynebacterium variabile DSM 44702	PRJNA62003	Complete	Soft smear-ripened cheese
Kocuria varians Mu208	PRJEB326	Permanent draft	Soft smear-ripened cheese
Leucobacter komagatae 1L36	PRJEB246	Permanent draft	Soft smear-ripened cheese
Leucobacter sp. ER15_166_BHI15	PRJEB301	Permanent draft	Uncooked semi-hard cheese with cow milk
Luteococcus japonicus LSP_Lj1	PRJEB313	Permanent draft	Cheese
Microbacterium foliorum C45	PRJEB276	Permanent draft	Soft smear-ripened cheese
Microbacterium gubbeenense DSM 15944	PRJNA185602	Permanent draft	Surface of a smear-ripened cheese
Microbacterium gubbeenense Mu132	PRJEB325	Permanent draft	Soft smear-ripened cheese
Micrococcus luteus Mu201	PRJEB237	Permanent draft	Soft smear-ripened cheese with raw milk
Propionibacterium acidipropinici ATCC 4875	PRJNA158149	Permanent draft	Emmental cheese
Propionibacterium freudenreichii CIRM-BA1	PRJNA49535	Complete	isolated from Swiss cheese
Propionibacterium freudenreichii ITG P20	PRJEB4826	In progress	Cheese
Propionibacterium thoenii DSM 20276	PRJNA185646	Permanent draft	Emmental cheese
Bacteroidetes			
Chryseobacterium bovis Pi_18	PRJEB34	Permanent draft	Uncooked semi-hard cheese with cow milk
Chryseobacterium ginsengisoli M17	PRJEB316	Permanent draft	Uncooked semi-hard cheese with cow milk
Firmicutes			
Alkalibacterium kapii FAM208_38	PRJEB304	Permanent draft	Surface of pressed cheese
Bacillus altitudinis 263	PRJEB231	Permanent draft	Mould-ripened soft cheese
Bacillus altitudinis ATCC 10987	PRJNA241431	Permanent draft	Cheese
Bacillus altitudinis m1293 (BPS-2)	PRJNA55163	Permanent draft	Cream cheese
Bavariicoccus seileri DSM 19936	PRJNA188834	Permanent draft	Surface of smear-ripened cheese
Bavariicoccus seileri WCC_4188	PRJEB359	Permanent draft	Soft smear-ripened cheese
Brevibacillus parabrevis CIP 110335	PRJEB288	Permanent draft	Hard cheese
Brochothrix thermosphacta cH814	PRJEB279	Permanent draft	Uncooked semi-hard cheese with cow milk
Carnobacterium maltaromaticum 38b	PRJEB254	Permanent draft	Soft smear-ripened cheese
Carnobacterium maltaromaticum LMA28	PRJNA179370	Complete	Mould-ripened soft cheese (Brie)
Clostridium tyrobutyricum UC7086	PRJNA170963	Permanent draft	Hard cheese (Gran Padano)
Enterococcus durans IPLA_655	PRJNA188163	Permanent draft	Cheese
Enterococcus faecium CRL1879	PRJNA191091	Permanent draft	Artisanal cheese
Enterococcus italicus DSM 15952	PRJNA53039	Permanent draft	Cheese, Italian Toma, from bovine milk
Enterococcus malodoratus ATCC 43197	PRJNA191903	Permanent draft	Gouda cheese
Enterococcus malodoratus FAM208_55	PRJEB305	Permanent draft	Surface of pressed cheese (Gouda)
Exiguobacterium acetylicum 180	PRJEB230	Permanent draft	Cheese
Facklamia tabacinasalis FAM208_56	PRJEB306	Permanent draft	Surface of pressed cheese
Jeotgalicoccus psychrophilus CRBM_D21	PRJEB298	Permanent draft	Uncooked semi-hard cheese with cow milk

Table 1. (*Continued*).

Strain	NCBI Bioproject ID.	Genome status	Source
Leuconostoc lactis 1283	PRJEB235	Permanent draft	Cheese
Leuconostoc mesenteroides TIFN8	PRJNA175676	Permanent draft	Natural cheese starter culture
Listeria innocua Clip11262	PRJNA86	Complete	Cheese, Morocco
Marinilactibacillus psychrotolerans 42ea	PRJEB266	Permanent draft	Soft smear-ripened cheese with raw milk
Marinilactibacillus psychrotolerans FAM208_59	PRJEB307	Permanent draft	Soft smear-ripened cheese
Ornithinibacillus bavariensis CIP 109287	PRJEB287	Permanent draft	Cheese
Paenibacillus sp. 3M17	PRJEB363	Permanent draft	Soft smear-ripened cheese
Staphylococcus equorum Mu2	PRJEA8889	Permanent draft	French smear-ripened cheese (Munster)
Staphylococcus fleuretti CIP 106114	PRJEB283	Permanent draft	Cheese made with goat milk
Staphylococcus lentus Ca2	PRJEB278	Permanent draft	Mould-ripened soft cheese
Staphylococcus vitulinus Ma1	PRJEB320	Permanent draft	Soft smear-ripened cheese with raw milk
Staphylococcus warnerii 1445	PRJEB241	Permanent draft	Uncooked semi-hard cheese with cow milk
Vagococcus fluvialis bH819	PRJEB275	Permanent draft	Uncooked semi-hard cheese with cow milk
Vagococcus lutrae FAM208	PRJEB303	Permanent draft	Surface of pressed cheese
Proteobacteria			
Acinetobacter johnsonii 3M05	PRJEB262	Permanent draft	Soft smear-ripened cheese
Alcaligenes faecalis 2L10	PRJEB250	Permanent draft	Soft smear-ripened cheese
Alcaligenes sp. 2L29	PRJEB251	Permanent draft	Soft smear-ripened cheese
Brevundimonas diminuta 3F5N	PRJEB260	Permanent draft	soft smear-ripened cheese with raw milk
Citrobacter freundii Pi_15	PRJEB346	Permanent draft	Uncooked semi-hard cheese with cow milk
Hafnia alvei GB001	PRJEB6257	Permanent draft	Cheese
Halomonas alkaliphila 3A7M	PRJEB256	Permanent draft	Soft smear-ripened cheese
Halomonas sp. 1M45	PRJEB249	Permanent draft	Soft smear-ripened cheese
Halomonas sp. 3F2F	PRJEB259	Permanent draft	Soft smear-ripened cheese
Halomonas venusta 3D7M	PRJEB258	Permanent draft	Soft smear-ripened cheese
Klebsiella oxytoca Pi_20	PRJEB348	Permanent draft	Uncooked semi-hard cheese with cow milk
Kluyvera intermedia TL336_A	PRJEB358	Permanent draft	Uncooked semi-hard cheese with cow milk
Morganella morganii 3A5A	PRJEB255	Permanent draft	Soft smear-ripened cheese with raw milk
Morganella psychrotolerans 925	PRJEB233	Permanent draft	Soft smear-ripened cheese
Proteus hauseri 1M10	PRJEB247	Permanent draft	Soft smear-ripened cheese
Proteus vulgaris 1M25	PRJEB248	Permanent draft	Soft smear-ripened cheese
Providencia alcalifaciens GM3	PRJEB309	Permanent draft	Soft smear-ripened cheese
Providencia heimbachae GR4	PRJEB310	Permanent draft	Soft smear-ripened cheese
Providencia rettgeri 947	PRJEB234	Permanent draft	Soft smear-ripened cheese
Pseudomonas fragi 1E26	PRJEB244	Permanent draft	Soft smear-ripened cheese
Pseudomonas sp. 1E44	PRJEB245	Permanent draft	Soft smear-ripened cheese
Psychrobacter aquimaris ER15_174_BHI7	PRJEB302	Permanent draft	Uncooked semi-hard cheese with cow milk
Psychrobacter celer 91	PRJEB270	Permanent draft	Mould-ripened soft cheese with raw milk
Psychrobacter faecalis H5	PRJEB280	Permanent draft	Soft smear-ripened cheese
Psychrobacter immobilis PG1	PRJEB345	Permanent draft	Soft smear-ripened cheese
Psychrobacter namhaensis 1439	PRJEB240	Permanent draft	Mould-ripened soft cheese with raw milk
Raoultella ornithinolytica TL332	PRJEB357	Permanent draft	Uncooked semi-hard cheese with cow milk
Raoultella planticola 3M45	PRJEB264	Permanent draft	Soft smear-ripened cheese
Serratia marcescens 448	PRJEB267	Permanent draft	Soft smear-ripened cheese
Serratia proteamaculans 1C2F	PRJEB243	Permanent draft	Soft smear-ripened cheese with raw milk
Serratia rubidaea TA_26	PRJEB355	Permanent draft	Uncooked semi-hard cheese with cow milk
Stenotrophomonas maltophilia Pi1	PRJEB349	Permanent draft	Uncooked semi-hard cheese with cow milk
Stenotrophomonas rhizophila PCA13	PRJEB334	Permanent draft	Uncooked semi-hard cheese with cow milk
Vibrio litoralis B4	PRJEB274	Permanent draft	Soft smear-ripened cheese

*Lactic acid bacteria belonging to the genera *Lactobacillus*, *Lactococcus* and *Streptococcus* were not included in this table.
†formerly *Brevibacterium linens* BL2.

LaPointe and Roy 2014) and even by high-throughput RNA sequencing (Lessard *et al.*, 2014).

The manufacture of fresh cheese curd generally takes from about 5 to 24 h. It involves the acidification of milk by lactic acid bacteria and the action of rennet, resulting in milk coagulation. The gel is then cut and the drained curds are transferred to moulds. Cheeses are then salted and transferred to ripening rooms. The ripening time for surface-ripened cheeses is typically 2–4 weeks. During that time, there is an intense growth and activity of aerobic microorganisms at the surface of the cheeses. Growth of the cheese surface microorganisms corresponds to a colonization of a nutrient-rich environment, followed by a stationary growth phase and sometimes by a decline in population. The ability of microorganisms to establish themselves on the surface of cheeses depends on several factors. One of them is the ability to use cheese as an efficient growth medium. The composition and structure of this medium change throughout ripening. In addition, the microorganisms have to adapt

themselves to the presence of other microorganisms with which they may have positive or negative interactions. As an example, a scheme has been proposed for the dynamics of the principal Livarot yeasts (Larpin *et al.,* 2006): *Kluyveromyces lactis* grows at the very beginning of ripening and contributes to lactose uptake. It is then inhibited by salting; *Geotrichum candidum* and *Debaryomyces hansenii* contribute to deacidification of the curd by preferentially consuming lactate and amino acids, favoring the growth of aerobic acido-sensitive ripening bacteria; *Yarrowia lipolytica,* a strongly lipolytic species, grows primarily during the latter half of ripening and limits the mycelium development of *G. candidum*. Abiotic conditions such as temperature and relative humidity also influence the growth of microorganisms at the surface of the cheese. The ability to survive in the cheese manufacturing environment is another important feature since it favors the subsequent recontamination of the cheese.

EVOLUTIONARY PROCESSES IN THE CHEESE HABITAT

The genomes of cheese surface microorganisms may contain signatures of 'domestication' due to genetic events that contribute to a better adaptation to the cheese habitat. Several types of events may be distinguished. In bacteria, genes that have no beneficial function tend to be eliminated due to the energy required for their maintenance. They can be eliminated by recombination, which is favored by the presence of mobile elements such as insertion sequences. Numerous insertion sequences and pseudogenes are found in the genome strain *Arthrobacter arilaitensis* Re117 that originates in cheese, showing that there is process of reductive evolution in this species that is still going on (Monnet *et al.,* 2010). Comparisons with *Arthrobacter* strains that originate in the soil showed that many genes involved in the transport and catabolism of substrates have been lost in the cheese strain, probably due to the lower diversity of substrates in cheeses.

In bacteria, horizontal gene transfers may occur via three main mechanisms: transformation, transduction and conjugation. Such transfers may confer a selective advantage to the recipient cell, as observed for *A. arilaitensis* Re117, whose genome contains a gene cluster involved in the catabolism of D-galactonate that probably originated from a *Pseudomonas* strain. The importance of horizontal transfers in the eukaryotic kingdom is thought by many to be anecdotal. However, the recent sequencing of the genome of a *Penicillium roqueforti* and a *P. camemberti* strain showed the presence of a large region (~500 kb) known as *Wallaby*, which resulted from horizontal transfers (Cheeseman *et al.,* 2014). *Wallaby* was found almost exclusively in species from the food environment. It has been independently acquired in some *Penicillium* species, and the perfect identity of the sequences implies recent transfer events. Such transfers may be facilitated by the ability of fungi such as *Penicillium* to form somatic fusions between mycelia. The function of *Wallaby* has not yet been well established, but the presence of genes predicted to be involved in the regulation of conidiation or in antimicrobial activities suggests a functional advantage associated with competition with other cheese microorganisms. Horizontal gene transfer events also occur in yeasts such as *Y. lipolytica*, *D. hansenii* and *K. lactis* (Dujon *et al.,* 2004), but this has not yet been investigated for cheese isolates.

Multilocus sequence typing analyses have shown that *G. candidum* strains can be separated into two populations: the cheese and the environment isolates, suggesting an adaptation to the cheese habitat (Morel 2012). The even distribution of mating types suggests that mating events occur in cheese and may contribute to gene exchanges. Diploid strains have been isolated from cheeses, revealing that mating also occurs for *D. hansenii* (Jacques *et al.,* 2010). Yeast diploids and hybrids display robust characteristics such as tolerance to environmental stress, which could be beneficial in the cheese surface habitat.

The rate at which microorganisms evolve to adapt to the cheese habitat is not known. However, the propagation of a *Lactococcus lactis* strain isolated from fermented plant material for only 1000 generations in milk resulted in several genetic modifications that consisted of point mutations and gene deletions (Bachmann *et al.,* 2012). There is evidence that herders in Europe were producing cheese between 6800 and 7400 years ago (Salque *et al.,* 2013), and it may thus be assumed that some cheese microorganisms result from extensive genetic adaptations that could even result in new species. Even if there is evidence of the evolution of cheese-specific strains, other habitats such as skin or soil also lead to selective pressure similar to that found on the cheese surface (e.g. osmotic stress, desiccation, iron-restriction), and some important properties of cheese strains may thus have arisen from evolutionary selection on the skin and in the soil (Monnet *et al.,* 2015). Ropars *et al.* (2012) distinguished two types of cheese molds. The first corresponds to ubiquitous fungi such as *P. roqueforti*, *Scopulariopsis candida* and *S. fusca*. *Penicillium roqueforti* has been frequently isolated from silage, from food products and from forest soil or wood. *Scopulariopsis candida* and *S. fusca* have diverse origins, from soil as well as plant and human pathogens. These molds seem to have only recently adapted to cheese. The second type corresponds to species such as *P. camemberti*, *Fusarium domesticum*, *S. flava* and *Sporendonema casei*, which are only found in cheese and are well adapted to this habitat. *Penicillium camemberti* is related to *P. cavernicola*, which can be isolated from the walls of natural caves and is also able to develop on fat-rich products. Cheeses were originally made in natural caves and cellars and it is conceivable that the capacity to live in fat-rich products has been conserved from a common ancestor. Adaptation of *F. domesticum* to the cheese surface may be related to the ability of a common ancestor of *F. dimerium* to establish itself in biofilms.

FACTORS AND FUNCTIONS INVOLVED IN THE GROWTH OF MICROORGANISMS ON THE CHEESE SURFACE

pH, temperature and moisture

Several factors important for the growth of cheese surface microorganisms are listed in Table 2. At the beginning of ripening, the pH value is typically around 5. On the surface, this low pH favors the growth of aerobic yeasts and molds that are more tolerant to acidity than bacteria. The ability of yeasts to tolerate acid pH values seems to be related to the activity of plasma membrane ATPase that regulates intracellular pH by exporting protons (Praphailong and Fleet 1997). Presumably, yeasts that tolerate low pH values have a more efficient or stable plasma membrane ATPase system. Cheese yeasts and molds contribute to the increase in the pH of the cheese surface by transforming lactate to CO_2 and also by producing ammonia from amino acids. Coagulase-negative staphylococci such as *Staphylococcus equorum*, *S. sciuri* and *S. xylosus* can grow at pH 5.5 and even below (Bockelmann *et al.,* 1997), which may explain their ability to grow early during ripening. Other surface bacteria such as *Arthrobacter* sp., *Brevibacterium* sp. and *Corynebacterium* sp. are

Table 2. Factors acting on the growth of microorganisms at the cheese surface

Factor	Significance for the cheese surface microorganisms	Comments
pH	Acido-tolerant strains grow at the early stages of ripening; acido-sensitive bacteria are more abundant at the end of ripening	pH changes are due to the activity of the microbial community
Oxygen	Aerobic strains grow under the surface of the curd (~2–5 mm) or form a mycelium cover over the curd	Regular turning of the cheese ensures that oxygen is supplied to all cheese surfaces
Salt concentration	Selective pressure for salt-tolerant strains	Salt concentration depends on manufacturing practices (amount and type of salt supply)
Temperature	Selective pressure for strains growing at 10–15°C	Temperature profile depends on manufacturing practices
Moisture	Selective pressure for strains able to grow in conditions of high or low moisture of rind	Moisture mainly depends on manufacturing practices (type of curd, washing, relative humidity of ripening cellar)
Energy substrates	Selective pressure for strains able to use the main energy sources from cheese (lactose, galactose, lactate, amino acids, lipids)	Changes in energy substrates present in the curd are due to the activity of the microbial community
Inhibitors	Selective pressure for strains able to tolerate inhibitors produced by the surface microflora, such as ammonia, free fatty acids and bacteriocins	These inhibitors are produced by the cheese microflora
Iron	Selective pressure for strains with efficient iron acquisition systems	Iron availability depends on several factors such as pH, proteolysis, diffusion in the solid cheese matrix and the presence of microbial iron chelators
Phages	Possible density-dependent predation of bacteria by phages	No information available about the presence of phages of cheese surface bacteria

more acido-sensitive and begin to grow when the pH is around 5.5 to 6.0. At the end of ripening, the pH at the cheese surface can reach a value higher than 7.5. It can be considered that the general trend towards yeast domination during the early stages of ripening in smear-ripened cheeses (i.e. when the pH is low), followed by bacterial domination at the end of ripening, is due to the fact that the yeasts are more adapted to acidic pH than the bacteria.

Cheese ripening typically occurs at a temperature of 10–15°C. Microorganisms that grow under these conditions include mesophiles that have a large tolerance of temperature range, psychrophiles and psychrotrophes. Optimal growth temperature for yeasts is between 20 and 30°C, but their ability to grow and have proteolytic and lipolytic activities at low temperatures favors their colonization of the cheese surface, as observed for *Candida* spp. and *Y. lipolytica* (Corbo *et al.*, 2001). The growth of *D. hansenii* is greater at 20°C than at 10 or 15°C, but the effect of NaCl is less pronounced at low temperatures (Masoud and Jakobsen 2005). For some cheese varieties, the temperature is set to ~20°C at the beginning of ripening to promote the growth and deacidifying activity of the yeasts.

The relative humidity of the ripening room also has an influence on the growth of microorganisms at the cheese surface. For example, *P. camemberti* grows less well at a relative humidity greater than 95% (Leclercq-Perlat *et al.*, 2013), and in smear-ripened cheeses, high humidity in ripening rooms, combined with repeated brushing of the cheeses with salt water, prevents the growth of molds. Correlations have been found between cheese rind moisture and community composition of 137 different rinds (Wolfe *et al.*, 2014). In this study, the fungus *Galactomyces* and four genera of *Proteobacteria* (*Psychrobacter*, *Vibrio*, *Pseudomonas* and *Pseudoalteromonas*) were positively correlated with rind moisture, whereas *Scopulariopsis*, *Aspergillus*, *Actinobacteria* and *Staphylococcus* were negatively associated with moisture. The impact of moisture on the growth of cheese rind genera was also observed in reconstituted *in vitro* microbial communities, where dry environments were enriched with *Debaryomyces*, *Staphylococcus* and *Penicillium*.

Salt tolerance

Cheeses are salted by applying salt to their surface or by submerging them in a saturated salt brine. *Debaryomyces hansenii* strains are able to grow at 16% NaCl (Masoud and Jakobsen 2005). This species is frequently isolated in salty environments such as seawater, brines and salted food products, and is one of the most salt-tolerant yeast species. It has mechanisms for extrusion of sodium ions, accumulates glycerol as a compatible solute when exposed to high NaCl concentrations (Prista *et al.*, 2005) and has a high intrinsic resistance to the toxic effects of sodium and potassium ions. *Geotrichum candidum* is considered to be less tolerant to salt than *D. hansenii* (Boutrou and Gueguen 2005). In mould-ripened cheeses, if the salt content is too low, *G. candidum* can outcompete *P. camemberti* and form a surface defect known as 'toad skin'. On the other hand, when the salt concentration is too high, the growth of *G. candidum* will be suppressed and excessive growth of *P. camemberti* may then lead to excessive proteolysis and bitterness defects (Spinnler and Gripon 2004).

Brevibacterium linens and *Corynebacterium* spp. are stimulated by 4% NaCl and are able to grow in the presence of 12% NaCl, and some strains even tolerate 16% NaCl (Masoud and Jakobsen 2005). The *Brevibacterium*, *Corynebacterium* and *Arthrobacter* strains originating from cheese whose genomes have been sequenced are well equipped with genes that offer protection from high salt concentrations. One protection mechanism is the accumulation of osmoprotectants such as ectoine, proline and glycine betaine in the cytoplasm. Fifteen genes involved in the transport of glycine betaine and related osmolytes were identified in *A. arilaitensis* Re117, whereas a lower number—from six to nine genes—are present in *Arthrobacter* strains from

soil, which are less tolerant to high salt concentrations (Monnet *et al.*, 2010). One particularity of the *B. aurantiacum* ATCC 9174 genome is the large number of osmoprotectant transporters. Indeed, nine different betaine/carnitine/choline family transporters have been identified in this strain, whereas the mean number in other *Actinomycetales* is 1.5 (analysis performed with the Integrated Microbial Database, http://img.jgi.doe.gov/). The *Corynebacterium variabile* DSM 44702 genome contains the *ectP* gene encoding an ectoine transporter, *proP,* which encodes an osmoregulated proline transporter, and six genes encoding proteins of the betaine/carnitine/choline transporter family (Schröder *et al.*, 2011). High salt concentrations on the surface of smear-ripened cheeses may also explain the presence of bacteria usually found in marine environments, e.g. *Halomonas* sp., *Marinilactibacillus psychrotolerans*, *Pseudoalteromonas* sp. and *Vibrio* sp. (Feurer *et al.*, 2004; Ishikawa *et al.*, 2007; Roth *et al.*, 2011; Wolfe *et al.*, 2014).

Iron acquisition

Cheese is a highly iron-restricted habitat because milk is poor in iron (0.2–0.4 mg l^{-1}) and contains lactoferrin, a protein that has an antibacterial effect due to its ability to chelate iron. In model cheeses involving a lactic acid bacterium (*L. lactis*), a yeast (*D. hansenii*) and different ripening bacteria (*Arthrobacter*, *Corynebacterium* or *Brevibacterium*), the addition of iron or siderophore enhanced the growth of ripening bacteria (Monnet, Back and Irlinger 2012). The genomes of the four bacteria originating in cheese, *C. variabile* DSM 44702, *C. casei* UCMA 3821, *A. arilaitensis* Re117 and *B. aurantiacum* ATCC 9174, are well equipped with genes involved in iron acquisition. In *A. arilaitensis* Re117, two siderophore biosynthesis gene clusters have been identified, one of which has no counterpart in the *Arthrobacter* strains from soil (Monnet *et al.*, 2010). In *C. variabile* DSM 44702, genes involved in the production of a catechol siderophore are present, some of which are located on a genomic island (Schröder *et al.*, 2011). In the taxonomic subline in which *C. variabile* is located (cluster 3), similar siderophore biosynthesis genes are only present in the genome of the pathogen *C. jeikeium*. Examination of the draft genome sequence of *B. aurantiacum* ATCC 9174 also revealed the presence of a gene cluster involved in the synthesis of a catechol-type siderophore (locus BlinB01002486 to BlinB01002493). Three of these genes (BlinB01002490 to BlinB01002492) result from a horizontal gene transfer since the closest orthologs are found in Gram-negative species. Furthermore, most of the *Brevibacterium* strains of dairy origin investigated by Noordman *et al.* (2006) were able to produce siderophores. In *S. aureus*, the *sbnABCDEFGHI* operon encodes proteins involved in the biosynthesis of the siderophore staphylobactin, which enhances virulence. This operon is also present in the genome of *S. equorum* Mu2, where it probably contributes to the supply of iron during growth at the cheese surface (Irlinger *et al.*, 2012). The number of ABC-type iron-siderophore transport components is higher in *Actinomycetales* from cheeses (from 20 to 56 genes) than the mean number (13 genes) found in *Actinomycetales* from other environments (Monnet, Back and Irlinger 2012). In addition, cheese *Actinomycetales* have fewer proteins with iron–sulfur cluster domains, and it has been hypothesized that these strains decreased their need for iron by eliminating proteins that require iron.

Pezizomycotina fungi such as *Penicillium* spp. are able to produce siderophores (Ong and Neilands 1979; Winkelmann 2007), whereas Saccharomycotina such as *K. lactis*, *D. hansenii*, *G. candidum* and *Y. lipolytica* cannot produce them but are able to import siderophores produced by other microorganisms (Haas, Eisendle and Turgeon 2008; Blaiseau *et al.*, 2010). It is possible that iron acquisition is at the origin of some interactions between the microorganisms that grow on the surface of cheese, as suggested for microorganisms from marine sediments (D'Onofrio *et al.*, 2010).

Energy substrates

Even if lactic acid bacteria are found at the cheese surface, they do not represent a large fraction of the bacterial population in many cases. This is due to the competition with the aerobic microorganisms that catabolize important energy sources such as lipids, lactic acid and amino acids, which are rarely used by the fermentative lactic acid bacteria. Surface-ripened cheeses have a high surface area/volume ratio, which favors the growth and activity of the aerobic microflora. The main substrates catabolized by cheese surface microorganisms to produce energy are lactose, galactose, lactate, amino acids, proteins and lipids. The extent and chronology of substrate utilization depends on the cheese variety and microorganisms.

In general, only limited amounts of lactose are available at the beginning of ripening. It is catabolized by yeasts such as *K. lactis* and *K. marxianus*, which have a fermentative lactose catabolism, by *D. hansenii* that performs a complete oxidation to CO_2, but not by *G. candidum* (Boutrou and Gueguen 2005). In Camembert-type cheeses, it was suggested that lactose is a major energy source for the mycelial growth of *P. camemberti* at the beginning of ripening. Its depletion might provoke stress, resulting in slower growth and spore production (Leclercq-Perlat *et al.*, 2013). Most aerobic ripening bacteria do not catabolize lactose (Mounier *et al.*, 2007), which may be due to the fact that they mostly grow during the second part of ripening, after lactose exhaustion. In cheeses manufactured with thermophilic lactic acid bacteria, such as Reblochon, galactose is frequently present at the beginning of ripening, and is then catabolized by *G. candidum* and *D. hansenii*.

Lactate is another important energy substrate for yeasts and molds present at the surface of cheeses, and also for most aerobic ripening bacteria (Mounier *et al.*, 2007). Catabolism of lactate involves the activity of lactate transporters and lactate dehydrogenases, which results in the production of pyruvate that is later catabolized through the TCA cycle. The genomes of the strains *S. equorum* Mu2, *B. aurantiacum* ATCC 9174, *A. arilaitensis* Re117, *C. casei* UCMA 3821 and *C. variabile* DSM 44702 originating from cheese encode lactate transporters and lactate dehydrogenases. Metatranscriptome analyses of Camembert-type cheeses revealed an early expression of fungal genes involved in the catabolism of lactate (Lessard *et al.*, 2014).

Many bacteria and fungi that live on the cheese surface are proteolytic, which results in the production of amino acids from caseins. Amino acids are frequently used as an energy substrate after lactate exhaustion (Mounier *et al.*, 2007). There is a wide diversity in proteolytic and peptidolytic activity between species and strains of the same species. However, it is generally recognized that *Y. lipolytica* and *K. marxianus* are more proteolytic than *D. hansenii*. Production of extracellular proteases has been shown in typical cheese surface bacteria such as *B. linens*, *M. gubbeenense* and *A. nicotianae* (Ghosh, Bockelmann and Heller 2009). Glutamate is among the most abundant amino acids in caseins and is frequently the most abundant free amino acid in cheeses (Rosenberg and Altemueller 2001). It is mainly catabolized by cheese surface microorganisms through glutamate dehydrogenase, yielding NADH,

ammonia and alpha-ketoglutarate, which can enter into the TCA cycle. Alpha-ketoglutarate is also a substrate for transamination reactions and favors the catabolism of other amino acids. A bifunctional proline, dehydrogenase/pyrroline-5-carboxylate dehydrogenase [PutA], which catalyzes the oxidation of proline to glutamate using a membrane-bound quinone and NAD as an electron acceptor, is present in the genome of the strains *A. arilaitensis* Re117 and *C. variabile* DSM 44702 originating in cheeses (Monnet *et al.*, 2010; Schröder *et al.*, 2011).

Cow's milk contains an average of 35 g l^{-1} of lipids, mainly triglycerides. Lipolysis results in the production of free fatty acids that constitute energy substrates for many cheese microorganisms. High levels of lipolysis are observed in mold-ripened cheeses. This is due to the high lipolytic activity of *Penicillium* strains. *Yarrowia lipolytica* is considered to be the most lipolytic cheese yeast (Lanciotti *et al.*, 2005). In some cheese varieties, intense lipolysis is the result of the activity of *G. candidum* strains (Boutrou and Gueguen 2005). Staphylococci have higher lipolytic activity than other surface bacteria (Curtin, Gobbetti and McSweeney 2002). The genome of the cheese bacterium *A. arilaitensis* Re117 encodes a secretory triacylglycerol lipase and has several acyl-CoA dehydrogenases and fatty acid-CoA ligases with no counterpart in environmental *Arthrobacter* strains, which may be the result of the adaptation of this strain to the fatty acids present in cheeses (Monnet *et al.*, 2010). Interestingly, in *C. variabile* DSM 44702, there is also a wide diversity of fatty acid-CoA ligases, which would enable the strain to utilize a broader range of fatty acid substrates in its natural cheese environment (Schröder *et al.*, 2011). Free fatty acids are known to have inhibitory effects on a wide range of microorganisms (Altieri, Cardillo and Sinigaglia 2005), but their impact on the growth of microorganisms on the cheese surface has not yet been evaluated.

Apart from interactions linked to the supply of energy sources, it is likely that many other nutritional interactions occur between cheese surface microorganisms. However, except for evidence of the stimulation of smear bacteria by vitamins (pantothenic acid, niacin and riboflavin) produced by yeasts (Purko, Nelson and Wood 1951), there is a lack of knowledge concerning this topic.

Bacteriocins and phages

Some cheese surface bacteria produce bacteriocins such as linocin M18, linecin A, linenscin OC2 and micrococcin P1 (Kato *et al.*, 1991; Valdés-Stauber and Scherer 1994; Maisnier-Patin and Richard 1995; Carnio *et al.*, 2000). Bacteriocin production by these bacteria has mainly been investigated in order to select strains able to prevent the growth of *Listeria*. However, bacteriocins may inhibit other microbial groups found on the cheese surface and, therefore, confer a selective advantage to producers.

In contrast to lactic acid bacteria, very little is known about the importance of phages on cheese surface bacteria. To our knowledge, no cases of growth inhibition during cheese manufacturing have been reported in the literature. The *C. variabile* DSM 44702 genome contains a putative prophage that comprises 60 genes (Schröder *et al.*, 2011). In the *C. casei* UCMA 3821 genome, 85 genes have been annotated as phage proteins and four CRISPR (clustered regularly interspaced short palindromic repeats) loci were identified (Monnet *et al.*, 2012). CRISPR loci provide acquired resistance to bacteriophages (Sorek, Kunin and Hugenholtz 2008). This indicates that phages may exert an influence on the development of cheese surface bacteria during ripening.

CONCLUSION

During the last 10 years, the characterization of the microbial composition of these communities has been facilitated by the development of culture-independent methods based on molecular fingerprints or on high-throughput sequencing of amplicons. In addition, the sequencing of the genome of typical species found on the cheese surface and comparative genomic analyses revealed signatures of 'domestication' and other interesting features related to their ability to grow and to survive in their habitat. Such investigations should expand in the future due to the rapid growth of the number of sequenced microbial genomes. However, these studies have to be complemented by *in situ* analyses in cheeses, especially by gene expression profiling. Considerable progress has been made for the quantification of mRNA targets in cheeses by reverse transcription real-time PCR. Metatranscriptomic analyses will provide a more global picture of the functioning of cheese surface communities. These analyses are beginning to be applied to cheeses or model cheeses, but some limitations still have to be overcome, such as the elimination of ribosomal RNA and the need for a higher throughput for taking the majority of the population into account. In addition, it is important to more effectively link physiological traits of microorganisms to their genes within their natural environment. The massive sequencing of transposon libraries offers very interesting perspectives for investigating cheese surface microorganisms. This technique involves the inoculation of pools of mutants in a representative medium, followed by the monitoring of the frequency of mutations by massive sequencing (van Opijnen and Camilli 2013), making it possible to evaluate the impact of each gene on strain fitness. This will require the development of efficient mutagenesis systems that are currently lacking for the typical cheese surface microorganisms.

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