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1 **Growth and adaptation of microorganisms on the cheese surface**

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15
16 **Running title:** Cheese surface microorganisms

17 **Keywords:** cheese rind, smear-ripened cheese, ripening, *Brevibacterium*, *Arthrobacter*,
18 *Geotrichum candidum*, *Debaryomyces hansenii*

19
20
21 **Abstract**

22
23 Microbial communities living on cheese surfaces are composed of various bacteria, yeasts and
24 molds that interact together, thus generating the typical sensory properties of a cheese.
25 Physiological and genomic investigations have revealed important functions involved in the
26 ability of microorganisms to establish themselves at the cheese surface. These functions
27 include the ability to use the cheese's main energy sources, to acquire iron, to tolerate low pH
28 at the beginning of ripening, and to adapt to high salt concentrations and moisture levels.
29 Horizontal gene transfer events involved in the adaptation to the cheese habitat have been
30 described, both for bacteria and fungi. In the future, *in situ* microbial gene expression
31 profiling and identification of genes that contribute to strain fitness by massive sequencing of
32 transposon libraries will help us to better understand how cheese surface communities
33 function.

35 **Introduction**

36

37 Surface-ripened cheeses are characterized by a complex surface microflora composed of
38 various types of bacteria, yeasts and molds. Until ten years ago, very few investigation
39 strategies were available for studying typical microorganisms that live on cheese surfaces.
40 Most studies concerned the monitoring of the growth of selected species in model cheeses and
41 the assay of enzymatic activities or substrates and metabolic products. However, the rapid
42 development of microbial genome sequencing has offered new investigation opportunities.
43 Comparative genomic analyses help to identify genetic determinants specific to the cheese
44 habitat and to understand the emergence of species adapted to the cheese surface. Cheese-
45 originating strains whose genome sequences are available or for which there is a genome
46 sequencing project are listed in Table 1. Functional metagenomics provides avenues for a
47 deeper understanding of microbial communities living on cheese surfaces (Wolfe *et al.*,
48 2014). In addition, over the last years, considerable progress has been achieved for the *in situ*
49 quantification of mRNA transcripts by reverse transcription-quantitative PCR (Duquenne *et*
50 *al.*, 2010, Falentin *et al.*, 2012, Monnet *et al.*, 2013, Desfossés-Foucault *et al.*, 2014) and even
51 by high-throughput RNA sequencing (Lessard *et al.*, 2014).

52 The manufacture of fresh cheese curd generally takes from about five to 24 hours. It
53 involves the acidification of milk by lactic acid bacteria and the action of rennet, resulting in
54 milk coagulation. The gel is then cut and the drained curds are transferred to moulds. Cheeses
55 are then salted and transferred to ripening rooms. The ripening time for surface-ripened
56 cheeses is typically 2-4 weeks. During that time, there is an intense growth and activity of
57 aerobic microorganisms at the surface of the cheeses. Growth of the cheese surface
58 microorganisms corresponds to a colonization of a nutrient-rich environment, followed by a
59 stationary growth phase and sometimes by a decline in population. The ability of
60 microorganisms to establish themselves on the surface of cheeses depends on several factors.
61 One of them is the ability to use cheese as an efficient growth medium. The composition and
62 structure of this medium change throughout ripening. In addition, the microorganisms have to
63 adapt themselves to the presence of other microorganisms with which they may have positive
64 or negative interactions. As an example, a scheme has been proposed for the dynamics of the
65 principal Livarot yeasts (Larpin *et al.*, 2006): *Kluyveromyces lactis* grows at the very
66 beginning of ripening and contributes to lactose uptake. It is then inhibited by salting;
67 *Geotrichum candidum* and *Debaryomyces hansenii* contribute to deacidification of the curd
68 by preferentially consuming lactate and amino acids, favoring the growth of aerobic acido-

69 sensitive ripening bacteria; *Yarrowia lipolytica*, a strongly lipolytic species, grows primarily
70 during the latter half of ripening and limits the mycelium development of *G. candidum*.
71 Abiotic conditions such as temperature and relative humidity also influence the growth of
72 microorganisms at the surface of the cheese. The ability to survive in the cheese
73 manufacturing environment is another important feature since it favors the subsequent
74 recontamination of the cheese.

75

76 **Evolutionary processes in the cheese habitat**

77

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

89 In bacteria, horizontal gene transfers may occur via three main mechanisms:
90 transformation, transduction and conjugation. Such transfers may confer a selective advantage
91 to the recipient cell, as observed for *A. arilaitensis* Re117, whose genome contains a gene
92 cluster involved in the catabolism of D-galactonate that probably originated from a
93 *Pseudomonas* strain. The importance of horizontal transfers in the eukaryotic kingdom is
94 thought by many to be anecdotal. However, the recent sequencing of the genome of a
95 *Penicillium roqueforti* and a *Penicillium camemberti* strain showed the presence of a large
96 region (~500 kb) known as *Wallaby*, which resulted from horizontal transfers (Cheeseman *et*
97 *al.*, 2014). *Wallaby* was found almost exclusively in species from the food environment. It has
98 been independently acquired in some *Penicillium* species, and the perfect identity of the
99 sequences implies recent transfer events. Such transfers may be facilitated by the ability of
100 fungi such as *Penicillium* to form somatic fusions between mycelia. The function of *Wallaby*
101 has not yet been well established, but the presence of genes predicted to be involved in the
102 regulation of conidiation or in antimicrobial activities suggests a functional advantage

103 associated with competition with other cheese microorganisms. Horizontal gene transfer
104 events also occur in yeasts such as *Y. lipolytica*, *D. hansenii* and *K. lactis* (Dujon *et al.*, 2004),
105 but this has not yet been investigated for cheese isolates.

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

113 The rate at which microorganisms evolve to adapt to the cheese habitat is not known.
114 However, the propagation of a *Lactococcus lactis* strain isolated from fermented plant
115 material for only 1000 generations in milk resulted in several genetic modifications that
116 consisted of point mutations and gene deletions (Bachmann *et al.*, 2012). There is evidence
117 that herders in Europe were producing cheese between 6,800 and 7,400 years ago (Salque *et*
118 *al.*, 2013) and it may thus be assumed that some cheese microorganisms result from extensive
119 genetic adaptations that could even result in new species. Even if there is evidence of the
120 evolution of cheese-specific strains, other habitats such as skin or soil also lead to selective
121 pressure similar to that found on the cheese surface (e.g., osmotic stress, desiccation, iron-
122 restriction), and some important properties of cheese strains may thus have arisen from
123 evolutionary selection on the skin and in the soil (Monnet *et al.*, in press). Ropars *et al.* (2012)
124 distinguished two types of cheese molds. The first corresponds to ubiquitous fungi such as
125 *P. roqueforti*, *Scopulariopsis candida* and *Scopulariopsis fusca*. *Penicillium roqueforti* has
126 been frequently isolated from silage, from food products and from forest soil or wood.
127 *Scopulariopsis candida* and *S. fusca* have diverse origins, from soil as well as plant and
128 human pathogens. These molds seem to have only recently adapted to cheese. The second
129 type corresponds to species such as *P. camemberti*, *Fusarium domesticum*, *Scopulariopsis*
130 *flava* and *Sporendonema casei*, which are only found in cheese and are well adapted to this
131 habitat. *Penicillium camemberti* is related to *P. cavernicola*, which can be isolated from the
132 walls of natural caves and is also able to develop on fat-rich products. Cheeses were originally
133 made in natural caves and cellars and it is conceivable that the capacity to live in fat-rich
134 products has been conserved from a common ancestor. Adaptation of *F. domesticum* to the
135 cheese surface may be related to the ability of a common ancestor of *Fusarium dimerium* to
136 establish itself in biofilms.

137

138 **Factors and functions involved in the growth of microorganisms on the cheese surface**

139

140 pH, temperature and moisture

141 Several factors important for the growth of cheese surface microorganisms are listed in
142 Table 2. At the beginning of ripening, the pH value is typically around 5. On the surface, this
143 low pH favors the growth of aerobic yeasts and molds that are more tolerant to acidity than
144 bacteria. For example, growth of *Penicillium camemberti* can take place in the pH range of
145 3.5-6.5 (Abbas & Dobson, 2011). The ability of yeasts to tolerate acid pH values seems to be
146 related to the activity of plasma membrane ATPase that regulates intracellular pH by
147 exporting protons (Praphailong & Fleet, 1997). Presumably, yeasts that tolerate low pH values
148 have a more efficient or stable plasma membrane ATPase system. Cheese yeasts and molds
149 contribute to the increase in the pH of the cheese surface by transforming lactate to CO₂ and
150 also by producing ammonia from amino acids. Coagulase-negative staphylococci such as
151 *Staphylococcus equorum*, *Staphylococcus sciuri* and *Staphylococcus xylosus* can grow at pH
152 5.5 and even below (Bockelmann *et al.*, 1997), which may explain their ability to grow early
153 during ripening. Other surface bacteria such as *Arthrobacter* sp., *Brevibacterium* sp. and
154 *Corynebacterium* sp. are more acido-sensitive and begin to grow when the pH is around 5.5 to
155 6.0. At the end of ripening, the pH at the cheese surface can reach a value higher than 7.5. It
156 can be considered that the general trend towards yeast domination during the early stages of
157 ripening in smear-ripened cheeses (i.e., when the pH is low), followed by bacterial
158 domination at the end of ripening, is due to the fact that the yeasts are more adapted to acidic
159 pH than the bacteria.

160 Cheese ripening typically occurs at a temperature of 10-15°C. Microorganisms that
161 grow under these conditions include mesophiles that have a large tolerance of temperature
162 range, psychrophiles and psychrotrophes. Optimal growth temperature for yeasts is between
163 20 and 30°C, but their ability to grow and have proteolytic and lipolytic activities at low
164 temperatures favors their colonization of the cheese surface, as observed for *Candida* spp. and
165 *Y. lipolytica* (Corbo *et al.*, 2001). The growth of *D. hansenii* is greater at 20°C than at 10 or
166 15°C, but the effect of NaCl is less pronounced at low temperatures (Masoud & Jakobsen,
167 2005). For some cheese varieties, the temperature is set to ~20°C at the beginning of ripening
168 to promote the growth and deacidifying activity of the yeasts.

169 The relative humidity of the ripening room also has an influence on the growth of
170 microorganisms at the cheese surface. For example, *P. camemberti* grows less well at a

171 relative humidity greater than 95% (Leclercq-Perlat *et al.*, 2013), and in smear-ripened
172 cheeses, high humidity in ripening rooms, combined with repeated brushing of the cheeses
173 with salt water, prevents the growth of molds. Correlations have been found between cheese
174 rind moisture and community composition of 137 different rinds (Wolfe *et al.*, 2014). In this
175 study, the fungus *Galactomyces* and four genera of *Proteobacteria* (*Psychrobacter*, *Vibrio*,
176 *Pseudomonas* and *Pseudoalteromonas*) were positively correlated with rind moisture,
177 whereas *Scopulariopsis*, *Aspergillus*, *Actinobacteria* and *Staphylococcus* were negatively
178 associated with moisture. The impact of moisture on the growth of cheese rind genera was
179 also observed in reconstituted *in vitro* microbial communities, where dry environments were
180 enriched with *Debaryomyces*, *Staphylococcus* and *Penicillium*.

181

182 Salt tolerance

183 Cheeses are salted by applying salt to their surface or by submerging them in a saturated salt
184 brine. *Debaryomyces hansenii* strains are able to grow at 16% NaCl (Masoud & Jakobsen,
185 2005). This species is frequently isolated in salty environments such as seawater, brines and
186 salted food products, and is one of the most salt-tolerant yeast species. It has mechanisms for
187 extrusion of sodium ions, accumulates glycerol as a compatible solute when exposed to high
188 NaCl concentrations (Prista *et al.*, 2005), and has a high intrinsic resistance to the toxic effects
189 of sodium and potassium ions. *Geotrichum candidum* is considered to be less tolerant to salt
190 than *D. hansenii* (Boutrou & Gueguen, 2005). In mould-ripened cheeses, if the salt content is
191 too low, *G. candidum* can outcompete *Penicillium camemberti* and form a surface defect
192 known as "toad skin". On the other hand, when the salt concentration is too high, the growth
193 of *G. candidum* will be suppressed and excessive growth of *P. camemberti* may then lead to
194 excessive proteolysis and bitterness defects (Spinnler & Gripon, 2004).

195 *Brevibacterium linens* and *Corynebacterium* spp. are stimulated by 4% NaCl, are able
196 to grow in the presence of 12% NaCl, and some strains even tolerate 16% NaCl (Masoud &
197 Jakobsen, 2005). The *Brevibacterium*, *Corynebacterium* and *Arthrobacter* strains originating
198 from cheese whose genomes have been sequenced are well equipped with genes that offer
199 protection from high salt concentrations. One protection mechanism is the accumulation of
200 osmoprotectants such as ectoine, proline and glycine betaine in the cytoplasm. Fifteen genes
201 involved in the transport of glycine betaine and related osmolytes were identified in *A.*
202 *arilaitensis* Re117, whereas a lower number - from six to nine genes - are present in
203 *Arthrobacter* strains from soil, which are less tolerant to high salt concentrations (Monnet *et*
204 *al.*, 2010). One particularity of the *Brevibacterium aurantiacum* ATCC 9174 genome is the

205 large number of osmoprotectant transporters. Indeed, nine different betaine/carnitine/choline
206 family transporters have been identified in this strain, whereas the mean number in other
207 *Actinomycetales* is 1.5 (analysis performed with the Integrated Microbial Database,
208 <http://img.jgi.doe.gov/>). The *Corynebacterium variabile* DSM 44702 genome contains the
209 *ectP* gene encoding an ectoine transporter, *proP*, which encodes an osmoregulated proline
210 transporter, and six genes encoding proteins of the betaine/carnitine/choline transporter family
211 (Schröder *et al.*, 2011). High salt concentrations on the surface of smear-ripened cheeses may
212 also explain the presence of bacteria usually found in marine environments, e.g.,
213 *Halomonas* sp., *Marinilactibacillus psychrotolerans*, *Pseudoalteromonas* sp. and *Vibrio* sp.
214 (Feurer *et al.*, 2004, Ishikawa *et al.*, 2007, Roth *et al.*, 2011, Wolfe *et al.*, 2014).

215

216 Iron acquisition

217 Cheese is a highly iron-restricted habitat because milk is poor in iron (0.2-0.4 mg/l) and
218 contains lactoferrin, a protein that has an antibacterial effect due to its ability to chelate iron.
219 In model cheeses involving a lactic acid bacterium (*L. lactis*), a yeast (*D. hansenii*) and
220 different ripening bacteria (*Arthobacter*, *Corynebacterium* or *Brevibacterium*), the addition of
221 iron or siderophore enhanced the growth of ripening bacteria (Monnet *et al.*, 2012). The
222 genomes of the four bacteria originating in cheese, *C. variabile* DSM 44702,
223 *Corynebacterium casei* UCMA 3821, *A. arilaitensis* Re117 and *B. aurantiacum* ATCC 9174,
224 are well equipped with genes involved in iron acquisition. In *A. arilaitensis* Re117, two
225 siderophore biosynthesis gene clusters have been identified, one of which has no counterpart
226 in the *Arthrobacter* strains from soil (Monnet *et al.*, 2010). In *C. variabile* DSM 44702, genes
227 involved in the production of a catechol siderophore are present, some of which are located on
228 a genomic island (Schröder *et al.*, 2011). In the taxonomic sub-line in which *C. variabile* is
229 located (cluster 3), similar siderophore biosynthesis genes are only present in the genome of
230 the pathogen *Corynebacterium jeikeium*. Examination of the draft genome sequence of
231 *B. aurantiacum* ATCC 9174 also revealed the presence of a gene cluster involved in the
232 synthesis of a catechol-type siderophore (locus BlinB01002486 to BlinB01002493). Three of
233 these genes (BlinB01002490 to BlinB01002492) result from a horizontal gene transfer since
234 the closest orthologs are found in Gram-negative species. Furthermore, most of the
235 *Brevibacterium* strains of dairy origin investigated by Noordman *et al.* (Noordman *et al.*,
236 2006) were able to produce siderophores. In *Staphylococcus aureus*, the *sbnABCDEFGHI*
237 operon encodes proteins involved in the biosynthesis of the siderophore staphylobactin, which
238 enhances virulence. This operon is also present in the genome of *S. equorum* Mu2, where it

239 probably contributes to the supply of iron during growth at the cheese surface (Irlinger *et al.*,
240 2012). The number of ABC-type iron-siderophore transport components is higher in
241 *Actinomycetales* from cheeses (from 20 to 56 genes) than the mean number (13 genes) found
242 in *Actinomycetales* from other environments (Monnet *et al.*, 2012). In addition, cheese
243 *Actinomycetales* have fewer proteins with iron-sulfur cluster domains, and it has been
244 hypothesized that these strains decreased their need for iron by eliminating proteins that
245 require iron.

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

253

254 Energy substrates

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

263 In general, only limited amounts of lactose are available at the beginning of ripening.
264 It is catabolized by yeasts such as *K. lactis* and *Kluyveromyces marxianus*, which have a
265 fermentative lactose catabolism, by *D. hansenii* that performs a complete oxidation to CO₂,
266 but not by *G. candidum* (Boutrou & Gueguen, 2005). In Camembert-type cheeses, it was
267 suggested that lactose is a major energy source for the mycelial growth of *P. camemberti* at
268 the beginning of ripening. Its depletion might provoke stress, resulting in slower growth and
269 spore production (Leclercq-Perlat *et al.*, 2013). Most aerobic ripening bacteria do not
270 catabolize lactose (Mounier *et al.*, 2007), which may be due to the fact that they mostly grow
271 during the second part of ripening, after lactose exhaustion. In cheeses manufactured with

272 thermophilic lactic acid bacteria, such as *Reblochon*, galactose is frequently present at the
273 beginning of ripening, and is then catabolized by *G. candidum* and *D. hansenii*.

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

283 Many bacteria and fungi that live on the cheese surface are proteolytic, which results
284 in the production of amino acids from caseins. Amino acids are frequently used as an energy
285 substrate after lactate exhaustion (Mounier *et al.*, 2007). There is a wide diversity in
286 proteolytic and peptidolytic activity between species and strains of the same species.
287 However, it is generally recognized that *Y. lipolytica* and *K. marxianus* are more proteolytic
288 than *D. hansenii*. Production of extracellular proteases has been shown in typical cheese
289 surface bacteria such as *B. linens*, *M. gubbeenense* and *Arthrobacter nicotianae* (Ghosh *et al.*,
290 2009). Glutamate is among the most abundant amino acids in caseins and is frequently the
291 most abundant free amino acid in cheeses (Rosenberg & Altemueller, 2001). It is mainly
292 catabolized by cheese surface microorganisms through glutamate dehydrogenase, yielding
293 NADH, ammonia and alpha-ketoglutarate, which can enter into the TCA cycle. Alpha-
294 ketoglutarate is also a substrate for transamination reactions and favors the catabolism of
295 other amino acids. A bifunctional proline, dehydrogenase/pyrroline-5-carboxylate
296 dehydrogenase [PutA], which catalyzes the oxidation of proline to glutamate using a
297 membrane-bound quinone and NAD as an electron acceptor, is present in the genome of the
298 strains *A. arilaitensis* Re117 and *C. variable* DSM 44702 originating in cheeses (Monnet *et*
299 *al.*, 2010, Schröder *et al.*, 2011).

300 Cow's milk contains an average of 35 g/l of lipids, mainly triglycerides. Lipolysis
301 results in the production of free fatty acids that constitute energy substrates for many cheese
302 microorganisms. High levels of lipolysis are observed in mold-ripened cheeses. This is due to
303 the high lipolytic activity of *Penicillium* strains. *Yarrowia lipolytica* is considered to be the
304 most lipolytic cheese yeast (Lanciotti *et al.*, 2005). In some cheese varieties, intense lipolysis
305 is the result of the activity of *G. candidum* strains (Boutrou & Gueguen, 2005). Staphylococci

306 have higher lipolytic activity than other surface bacteria (Curtin *et al.*, 2002). The genome of
307 the cheese bacterium *A. arilaitensis* Re117 encodes a secretory triacylglycerol lipase and has
308 several acyl-CoA dehydrogenases and fatty acid-CoA ligases with no counterpart in
309 environmental *Arthrobacter* strains, which may be the result of the adaptation of this strain to
310 the fatty acids present in cheeses (Monnet *et al.*, 2010). Interestingly, in *C. variabile* DSM
311 44702, there is also a wide diversity of fatty acid-CoA ligases, which would enable the strain
312 to utilize a broader range of fatty acid substrates in its natural cheese environment (Schröder
313 *et al.*, 2011). Free fatty acids are known to have inhibitory effects on a wide range of
314 microorganisms (Altieri *et al.*, 2005), but their impact on the growth of microorganisms on
315 the cheese surface has not yet been evaluated.

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

321

322 Bacteriocins and phages

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

329 In contrast to lactic acid bacteria, very little is known about the importance of phages
330 on cheese surface bacteria. To our knowledge, no cases of growth inhibition during cheese
331 manufacturing have been reported in the literature. The *C. variabile* DSM 44702 genome
332 contains a putative prophage that comprises 60 genes (Schröder *et al.*, 2011). In the *C. casei*
333 UCMA 3821 genome, 85 genes have been annotated as phage proteins and four CRISPR
334 (clustered regularly interspaced short palindromic repeats) loci were identified (Monnet *et al.*,
335 2012). CRISPR loci provide acquired resistance to bacteriophages (Sorek *et al.*, 2008). This
336 indicates that phages may exert an influence on the development of cheese surface bacteria
337 during ripening.

338

339 **Conclusion**

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During the last ten 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 & 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.

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

Strain	NCBI Bioproject ID.	Genome status	Source
FUNGI			
Ascomycota			
<i>Penicillium camemberti</i> FM013	PRJEB4962	In progress	Cheese
<i>Penicillium roqueforti</i> FM164	PRJNA239656	Permanent draft	Cheese
<i>Geotrichum candidum</i> CILB 918	PRJEB5752	In progress	Milk from Pont l'Evêque cheese
BACTERIA			
Actinobacteria			
<i>Agrococcus casei</i> LMG_22410	PRJEB311	Permanent draft	Soft smear-ripened cheese
<i>Arthrobacter arilaitensis</i> Re117	PRJNA53509	Complete	Smeard slightly pressed cheese (Reblochon)
<i>Arthrobacter arilaitensis</i> 3M03	PRJEB261	Permanent draft	Soft smear-ripened cheese
<i>Arthrobacter arilaitensis</i> GMPA29	PRJEB354	Permanent draft	Soft smear-ripened cheese
<i>Arthrobacter bergerei</i> Ca106	PRJEB277	Permanent draft	Mould-ripened soft cheese
<i>Brachybacterium alimentarium</i> CNRZ925	PRJEB293	Permanent draft	Cooked hard cheese
<i>Brevibacterium antiquum</i> CNRZ918	PRJEB292	Permanent draft	Hard cheese
<i>Brevibacterium aurantiacum</i> ATCC 9174†	PRJNA54109	Permanent draft	Smear-ripened cheese (Romadur)
<i>Brevibacterium casei</i> CIP 102111	PRJEB281	Permanent draft	Cheddar Cheese
<i>Brevibacterium linens</i> ATCC 9172	PRJEB273	Permanent draft	Cheese (Harzerkase)
<i>Corynebacterium ammoniagenes</i> ws_2211	PRJEB360	Permanent draft	Cheese surface
<i>Corynebacterium casei</i> LMG S-19264	PRJNA186910	Complete	Surface of a smear-ripened cheese
<i>Corynebacterium casei</i> UCMA 3821	PRJNA78139	Permanent draft	Soft smear-ripened cheese (Livarot)
<i>Corynebacterium flavescens</i> Mu128	PRJEB324	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Corynebacterium flavescens</i> OJ8	PRJNA242338	In progress	Cheese
<i>Corynebacterium variabile</i> DSM 44702	PRJNA62003	Complete	Soft smear-ripened cheese
<i>Kocuria varians</i> Mu208	PRJEB326	Permanent draft	Soft smear-ripened cheese
<i>Leucobacter komagatae</i> 1L36	PRJEB246	Permanent draft	Soft smear-ripened cheese
<i>Leucobacter</i> sp. ER15_166_BHI15	PRJEB301	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Luteococcus japonicus</i> LSP_Lj1	PRJEB313	Permanent draft	Cheese
<i>Microbacterium foliorum</i> C45	PRJEB276	Permanent draft	Soft smear-ripened cheese
<i>Microbacterium gubbeenense</i> DSM 15944	PRJNA185602	Permanent draft	Surface of a smear-ripened cheese
<i>Microbacterium gubbeenense</i> Mu132	PRJEB325	Permanent draft	Soft smear-ripened cheese
<i>Micrococcus luteus</i> Mu201	PRJEB237	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Propionibacterium acidipropionici</i> ATCC 4875	PRJNA158149	Permanent draft	Emmental cheese
<i>Propionibacterium freudenreichii</i> CIRM-BA1	PRJNA49535	Complete	isolated from Swiss cheese
<i>Propionibacterium freudenreichii</i> ITG P20	PRJEB4826	In progress	Cheese
<i>Propionibacterium thoenii</i> DSM 20276	PRJNA185646	Permanent draft	Emmental cheese
Bacteroidetes			
<i>Chryseobacterium bovis</i> Pi_18	PRJEB34	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Chryseobacterium ginsengisoli</i> M17	PRJEB316	Permanent draft	Uncooked semi-hard cheese with cow milk
Firmicutes			
<i>Alkalibacterium kapii</i> FAM208_38	PRJEB304	Permanent draft	Surface of pressed cheese
<i>Bacillus altitudinis</i> 263	PRJEB231	Permanent draft	Mould-ripened soft cheese
<i>Bacillus altitudinis</i> ATCC 10987	PRJNA241431	Permanent draft	Cheese
<i>Bacillus altitudinis</i> m1293 (BPS-2)	PRJNA55163	Permanent draft	Cream cheese
<i>Bavariicoccus seileri</i> DSM 19936	PRJNA188834	Permanent draft	Surface of smear-ripened cheese
<i>Bavariicoccus seileri</i> WCC_4188	PRJEB359	Permanent draft	Soft smear-ripened cheese
<i>Brevibacillus parabrevis</i> CIP 110335	PRJEB288	Permanent draft	Hard cheese
<i>Brochothrix thermosphacta</i> cH814	PRJEB279	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Carnobacterium maltaromaticum</i> 38b	PRJEB254	Permanent draft	Soft smear-ripened cheese
<i>Carnobacterium maltaromaticum</i> LMA28	PRJNA179370	Complete	Mould-ripened soft cheese (Brie)
<i>Clostridium tyrobutyricum</i> UC7086	PRJNA170963	Permanent draft	Hard cheese (Gran Padano)
<i>Enterococcus durans</i> IPLA_655	PRJNA188163	Permanent draft	Cheese
<i>Enterococcus faecium</i> CRL1879	PRJNA191091	Permanent draft	Artisanal cheese
<i>Enterococcus italicus</i> DSM 15952	PRJNA53039	Permanent draft	Cheese, Italian Toma, from bovine milk
<i>Enterococcus malodoratus</i> ATCC 43197	PRJNA191903	Permanent draft	Gouda cheese
<i>Enterococcus malodoratus</i> FAM208_55	PRJEB305	Permanent draft	Surface of pressed cheese (Gouda)
<i>Exiguobacterium acetyllicum</i> 180	PRJEB230	Permanent draft	Cheese
<i>Facklamia tabacinasalis</i> FAM208_56	PRJEB306	Permanent draft	Surface of pressed cheese
<i>Jeotgalicoccus psychrophilus</i> CRBM_D21	PRJEB298	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Leuconostoc lactis</i> 1283	PRJEB235	Permanent draft	Cheese

<i>Leuconostoc mesenteroides</i> TIFN8	PRJNA175676	Permanent draft	Natural cheese starter culture
<i>Listeria innocua</i> Clip11262	PRJNA86	Complete	Cheese, Morocco
<i>Marinilactibacillus psychrotolerans</i> 42ea	PRJEB266	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Marinilactibacillus psychrotolerans</i> FAM208_59	PRJEB307	Permanent draft	Soft smear-ripened cheese
<i>Ornithinibacillus bavariensis</i> CIP 109287	PRJEB287	Permanent draft	Cheese
<i>Paenibacillus</i> sp. 3M17	PRJEB363	Permanent draft	Soft smear-ripened cheese
<i>Staphylococcus equorum</i> Mu2	PRJEA8889	Permanent draft	French smear-ripened cheese (Munster)
<i>Staphylococcus fleuretti</i> CIP 106114	PRJEB283	Permanent draft	Cheese made with goat milk
<i>Staphylococcus lentus</i> Ca2	PRJEB278	Permanent draft	Mould-ripened soft cheese
<i>Staphylococcus vitulinus</i> Ma1	PRJEB320	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Staphylococcus warnerii</i> 1445	PRJEB241	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Vagococcus fluvialis</i> bH819	PRJEB275	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Vagococcus lutrae</i> FAM208	PRJEB303	Permanent draft	Surface of pressed cheese

Proteobacteria

<i>Acinetobacter johnsonii</i> 3M05	PRJEB262	Permanent draft	Soft smear-ripened cheese
<i>Alcaligenes faecalis</i> 2L10	PRJEB250	Permanent draft	Soft smear-ripened cheese
<i>Alcaligenes</i> sp. 2L29	PRJEB251	Permanent draft	Soft smear-ripened cheese
<i>Brevundimonas diminuta</i> 3F5N	PRJEB260	Permanent draft	soft smear-ripened cheese with raw milk
<i>Citrobacter freundii</i> Pi_15	PRJEB346	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Hafnia alvei</i> GB001	PRJEB6257	Permanent draft	Cheese
<i>Halomonas alkaliphila</i> 3A7M	PRJEB256	Permanent draft	Soft smear-ripened cheese
<i>Halomonas</i> sp. 1M45	PRJEB249	Permanent draft	Soft smear-ripened cheese
<i>Halomonas</i> sp. 3F2F	PRJEB259	Permanent draft	Soft smear-ripened cheese
<i>Halomonas venusta</i> 3D7M	PRJEB258	Permanent draft	Soft smear-ripened cheese
<i>Klebsiella oxytoca</i> Pi_20	PRJEB348	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Kluyvera intermedia</i> TL336_A	PRJEB358	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Morganella morganii</i> 3A5A	PRJEB255	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Morganella psychrotolerans</i> 925	PRJEB233	Permanent draft	Soft smear-ripened cheese
<i>Proteus hauseri</i> 1M10	PRJEB247	Permanent draft	Soft smear-ripened cheese
<i>Proteus vulgaris</i> 1M25	PRJEB248	Permanent draft	Soft smear-ripened cheese
<i>Providencia alcalifaciens</i> GM3	PRJEB309	Permanent draft	Soft smear-ripened cheese
<i>Providencia heimbachae</i> GR4	PRJEB310	Permanent draft	Soft smear-ripened cheese
<i>Providencia rettgeri</i> 947	PRJEB234	Permanent draft	Soft smear-ripened cheese
<i>Pseudomonas fragi</i> 1E26	PRJEB244	Permanent draft	Soft smear-ripened cheese
<i>Pseudomonas</i> sp. 1E44	PRJEB245	Permanent draft	Soft smear-ripened cheese
<i>Psychrobacter aquimaris</i> ER15_174_BH17	PRJEB302	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Psychrobacter celer</i> 91	PRJEB270	Permanent draft	Mould-ripened soft cheese with raw milk
<i>Psychrobacter faecalis</i> H5	PRJEB280	Permanent draft	Soft smear-ripened cheese
<i>Psychrobacter immobilis</i> PG1	PRJEB345	Permanent draft	Soft smear-ripened cheese
<i>Psychrobacter namhaensis</i> 1439	PRJEB240	Permanent draft	Mould-ripened soft cheese with raw milk
<i>Raoultella ornithinolytica</i> TL332	PRJEB357	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Raoultella planticola</i> 3M45	PRJEB264	Permanent draft	Soft smear-ripened cheese
<i>Serratia marcescens</i> 448	PRJEB267	Permanent draft	Soft smear-ripened cheese
<i>Serratia proteamaculans</i> 1C2F	PRJEB243	Permanent draft	Soft smear-ripened cheese with raw milk
<i>Serratia rubidaea</i> TA_26	PRJEB355	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Stenotrophomonas maltophilia</i> Pi1	PRJEB349	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Stenotrophomonas rhizophila</i> PCA13	PRJEB334	Permanent draft	Uncooked semi-hard cheese with cow milk
<i>Vibrio littoralis</i> B4	PRJEB274	Permanent draft	Soft smear-ripened cheese

373 * Lactic acid bacteria belonging to the genera *Lactobacillus*, *Lactococcus* and *Streptococcus*

374 were not included in this table

375 † formerly *Brevibacterium linens* BL2

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

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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

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