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Drivers that establish and assembly the lactic acid bacteria biota in cheeses

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Abstract

Background Cheeses are inherently microbiologically and biochemically dynamic. Numerous biotic and abiotic drivers govern the establishment and assembly of a core microbiota in cheese, which, for internally-ripened cheeses, having an intermediate to long period of ripening, consists of starter and non-starter lactic acid bacteria (SLAB and NSLAB). The management of this dynamic ecosystem has to consider this core as a super-organism, which results from the sums of microbial metabolisms and interactions among individual microbes. Scope and Approach This review focuses on all presumptive drivers, raw and pasteurized milk, farming system and house microbiota, and intrinsic and extrinsic factors during cheese manufacture and ripening, which influence the populations of SLAB and NSLAB. The interactions between these two microbial groups are described also. Key Findings and Conclusions Although less complex than natural environments, the cheese ecosystem shows a variable flux of its core microbiota during time and through space. Many and diverse drivers establish and assembly the lactic acid bacteria biota. If such drivers are efficient to guarantee microbial and cheese diversities, on the other hand, their control is the fundamental pre-requisite to synchronize and balance microbiological events. The methodological approaches (e.g., omics techniques and integrated system biology) have markedly improved to concretize this ambitious goal. Facing and improving the knowledge on the main drivers, the current step should focus on a unique puzzle of coexisting species/biotypes likely a super-organism, whose guide has to consider all casehardened microbial elements.

Keywords	Cheeses; drivers; core microbiota; ecosystem; lactic acid bacteria biota
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Bari, May, 5, 2018

Dear Editor,

please find included the revised manuscript “Drivers that establish and assembly the lactic acid bacteria biota in cheeses” by Gobbetti et al. that we would like to re-submit for publication on Trends in Food Science & Technology Journal.

The corresponding author is:

Prof. Marco Gobbetti, e-mail address: marco.gobbetti@unibz.it; Free University of Bolzano, Faculty of Science and Technology, Piazza Università 5, 39100, Bolzano, Italy, Tel.: +39 0471 01721.

I suggest you reviewers whit specific expertise on the subject of the manuscript. I briefly stated the appropriate expertise of each reviewers and for each I included details of two recent relevant research or review papers. Full details were also provided.

1) Professor Sylvie Lortal

French National Institute for Agricultural Research | INRA · Department of Microbiology and the Food Chain, France

email: sylvie.lortal@rennes.inra.fr

Sylvie Lortal is Research Director at INRA –National Institute for Agricultural Research, France. Her key topics and expertises were the mechanisms and issues from milk and egg transformation and the well-balanced skills and analytical devices in Biochemistry, Process and Microbiology. The personal scientific skills were Food science, Biochemistry, Molecular Biology, Microbiology, Lactic Acid Bacteria and Cheese Ecosystem.

- Boucher, C.L., Gagnaire, V., Briard-Bion, V., Jardin, J., Maillard, M.-B., Dervilly-Pinel, G., Le Bizec, B., Lortal, S., Jeanson, S., & Thierry, A. (2016). Spatial distribution of *Lactococcus lactis* colonies modulates the production of major metabolites during the ripening of a model cheese. *Applied and Environmental Microbiology*, 82(1), 202-210.
- Floury, J., El Mourdi, I., Silva, J.V., Lortal, S., Thierry, A., & Jeanson, S. (2015). Diffusion of solutes inside bacterial colonies immobilized in model cheese depends on their physicochemical properties: A time-lapse microscopy study. *Frontiers in Microbiology*, 6, 366.

2) Professor Paul McSweeney

School of Food & Nutritional Sciences, Room 234, Food Science Building, University College
Cork, Cork, Ireland

email: p.mcsweeney@ucc.ie

Paul McSweeney is Professor of Food Chemistry in the Department of Food and Nutritional Sciences, University College, Cork, Ireland (UCC). Overall theme of his research is Dairy Biochemistry with particular reference to factors affecting cheese flavour and texture, proteolysis during cheese maturation including the role of non-starter lactic acid bacteria and smear microorganisms, the ripening of hybrid and non-Cheddar varieties, the specificity of proteinases on the caseins, proteolysis and lipolysis in cheese during ripening and characterization of enzymes important to cheese ripening (proteinases, peptidases, amino acid catabolic enzymes).

- PLH McSweeney, PF Fox, PD Cotter, DW Everett – 2017. Cheese: Chemistry, Physics and Microbiology. Fourth Edition, Elsevier, Academic Press.
- Bertuzzi, A. S., Walsh, A. M., Sheehan, J. J., Cotter, P. D., Crispie, F., McSweeney, P. L. H., Kilcawley, K. N., & Rea, M. C. (2018). Omics-Based Insights into Flavor Development and Microbial Succession within Surface-Ripened Cheese. *mSystems* 3 (1), e00211-17; DOI: 10.1128/mSystems.00211-17.

3) Professor Effie Tsakalidou

Agricultural University of Athens, Department Food Science and Technology, Dairy Research
email: et@aua.gr

Effie Tsakalidou is Professor of Food Biochemistry in the Department of Food Science and Human Nutrition, Agricultural University of Athens. Her research interests lie in the field of lactic acid bacteria, with emphasis on taxonomy, metabolism, physiology, genetics, bioinformatics, antimicrobial peptides, probiotics and technological performance. She is Editor for the International Dairy Journal and member of the Editorial Board in several international scientific journals.

- Georgalaki, M., Zoumpopoulou, G., Mavrogonatou, E., (...), Papadimitriou, K., Tsakalidou, E. (2017). Evaluation of the antihypertensive angiotensin-converting enzyme inhibitory (ACE-I) activity and other probiotic properties of lactic acid bacteria isolated from traditional Greek dairy products. *International Dairy Journal*, 75,10-21.
- Papadimitriou, K., Mavrogonatou, E., Bolotin, A., Tsakalidou, E., & Renault, P. (2016). Whole-Genome Sequence of the Cheese Isolate *Streptococcus macedonicus*. *Genome Announcement*. 4(5), e01025-16.

4) Professor Eddy Smid

Department of Agrotechnology and Food Sciences, Food Microbiology Laboratory, 6700AA
WAGENINGEN

email: eddy.smid@wur.nl

Eddy Smid is associate professor at the Laboratory of Food Microbiology, Wageningen University where he is currently leading the research group Food Fermentation. He (co-)authored 112 scientific (peer reviewed) papers and 8 patent applications in the fields of microbial physiology, molecular biology, plant pathology, food microbiology, biotechnology, metabolic engineering, food fermentation and metabolic modeling.

- Van Mastrigt, O., Abee, T., Lillevang, S.K., & Smid, E.J. (2018). Quantitative physiology and aroma formation of a dairy *Lactococcus lactis* at near-zero growth rates. *Food Microbiology*, 73, 216-226.
- Spus, M., Liu, H., Wels, M., Abee, T., & Smid, E.J. (2017). Isolation and characterization of *Lactobacillus helveticus* DSM 20075 variants with improved autolytic capacity. *International Journal of Food Microbiology*, 241, 173-180.

Thank you very much for the re-consideration of this manuscript.

Best Regards

Marco Gobetti

Bari, May, 5, 2018

Dear Editor and reviewers,

please find included the revised manuscript “Drivers that establish and assembly the lactic acid bacteria biota in cheeses” by Gobbetti et al. that we would like to re-submit for publication on Trends in Food Science & Technology Journal.

Comments from the editors and reviewers: “Your manuscript has been checked with Ithenticate anticopy software revealing that 31% of the text is copied from other sources. Authors re-write the text to reduce such high % of copied text and re-submit.”

Response: The manuscript has been re-writed and checked with an anticopy programs and the % of text from other sources has been reduced.

Thank you very much for the re-consideration of this manuscript.

Best Regards

Marco Gobbetti

Highlights

- Cheeses are microbiologically and biochemically dynamic
- Biotic and abiotic drivers govern the establishment/assembly of a core microbiota
- The core microbiota consists of starter and non-starter lactic acid bacteria
- The sums of microbial metabolisms and interactions result in a super-organism to be driven
- All presumptive drivers that influence the core microbiota are described

Background

Cheeses are inherently microbiologically and biochemically dynamic. Numerous biotic and abiotic drivers govern the establishment and assembly of a core microbiota in cheese, which, for internally-ripened cheeses, having an intermediate to long period of ripening, consists of starter and non-starter lactic acid bacteria (SLAB and NSLAB). The management of this dynamic ecosystem has to consider this core as a super-organism, which results from the sums of microbial metabolisms and interactions among individual microbes.

Scope and Approach

This review focuses on all presumptive drivers, raw and pasteurized milk, farming system and house microbiota, and intrinsic and extrinsic factors during cheese manufacture and ripening, which influence the populations of SLAB and NSLAB. The interactions between these two microbial groups are described also.

Key Findings and Conclusions

Although less complex than natural environments, the cheese ecosystem shows a variable flux of its core microbiota during time and through space. Many and diverse drivers establish and assemble the lactic acid bacteria biota. If such drivers are efficient to guarantee microbial and cheese diversities, on the other hand, their control is the fundamental pre-requisite to synchronize and balance microbiological events. The methodological approaches (e.g., omics techniques and integrated system biology) have markedly improved to concretize this ambitious goal. Facing and improving the knowledge on the main drivers, the current step should focus on a unique puzzle of coexisting species/biotypes likely a super-organism, whose guide has to consider all case-hardened microbial elements.

1 **Drivers that establish and assembly the lactic acid bacteria biota in cheeses**

2

3 Marco Gobbetti^a, Raffaella Di Cagno^a, Maria Calasso^b, Erasmo Neviani^c, Patrick F. Fox^d, Maria
4 De Angelis^b

5

6 ^aFaculty of Science and Technology, Free University of Bolzano, Bolzano, Italy

7 ^bDepartment of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy

8 ^cFood and Drug Department, University of Parma, Parma, Italy

9 ^dDepartment of Food and Nutritional Sciences, University College, Western Road, Cork, Ireland

10

11

12 *Corresponding author. E-mail address: marco.gobbetti@unibz.it (Marco Gobbetti)

13 Full postal address: Faculty of Science and Technology, Free University of Bolzano, Piazza

14 Università 5, 39100, Bolzano, Italy

16 STRUCTURED ABSTRACT

17 *Background*

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36 improving the knowledge on the main drivers, the current step should focus on a unique puzzle
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38 casehardened microbial elements.

39 **Keywords**

40 Cheeses; drivers; core microbiota; ecosystem; lactic acid bacteria biota

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45 **1. Introduction**

46 Contrarily to most dairy products, which show biological, biochemical and physical-
47 chemical stability, cheeses are inherently unstable because of their microbiological and
48 biochemical dynamicity. Manufacture and ripening rely on numerous and subsequent
49 microbiological interventions, which, when balanced, allow to the production of cheeses with
50 pleasant sensory properties but when uncontrolled, give rise to off-flavors and -odors. Although
51 ingredients are simple (milk, rennet and salt) for most varieties, cheese microbiology is crucial in
52 tackling complex, dynamic and diverse ecosystems, with repercussions on the overall cheese
53 quality and authenticity. The diversity of coexisting species/biotypes in most food ecosystems
54 has been a major puzzle in recent decades.

55 Cheese microbes are deliberately added (starters) or they enter milk as contaminants.
56 Therefore, starters, including primary (natural or commercial), secondary or adjunct lactic acid
57 bacteria (SLAB) and the milk autochthonous microbiota (mainly consisting of non-starter lactic
58 acid bacteria, NSLAB) are the main players (ripening agents) in internally-ripened cheeses
59 having an intermediate to long period of ripening, which mainly explain the diversity of cheeses.
60 The literature of recent years has been considered comprehensive reviews (see, Montel et al.,
61 2014; Blaya, Barzideh, & LaPointe, 2018; McAuliffe, 2018; Gatti, Bottari, Lazzi, Neviani, &
62 Mucchetti, 2014; Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015), which covered
63 specific aspects of SLAB and NSLAB with the main aim of establishing their role in cheese
64 manufacture and overall quality. Unavoidably, microbiology is linked to technology and, in
65 particular, to all the interventions (e.g., creaming, pasteurization, cooking, brining) that affect its
66 biodiversity. A coherent and novel view dealing with the management of the lactic acid bacteria
67 biota in cheeses during manufacture and ripening must consider not individual players and their

68 cheese-related performance but the overall community (core). According to this concept, the core
69 microbiota in cheese is like a super-organism, consisting of the sums of microbial metabolisms
70 and interactions among individual microbes. This metabolic network must be understood,
71 assembled and driven in such a complex ecosystem. As well, knowledge on cell regulation and
72 metabolic dependencies has to improve.

73 Recently, data that has accumulated in the literature justifies this review, which aims at
74 describing the drivers that establish and assemble the lactic acid bacteria biota in cheeses. It is
75 necessary to elucidate the determinants of species/biotypes diversity in microbial ecosystems to
76 depict the dynamic scenario of SLAB and NSLAB populations. This review refers to bacterial
77 internally-ripened cheeses, giving insights on how drivers establish, assemble and control the
78 overall microbial community from milking to cheese manufacture and ripening.

79 **2. The methodology approach**

80 The methodology approach mainly concerns the choice of technique for bacterial
81 enumeration, identification (what is there?) and metabolic characterization (what is doing what?),
82 the model system to reproduce cheese ecosystems and, not last, the protocol to assess the
83 bacterial spatial distribution (what is distributed where?) and interaction into the cheese (what
84 interacts with?) (Fig. 1).

85 The techniques for characterizing the dynamics and assembly of the cheese microbiota have
86 used culture-dependent and -independent approaches; the latter have evolved rapidly regarding
87 the capacity to describe phyla, genera and species. Nowadays, molecular approaches based on
88 DNA or RNA combined with high-throughput sequencing (HTS) technologies supply
89 unprecedented opportunities to profile dominant and sub-dominant cheese microbiota (Parente et
90 al., 2016). Usually, the identification of bacterial populations in the microbiota is via prokaryotic

91 universal primer, or 16S ribosomal DNA. Nevertheless, most HTS studies may limit taxonomic
92 classification to the genus level due to technological (e.g., sequence length) and/or data
93 processing issues (e.g., sequence database availability). The current need is, therefore, to increase
94 taxonomic resolution (Dugat-Bony et al., 2016). On the contrary, culture-dependent approaches
95 have less resolution in depicting the overall microbial diversity, and fail to detect viable but-not-
96 cultivable cells and, in most cases, the sub-dominant populations. Currently, the best choice is
97 still to combine the two above approaches.

98 Analogously to other ecosystems (e.g., human microbiome), the multi-omics approach is the
99 best solution to combine dynamic microbial ecology and metabolism in cheesemaking. This
100 includes metagenome and metatranscriptome shotgun sequencing to explore the whole microbial
101 genome (Chen, Chen, & Lei, 2017) and, when combined to metabolome, it gives a cascade of
102 information on gene expressions, functions and metabolites. The so-called integrated systems
103 biology combines multiple perspectives of the post-genomics technologies and elucidates
104 metabolic interactions among microbes.

105 Because cheese microbial communities are not as complex as those of other natural
106 environments, they may be reproducible. This allows the setting up of model systems, which
107 closely mimic the cheese environment (Wolfe & Dutton, 2015). Undoubtedly, these systems are
108 decisive to investigate the interactions among microbes and biotic and abiotic factors.
109 Considering the protocols for the manufacture and the size of the cheese varieties, which, in turn,
110 determines pH, relative humidity, salt, temperature, water activity and chemical gradients,
111 various microbial niches populate the cheese curd. To date, two approaches, which differ in the
112 sampling procedure and technique for bacterial identification, may describe the spatial
113 distribution of bacteria in cheese. The first one is non-destructive and uses gel cassette or cryo-

114 sectioning models. Fluorescence in situ hybridization with labelled oligonucleotide probes is the
115 technique to detect bacteria (Jeanson et al., 2011). The second destructive approach is the most
116 common. Cheese sections are selected, followed by culture-dependent or -independent methods
117 to describe the microbial diversity (De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti,
118 2016). The microbiome results from deterministic drivers and functional traits of microbes,
119 which occur or disappear, depending on their capacity to thrive or not in the cheese environment.
120 Model cheese systems combining transcriptomic with proteomic approaches offer a useful
121 methodology for studying the mechanisms of metabolism regulation and adaptation of the lactic
122 acid bacteria biota.

123 **3. The players**

124 Considering its focus, this review addresses two main microbial players: SLAB and NSLAB.
125 SLAB may be mesophilic or thermophilic (Table 1). Mesophilic SLAB include mainly
126 *Lactococcus lactis*, and its subspecies, *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis*.
127 *Leuconostoc* sp. for citrate fermentation may be present in the formulation also. *Streptococcus*
128 *thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lb.*
129 *delbrueckii* subsp. *lactis* are the most common thermophilic SLAB. The manufacture of semi-
130 hard, hard and extra-hard Italian and Swiss cheese varieties use thermophilic SLAB. The above
131 distinction is losing some of its significance, since mesophilic and thermophilic SLAB species
132 are, sometimes, present together in both undefined and defined starters for making cheese
133 without or with low cooking. These mixtures may also contain some selected NSLAB species
134 (mainly mesophilic lactobacilli). The main activity of primary starters concerns the synthesis of
135 lactic acid from lactose, which takes place during early cheese manufacture. Usually, the pH of
136 the curd decreases to ca. 5.3, in 6 h at 30-37°C, depending on the manufacture protocol.

137 Commercial defined or undefined primary starters or natural primary starter cultures are
138 available. Two types of natural starter cultures, milk and whey cultures, distinguish depending on
139 the substrate and techniques used for reproduction. Their production occurs daily at the cheese
140 plant using back slopping protocols and/or selective treatments such heating, incubation at
141 relatively high temperature and acidification. The community of natural starter cultures faces
142 pressures by biotic and abiotic drivers. Even the partly controlled environmental conditions, these
143 undefined cultures show resilience and robustness with respect to defined cultures, which have
144 lower microbial diversity. Microbial contamination from raw milk or the cheesemaking
145 environment may occur as well as the control of other parameters (e.g., media and culture
146 conditions) is somewhat limited during natural starter preparation. Natural cultures may be stable
147 for long-time or they may evolve continuously, depending on the selective pressure used at any
148 cheese plant during propagation. If the biotype diversity decreases, cultures become more
149 sensitive to temperature, pH changes, carbon and nitrogen depletions, salt and bacteriophage
150 infection (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014).

151 Undoubtedly, NSLAB microbiota is more diverse than SLAB, consisting of lactococci,
152 pediococci, enterococci, *Leuconostoc* spp., thermophilic lactic acid bacteria and, especially,
153 mesophilic facultative and obligate hetero-fermentative lactobacilli (Table 2). A very large
154 diversity accompanies the most common cheese varieties, also depending on the protocol for
155 cheesemaking. Because of their importance during ripening, the load of NSLAB contamination
156 from raw milk, environment or natural starter cultures, often ensures the necessary level of
157 inoculum for several cheese varieties. Nevertheless, the need the needs to standardize
158 cheesemaking and to accelerate ripening has prompted the use of several NSLAB, often called as
159 secondary starters, adjuncts or attenuated adjuncts.

160 Speaking about players in cheese manufacture, bacteriophages have a fundamental role
161 because of their selective pressure within the bacterial community. Milk, whey and bacteria
162 theme-selves, containing temperate phage, are the primary entry routes for bacteriophages into
163 cheese environments. Overall, numbers from 10^2 PFU/m³ to 10^8 PFU/m³ are detectable in the air
164 of cheese manufacturing plants. Efforts to prevent phage infections in dairy plants include factory
165 design, sanitation, ventilation, process changes, modified media for SLAB cultivation, and
166 especially SLAB culture rotation. SLAB from natural cultures are more tolerant to phage
167 infection because their cultivation in an environment heavily contaminated with bacteriophages
168 favors the dominance of resistant biotypes (Marcò, Moineau, & Quiberoni, 2012). Overall, mixed
169 cultures have the highest degree of resistance against bacteriophage infections. Bacteriophages
170 become integral members of the microbial community and may drive the generation of a
171 phenotypic diversity, which enhances performance and robustness. The “kill the winner”
172 hypothesis is an attempt, also for cheese ecosystems, to state that bacteriophages prevent,
173 together with other biotic and abiotic factors, a winner from emerging, thus assembling
174 coexistent and diverse species. Consequently, each bacterial species/biotype shows consecutive
175 cycles of exponential growth, followed by abrupt declines (Xue & Goldenfeld, 2017). If this is
176 true, the level of surviving species is inversely related to modifications in their growth rates but
177 increases with the occurrence and extent of phage-induced collapses. The understanding of phage
178 diversity at cheese processing sites may shed light on population dynamics. Novel emerging
179 bacteriophages have been discovered continuously as in the case of *Str. thermophilus* 987 phages
180 (McDonnell et al., 2016), which show several common genetic features with lactococcal phage
181 (P335 group) and other *Str. thermophilus* phages. This relatedness suggests genetic exchanges,
182 thus resulting in emerging phage group. The usual practice of using mixture of SLAB, as in the

183 case of *Lc. lactis* and *Str. thermophilus* biotypes, may have been the cause for this bacteriophage
184 evolution. When bacteria and phages meet each other, they antagonize and follow an evolution
185 process. This antagonism relies on bilateral activities. From one side, bacterial mutants use a
186 number of strategies (e.g., mutation of receptors and Clustered Regularly Interspaced Short
187 Palindromic Repeats - CRISPR-Cas systems) to withstand phage infection and, from the other
188 side, bacteriophages try to break down antiviral barriers to propagate (Marcò, Moineau, &
189 Quiberoni, 2012).

190 **4. Raw vs. pasteurized milk**

191 Milk from a healthy udder is virtually sterile, even if prior contamination by mammary gland
192 is still controversial. Its microbial colonization occurs through numerous sources (e.g., teat
193 surface, machinery, farm staff and environment, feed and collection vessels) upon milking (Fig.
194 2). Stage of lactation and seasonality also influence the composition of the milk microbiota
195 (Siefarth & Buettner, 2014). Raw milk directly contaminates the vat milk with its microbiota or
196 indirectly enriches the house microbiota. The rough estimation is that almost 100 genera and 400
197 microbial species may be present in raw milk (Montel et al., 2014). *Staphylococcus* sp. and
198 coryneform bacteria dominate. Other groups concern *Pseudomonas* spp., especially during milk
199 refrigeration, and lactic acid bacteria and *Enterobacteriaceae*. The inter-farm variability of the
200 microbiota is wide, while the variability at intra-farm level is less, except for the influence of the
201 seasons. Biotype diversity in raw milk is also substantial. In France, almost 43 genotypes of *Lc.*
202 *lactis* were detectable in raw milk, and the number of genotypes varies from 1 to 11 per farm
203 (Montel et al., 2014). Strains may remain detectable at a farm for long-time, representing a link
204 with of a specific milk microbiota. When cheesemaking occurs at the same farm using raw milk
205 and short and small supply dairy chain, this favors microbial diversity in cheese. The

206 geographical origin also influences the raw milk microbiota (Perin, Savo Sardaro, Nero, Neviani,
207 & Gatti, 2017). A study on ca. 5,000 farms differing in size, management and geographical
208 localization is in progress at the author's laboratory (Gobbetti et al., 2018 unpublished data). The
209 cause-effect relationships between farm conditions and richness of milk lactic acid bacteria biota
210 have been established. Industrial cheesemaking tends to give higher microbial standardization,
211 reducing the variability in milk matrix and practices. The biological richness of the raw milk is
212 the driver to withstand technology interventions and to overcome unattended abiotic hurdles.

213 Refrigeration modifies the microbial balance in milk. The population shifts from Gram-
214 positive to Gram-negative bacteria. As the most common psychrotrophs, *Pseudomonas* spp.
215 dominate in raw milk, together with *Acinetobacter* ssp. and *Enterobacteriaceae* (e.g., *Hafnia*
216 *alvei*) (Fricker, Skanseng, Rudi, Stessl, & Ehling-Schulz, 2011). Psychrotrophic bacteria largely
217 grew in milk, increasing ca. 3.0 log cfu/mL during 3 days at 8°C or 7 days at 4°C (Rasolofo, St-
218 Gelais, LaPointe, & Roy, 2010). Small variations in storage temperature and duration led to a
219 different balance of species/biotype. Vat raw milk contains thiocyanate (SCN⁻), oxidation of
220 which by the lactoperoxidase (LP)-H₂O₂ system determines bactericidal or bacteriostatic effects
221 depending on Gram-negative or Gram-positive bacteria (Gatti, Bottari, Lazzi, Neviani, &
222 Mucchetti, 2014).

223 Natural skimming is a process of fat separation used to reduce its concentration in raw milk
224 for making Italian extra-hard cheese varieties (e.g., Parmigiano Reggiano and Grana Padano).
225 The cream naturally rises to the surface because of the difference on specific gravity, which
226 causes a decrease of the number of spore-forming bacteria that are concentrated in the cream
227 with the fat globules. A slight microbial acidification occurs during creaming. Depending on the
228 environmental temperature and duration of creaming, an increase of the raw milk mesophilic

229 microbiota occurs also. Slight proteolysis accompanies the natural skimming. The liberation of
230 short chain peptides favors the growth of SLAB (Gatti, Bottari, Lazzi, Neviani, & Mucchetti,
231 2014).

232 Pasteurization standardizes the microbial composition and improves the safety of milk by
233 reducing its microbial load and diversity. Psychrotrophic and mesophilic bacteria, including
234 NSLAB, decrease 2 log cycles or more. Spores and other heat resistant bacteria withstand
235 pasteurization and contaminate the cheese, together with other microbes (e.g., NSLAB), the
236 introduction of which is via post-pasteurization contamination (Gobbetti, De Angelis, Di Cagno,
237 Mancini, & Fox, 2015). NSLAB biotypes may withstand heat treatments, mainly entering a
238 viable but-non-cultivable state, but recovering and proliferating in the curd during ripening (De
239 Angelis et al., 2004).

240 **5. The house microbiota**

241 The dairy environment, comprising cattle, equipment, feed, feces, humans and air, harbors
242 and acts as a vector of resident microbes, defined as the house microbiota (Montel et al., 2014)
243 (Fig. 2). This unavoidably interacts with the microbial populations of raw milk, SLAB and
244 NSLAB, especially during milking and cheese manufacture. After milking, the first microbial
245 contamination is via the teat canal and surface, and dairy equipment (e.g., milking machine, milk
246 line and tank). The teat skin is the major source of microbial contamination for raw milk (Fréтин
247 et al., 2018). Staphylococci, coryneforms, *Enterobacteriaceae*, *Clostridium* ssp., lactic acid
248 bacteria (e.g., *Lc. lactis*) and *Pseudomonas* ssp. largely colonize the teat surface. Several phyla
249 are mainly detectable in teat canal such as Actinobacteria and Firmicutes (*Clostridiaceae*,
250 *Staphylococcaceae* and *Lactobacillaceae*, *Enterococcaceae* less frequently), followed by
251 Proteobacteria (Gill et al., 2006). Biofilms on milking equipment represent other sources of

252 microbial inoculum (Marchand et al., 2012). Mesophilic lactobacilli such as *Lactobacillus*
253 *plantarum* have the capability to form biofilms, which favors their growth after disinfecting
254 treatments. Milker, feed (grass, silage and hay), drinking and washing water, stable and milking
255 parlor air, feces are other sources of relevant contamination. The management system of the farm
256 certainly has an influence, but this makes it difficult to identify the effect of individual practices.
257 Besides, farm management is evolving rapidly towards high levels of automation and,
258 consequently, the importance of several practices undergoes fluctuation. Grazing systems
259 (extensive vs. semi-extensive) and seasonality (early vs. late summer) determine the variable
260 contamination of teat skin by *Atopobium*, *Bifidobacteriales*, *Clostridium*, *Corynebacteriales*,
261 *Coriobacteriia*, *Lachnospiraceae*, and lactic acid bacteria (Frétin et al., 2018). Feces are indirect
262 sources of *Enterobacteriaceae*, clostridia, yeasts, *Bifidobacterium* and various lactic acid bacteria
263 (Montel et al., 2014). The presence within the NSLAB microbiota of a typical intestinal species
264 such as *Lactobacillus reuteri* confirms the contamination from feces (Su, Oh, Walter, & Gänzle,
265 2012). The relationship between house and core microbiota was highlighted using Caciotta and
266 Caciocavallo Pugliese cheeses (Calasso et al., 2016). Mesophilic lactobacilli and lactococci, and,
267 especially, streptococci represented the highest number of detectable bacteria on dairy
268 equipment. As shown by 16S rRNA targeted metagenomics, *Str. thermophilus* dominated the
269 communities from knife surfaces and brine tank, and was the most frequent bacterium found in
270 draining table and ripening room. Other SLAB such as *Lc. lactis*, *Lb. delbrueckii* subsp. *lactis*
271 and *Lb. helveticus* showed low relative abundance in colonizing capability. Other genera/species
272 such as *Lactobacillus casei* and *Lb. plantarum* group also contribute to a heterogeneous house
273 microbiota. Dairy equipment (e.g., wooden vats and ripening shelves) used for the manufacture
274 of traditional cheeses (e.g., Ragusano) enrich the raw vat milk or the curd with certain lactic acid

275 bacteria (Licitra et al., 2007). The microbial composition of these biofilms is permanent during
276 seasons. Wooden vats act as source of microbial inoculum in milk. The number of lactic acid
277 bacteria increased consistently (ca. 50%) after few minutes from the contact (Settanni et al.,
278 2012). Nevertheless, the role of this population in cheesemaking still needs clarification.

279 **6. Cheese manufacture**

280 A complex and only partly understood network of interactions between biotic (e.g.,
281 microbial interactions) and abiotic (e.g., technology parameters, pH, water activity, redox
282 potential and chemical composition) factors determine continuous changes in the microbial
283 balance during cheese manufacture (Fig. 2). Apart from the variety, each internally-ripened
284 cheese has a specific, dense microbiota (ca. 8.0-9.0 log cells/g) consisting of a few to very
285 numerous species of SLAB and NSLAB (Dugat-Bony et al., 2016). Species/biotypes
286 contaminating vat milk grow, survive, decrease or dominate during cheesemaking. This depends
287 on the metabolic potential, which is species or even biotype specific. The first environmental
288 condition tackled by the microbiota concerns the biochemical composition of vat milk, followed
289 by the curd matrix as influenced by acidification and technological parameters (e.g., rennet
290 addition, temperature), and finally salting.

291 SLAB need to be active in the curd during cheese manufacture. Both acid production and
292 rapid growth (from ca. 6.0 log cfu/mL, in the inoculated milk, to ca. 8.0-9.0 log cfu/g, in the
293 curd) occur. The production of lactic acid decreases the pH, which contributes to curd formation
294 and inhibits pathogen growth. Milk citrate is used by some lactic acid bacteria (e.g., *Lc. lactis*
295 ssp., *Leuconostoc* sp., *Lb. casei* and *Lb. plantarum*) a secondary energy source. The co-
296 metabolism of citrate and lactose may occur also (Drici, Gilbert, Kihal, & Atlan, 2010). The rate
297 of pH decrease of Italian extra-hard varieties is according to temperature gradient and cheese

298 size, which inevitably affect the SLAB growth (Pecorari, Gambini, Reverberi, & Caroli, 2003).
299 The value of pH of the outer curd layer decreased to 5.1-5.2 within 5-6 h. The inner curd attains
300 almost the same pH only after 24 h because the temperature remains higher than 50°C for more
301 than 10 h. The highest growth of thermophilic SLAB is within 6 h in the outer layer, with *Lb.*
302 *helveticus* dominating, and between 6-24 h in the core, where *Lb. delbrueckii* ssp. prevails
303 (Giraffa, Rossetti, Mucchetti, Addeo, & Neviani, 1998; Gatti et al., 2008).

304 Cooking the curd is a typical technology intervention for making several Italian, Swiss and
305 other hard and extra-hard cheese varieties. It has a direct effect on whey separation and drainage
306 and, indirectly, affects the preparation of the whey natural cultures because of the relatively high
307 temperature that selects the composition of the resulting autochthonous lactic acid bacteria biota.
308 The intensity of heating varies from ca. 40-43°C up to 56°C. Curd cooking influences the early
309 steps of cheese manufacture and ripening. Thermal selection of NSLAB, which are naturally
310 present in the milk, and added SLAB, occurs and varies depending on the intensity of cooking.
311 Among SLAB, *Str. thermophilus* is less resistant to heating than *Lb. helveticus* (Sheehan,
312 Fenelon, Wilkinson, & McSweeney, 2007). The survival of *Leuconostoc lactis*, *Lc. lactis* and
313 psychrotrophic bacteria markedly decreased with curd temperature of 47-48°C (Giannino,
314 Marzotto, Dellaglio, & Feligini, 2009). Stretching is another technology option for making pasta
315 filata cheeses, which modifies the structure of the properly demineralized acid curd by the
316 addition of hot water (85-95°C or less). This operation induces curd melting. Heating the curd
317 under these conditions reduces the contaminating microbiota, including SLAB and NSLAB,
318 which results also because of the pH reduction. The dominating mesophilic microbiota of
319 Ragusano pasta filata cheese, which consists mainly of *Leuconostoc* spp. and *Lc. lactis*,
320 diminished during stretching (Randazzo, Torriani, Akkermans, de Vos, & Vaughan, 2002).

321 Besides, the scalding temperature, which controls the moisture in the non-fat substances,
322 decreases the viable microbiota of Dutch-type cheeses. It delays the growth of SLAB and their
323 metabolism, and enhances the growth of heat-tolerant bacteria at the expense of the mesophilic
324 biota (Porcellato & Skeie, 2016). After 12 to 24 h from curd extraction, SLAB continue to grow,
325 which further decreases the pH of extra-hard Italian cheese varieties (Santarelli et al., 2013a).

326 Salting is an important stage for most cheese varieties. Commonly, brine salting is used. The
327 duration of brining lasts few hours to numerous days, according to cheese size and shape, and
328 NaCl concentration and temperature. During brining, the osmotic pressure caused the Na⁺ and Cl⁻
329 movement from brine into the curd external layers. This osmotic pressure and gradient, and the
330 high salt concentration in the brine (15-23% NaCl) influence cell viability and growth, enzyme
331 activity and the development of ripening hotspots due to localized variations of salt in moisture
332 levels (Fox, Guinee, Cogan, & McSweeney, 2000). Transcriptomic analyses highlighted the
333 induction of bacterial genes and antioxidant enzymes in response to oxidative stresses, including
334 salt exposure (Tsuzuki et al., 2011). This leads to the generation of reactive oxygen species
335 (ROS). When ROS accumulation is higher than the bacterial scavenging capacity, breakage of
336 nucleic acids and proteins, enzyme inhibition and ultimately cell death occur. Usually,
337 mesophilic bacteria in brines used for semi-hard and hard Italian cheeses ranges from 2.0 to 6.5
338 log cfu/mL. Proteobacteria and Formicutes dominate, followed by Bacteroidetes and
339 Actinobacteria. Up to nine major genera populated each brine (Marino et al., 2017). Since the use
340 of brines for numerous cheesemaking batches, the microbial load from salt, curd and equipment
341 increase during time. The cheese surface harbors biofilms also composed of SLAB and NSLAB,
342 which originate from brine (Mounier et al., 2006). In several cases, bacteria entered in a viable
343 but-not-cultivable state (Gin & Goh, 2013). Compared to viability, SLAB autolysis, as measured

344 by the release of intracellular enzymes, decreases significantly with lower salt addition in
345 Cheddar cheese. The salt concentration also affects the level of cell permeabilization
346 (Yanachkina, McCarthy, Guinee, & Wilkinson, 2016).

347 **7. Cheese ripening**

348 The number of lactic acid bacteria present during cheese ripening results mainly from the
349 capacity to withstand heat (when applied) and acid stresses during manufacture, and to use
350 energy sources other than carbohydrates during mid to late ripening (Fig. 2). The diversity of
351 lactic acid bacteria in internally-ripened cheeses is wide. Literature data describe approximately
352 35main different species of lactic acid bacteria, spanning 7 genera (Montel et al., 2014). Usually,
353 the highest diversity and richness is reached within 2 months of ripening (Santarelli, Bottari,
354 Lazzi, Neviani, & Gatti, 2013b). In spite of increasing efforts to identify species, knowledge on
355 the diversity at the biotype level is low. Cell numbers of lactic acid bacteria remain elevated (ca.
356 8.0-9.0 log cfu/g) for several months during cheese ripening but species/biotypes change.
357 Overall, the high initial levels of cultivable SLAB decrease progressively. It reaches ca. 1% of
358 the initial load just after 1 month of cheese ripening (McSweeney et al., 1994). Contrarily, a few
359 numbers (2.0-3.0 log cfu/g) of NSLAB (if not used as secondary starters or adjuncts) increase
360 and reach a plateau (7.0-9.0 log cfu/g) after a few months of cheese ripening (Fitzsimons, Cogan,
361 Condon, & Beresford, 2001). During ripening, NSLAB have a generation time of approximately
362 8 days (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015). The cell number of SLAB and
363 NSLAB is mainly dependent on the type of starter culture, conditions of cheese manufacture and
364 time of ripening.

365 Examples concerning the dynamics of the SLAB and NSLAB are as follows. The lactic acid
366 bacteria biota of Parmigiano Reggiano and Grana Padano cheeses remains viable throughout

367 ripening. Depending on ripening time (12 to 24 months), viable counts are in the order of 4.5-6.0
368 log cfu/g (Santarelli, Bottari, Lazzi, Neviani, & Gatti, 2013b). Long-ripened Comté and Swiss-
369 type cheeses have almost the same levels (Depouilly, Dufrene, Beuvier, & Berthier, 2004;
370 Montel et al., 2014). SLAB such as *Lb. delbrueckii* ssp. *lactis* remained viable for 9 months in
371 extra-hard cheeses, and *Lb. helveticus* and *Lb. delbrueckii* ssp. *lactis* disappeared after 12 months
372 in Parmigiano Reggiano ripening (Gala et al., 2008; Solieri, Bianchi, & Giudici, 2012). A
373 combination of culture and hybridization techniques showed that *Lb. helveticus* dominated
374 experimental Grana Padano cheese during early ripening, while *Lb. delbrueckii* subsp. *lactis*
375 prevailed from 2 months onward (Zago et al., 2007). Cooking or stretching the curd have
376 repercussions on SLAB viability. As shown in 10-months ripened Pecorino Romano cheese,
377 thermophilic SLAB persist in aged cheeses, the curd for which underwent low cooking (e.g.,
378 48°C) (Mangia, Murgia, Garau, & Deiana, 2011). Uncooked, long-ripened Cheddar cheese
379 showed a quite stable persistence of mesophilic SLAB such as *Lc. lactis* (Crow, Curry, & Hayes,
380 2001). Heating the curd has less influence on NSLAB numbers and viability during ripening than
381 SLAB because the multiple sources of contamination. NSLAB remained viable at ca. 4.0 log
382 cfu/g in 24-months ripened Parmigiano Reggiano cheese (Coppola et al., 1997). For several cheese
383 varieties, dominant biotypes in the core of cheese were different from those found in raw milk,
384 suggesting that selective conditions occur during cheese manufacture (Montel et al., 2014).
385 Usually, NSLAB (e.g., *Lactobacillus paracasei*, *Lb. plantarum*, *Lb. casei* and *Lactobacillus*
386 *rhamnosus*) predominate over mesophilic and thermophilic SLAB after 10 to 30 months of
387 ripening (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014). The dominance of NSLAB with the
388 concomitant absence of thermophilic SLAB is a typical feature of high cooked and long-ripened
389 Gruyere and Comté cheeses (Depouilly, Dufrene, Beuvier, & Berthier, 2004; Casey, Häni,

390 Gruskovnjak, Schaeren, & Wechsler, 2006). *Lb. paracasei*, *Lb. rhamnosus*, *Lb. casei*, *Lb.*
391 *curvatus* and *Lb. plantarum* dominate in Cheddar cheese and reach ca. 8.0 log cfu/g within 3
392 months, remaining at almost the same level until 18 months of ripening (Crow, Curry, & Hayes,
393 2001). The diversity of NSLAB species was greatest during the early ripening of Cheddar cheese.
394 After two months of ripening, Irish Cheddar cheese contained *Lb. paracasei*, *Lb. plantarum* and
395 *Lb. curvatus*, but *Lb. paracasei* dominated from 9 to 24 months (Fitzsimons, Cogan, Condon, &
396 Beresford, 2001).

397 Cheese intrinsic (e.g., availability of substrates and co-factors, presence of inhibitor/activator
398 compounds, pH, water activity and redox potential) and extrinsic factors (e.g., oxygen,
399 temperature and environmental humidity) govern the spatial distribution of microbes within the
400 curd (Fig. 2). After coagulation, bacteria immobilize within the curd following a relatively
401 uniform but stochastic distribution. This event determines the distribution of colonies and forms
402 environmental micro-niches, which underwent to fluctuations throughout space and time. Few
403 studies dealt with bacterial distribution and movement in cheeses. Microscopy observations
404 reveal that bacteria occur mainly in whey pockets and at fat-protein interfaces, so they are not
405 fully homogeneously distributed (Hickey, Sheehan, Wilkinson, & Auty, 2015). Microbial counts
406 differ according to cheese spatial coordinates. Outer layers of Parmigiano Reggiano and Grana
407 Padano cheeses aged 9-12 to 18-20 months have lower values than inner layers (De Dea Lindner
408 et al., 2008). Gradients for moisture, salt, fat and protein were found in sub-blocks of Italian
409 ewes' milk hard cheeses (De Pasquale, Di Cagno, Buchin, De Angelis, & Gobbetti, 2016). While
410 the cell density did not differ substantially, the zones under the top and bottom rind harbored the
411 greatest diversity of lactic acid bacteria. The highest microbial diversity was statistically

412 correlated with the highest secondary proteolysis event and/or with the highest concentration of
413 volatile compounds.

414 Cell autolysis and/or cell permeabilization with the release of intracellular enzymes may take
415 place in cheese during ripening. The extent depends on species/biotypes and has repercussions on
416 microbial dynamics. Results concerning the autolysis of lactic acid bacteria are in part
417 contradictory. A microscopy-based count of viable cells present in Parmigiano Reggiano cheese
418 aged 12-20 months showed that the major part of dead SLAB cells did not lyse during ripening
419 (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014). Nevertheless, lysed cells of *Lb. helveticus*
420 and *Lb. delbrueckii* ssp. *lactis* became evident in Parmigiano Reggiano cheese only after 2
421 months of ripening, probably following the peak of acidification (Santarelli, Bottari, Lazzi,
422 Neviani, & Gatti, 2013b). Results for other cheese varieties showed that the substantial decrease
423 of the SLAB population during early ripening always accompanied lysis and release of
424 intracellular enzymes (Steele, Broadbent, & Kok, 2013). Differences at the microenvironment
425 level influence the rate of autolysis. The use of adjunct cultures with the capacity to synthesize
426 bacteriocins is a strategy for increasing the rate of SLAB cell lysis. Unlike to SLAB, the lysis of
427 NSLAB cells is rather infrequent (Gobbetti, De Angelis, Di Cagno, Mancini, & Fox, 2015).

428 **8. Interactions between SLAB and NSLAB**

429 The microbial interactions involved in cheese microbiota dynamics are numerous and only
430 partly elucidated. These interactions establish cheese intrinsic factors and, in turn, intrinsic and
431 extrinsic factors drive interactions. SLAB and NSLAB interact since the early stages of
432 manufacture to the cheese ripening process. Examples concerning nutrition, competition,
433 cooperation and stress response are as follow.

434 The growth of NSLAB in milk is sub-optimal, and depletion of lactose in the curd occurred
435 early during ripening. Under this condition, a variety of chemical compounds may act as
436 alternative energy sources. Only a few energy sources have a real *in situ* relevance. Microbiota
437 metagenomes from ripened cheeses, where NSLAB dominate, contained genes with an increased
438 number of functions on carbohydrate and protein metabolisms (Porcellato & Skeie, 2016). One
439 of the most accredited theory indicates that SLAB lysates provide energy sources for NSLAB.
440 NSLAB grew faster in Cheddar cheese manufactured SLAB that underwent fast lysis (Lane, Fox,
441 Walsh, Folkertsma, & McSweeney, 1997). Although genes responsible for autolysis do not
442 express during the non-cultivable state, bacterial autolysis often occurs after carbon depletion,
443 and in response to heating (if applied) and salt (Ganesan, Stuart, & Weimer, 2007). The cell
444 composition of SLAB (percentage of total dry weight) is approximately: 45% protein, 12-15%
445 polysaccharide, 10% teichoic acid, 6-8% RNA, 7% inorganic ions, 5.5% amino sugars, 4-4.3%
446 lipid and 3-3.3% DNA (Novak & Loubiere, 2000). Most NSLAB ferment ribose (Settanni &
447 Moschetti, 2010), which induces the acetate kinase route of the pentose pathway, gaining an
448 extra mole of ATP. Growing in a cheese model system, *Lb. rhamnosus* redirects its metabolism
449 toward acetate by pyruvate oxidase pathway and ribose degradation (Gatti et al., 2008). The same
450 capability was confirmed in cheese during ripening. Probably, lysing SLAB release pyruvate, as
451 intracellular metabolite, into the cheese matrix. Biotypes of *Lb. paracasei* grow well on N-
452 acetylglucosamine and sialic acid, which may be derived from nucleic acid and/or casein
453 deglycosylation (Adamberg et al., 2005). *Lactobacillus* sp. secrete nucleases and liberate
454 nucleotides, which either serve for the synthesis of DNA, RNA and coenzymes or as carbon and
455 nitrogen sources (Gatti et al., 2008). Under the limiting conditions of cheese ripening, SLAB
456 have the capability to synthesize oligosaccharides, which favor the NSLAB growth (Crow et al.,

457 2002). The spread of SLAB metabolite diffusion to NSLAB has been mathematically modelled
458 during Cheddar cheese ripening. Diffusion is limiting only for molecules having large size or
459 remaining immobilized into the cheese matrix. The diffusion of small molecules around NSLAB
460 niches takes place faster with respect to their release from cells. Therefore, the extent of NSLAB
461 growth in cheese during ripening depends more on the lysis rate of SLAB rather than on the
462 diffusion of metabolites (Czàràn, Rattray, Møller, & Christensen, 2018). Nevertheless, polymeric
463 components (e.g., polysaccharide, teichoic acids, RNA and DNA) of SLAB cells need hydrolysis
464 into their monomers (N-acetylglucosamine, N-acetylmuramic acid, ribose and deoxyribose) prior
465 diffusion. On the opposite, the use of cell-free cytoplasm extracts from NSLAB species (e.g., *Lb.*
466 *casei*) was not only useful to speed up cheese ripening but also enhanced the growth of SLAB
467 and NSLAB (Calasso et al., 2017).

468 Competition or cooperation between SLAB and NSLAB occurs for several metabolites
469 (Table 3). Upregulation of lactate dehydrogenase (*ldh*) and alcohol dehydrogenase (*adh*) genes
470 occurred in *Lc. lactis* subsp. *cremoris* when co-cultivated with *Lb. paracasei* (Desfossés-
471 Foucault, LaPointe, & Roy, 2014). The upregulation of the *ldh* gene occurred almost at the end of
472 cheese ripening under starvation conditions, thus indicating competition for substrates. On the
473 contrary, the downregulation of *ldh* gene took place in *Lb. paracasei*, which favored a switch to
474 mixed acid fermentation. Because of competition for carbohydrates under heat and salt stresses,
475 the upregulation of deoxyribose-phosphate aldolase and phosphofructokinase 1 genes occurred in
476 *Lb. paracasei* when associated to *Lc. lactis* subsp. *cremoris* during cheese manufacture.
477 Proteolysis during cheese ripening is an example of metabolic cooperation between SLAB and
478 NSLAB, which has an impact on cheese flavor and microbial dynamics. During manufacture and
479 ripening of Gouda cheese, the growth of *Leuconostoc mesenteroides* relies on the proteolysis by

480 *Lc. lactis* ssp., which provides small peptides and essential free amino acids (Smid &
481 Kleerebezem, 2014). The induction of peptidase genes in *Lb. casei* followed the initial
482 proteolysis by *Lc. Lactis* (Desfossés-Foucault, LaPointe, & Roy, 2014). Combinations of SLAB
483 with high peptidolytic *Lactobacillus* spp. are some of the most common tools used to accelerate
484 and diversify proteolysis during cheese ripening (Courtin et al., 2002). Metabolic pathways for
485 branched amino-acid catabolism and thioester formation (Smit, Smit, & Engels, 2005) are other
486 examples. Downregulation of the cystathionine- β -synthetase gene took place in single culture of
487 *Lc. lactis* ssp. On the contrary, the lactococcal upregulation of this gene occurred when in mixed
488 culture with *Lb. paracasei*. This interaction enables the use of methionine and cysteine.
489 Methionine is the major sulphur-containing amino acid in milk proteins and, among other S-
490 containing compounds, markedly contributes to cheese flavor (Desfossés-Foucault, LaPointe, &
491 Roy, 2014). Commonly, the liberation of free amino acids increases when mesophilic SLAB
492 (e.g., *Lc. lactis* ssp.) combine with glutamate-dehydrogenase positive NSLAB biotypes
493 (Kieronczyk, Skeie, Langsrud, & Yvon, 2003).

494 Despite a core gene expression, the response of lactic acid bacteria to stressful conditions
495 during cheese manufacture and ripening depends on biotypes and levels of microbial interactions.
496 Overall, responses heat shock and acid stresses rely mainly on chaperones (e.g., dnaK, dnaJ and
497 GroESL) and proteinase-chaperone complexes (e.g., ClpP, ClpX and FstH) (Parente, Cogan, &
498 Powell, 2017). Upregulation of *clpC*, *dnaJ*, and *groES* genes of *Lb. paracasei* occurred when
499 cultivated in co-presence of *Lc. lactis* ssp. *cremoris*. Nevertheless, *Lb. paracasei* showed a lower
500 expression of these genes during cheese ripening, which would be the consequence of decreased
501 viable cells of *L. lactis* ssp. *cremoris* SK11 over time. The hypothesis is that substrates are
502 released by lysing SLAB cells, and, in turn, NSLAB provide their catabolism (Desfossés-

503 Foucault, LaPointe, & Roy, 2014). Lactic acid bacteria have the concomitant capability to
504 synthesize, detect and respond to small signaling hormone-like molecules (e.g., autoinducer 2
505 and peptide pheromones), which have regulatory roles within complex communities (Gobbetti,
506 De Angelis, Di Cagno, Minervini, & Limitone, 2007). SLAB and NSLAB (e.g., *Lb. delbrueckii*
507 ssp, *Lb. rhamnosus*, *Lb. plantarum*) increased the synthesis of autoinducer 2 during acid
508 conditions, with *luxS* gene governing stress resistance and metabolic activities (Herve-Jimenez et
509 al., 2008). The association between *Str. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* is
510 rather common for making several cheese varieties. Biotypes of *Str. thermophilus* have two
511 distinct phases of growth in milk. The features of the second phase depend on the presence or
512 absence of *Lb. delbrueckii* ssp. *bulgaricus*. Comparative proteomic approaches showed variations
513 in the level of expression of enzymes related to amino acid biosynthesis, carbon and purine-
514 pyrimidine metabolisms, and response regulator RR05. The synthesis of cysteine from
515 glyceraldehydes 3-P, the trans-sulfuration and sulfhydrylation pathways, and the conversion of
516 homocysteine into methionine and, in turn, into cysteine increased. The level of the RR05
517 response regulator, which is part of the two component system (TCS), decreased during the late-
518 exponential phase of growth of *Str. thermophilus* (Herve-Jimenez et al., 2008). The TCS system
519 is indispensable to activate transcription and to synthesize peptide pheromones by lactic acid
520 bacteria. These include bacteriocins or bacteriocin-like peptides such as nisin by *Lc. lactis* and
521 plantaricin A by *Lb. plantarum*. Several quorum sensing-related proteins (e.g., PlnC activator and
522 AgrB-like proteins) increased during co-cultivation of *Lb. plantarum* with other lactobacilli. Co-
523 cultivation of *Lb. plantarum* biotypes with other NSLAB or in the presence of plantaricin A
524 induces proteins responsible for ABC transport and peptide uptake (OppA) (Calasso et al., 2013).
525 *Lc. lactis* shows an Opp system with specific pheromone binding activity. During growth in

526 whey, *Lb. helveticus* released signaling molecules (e.g., 2[5H]-furanones), which are responsible
527 for autolysis and release of enzymes (Ndagijimana, Vallicelli, Cocconcelli, Cappa, & Patrignani,
528 et al., 2006). Knowledge on extracellular signaling between SLAB and NSLAB under cheese
529 manufacture and ripening conditions explains the mechanisms of bacterial adaptation. Stress-
530 induced proteins are clear molecular markers for SLAB and NSLAB fitness.

531 **9. Conclusions**

532 Although less complex than natural environments, the cheese ecosystem shows a variable
533 flux of its core microbiota during time and through space. Many and diverse drivers establish and
534 assembly the lactic acid bacteria biota, intended as a consortium of SLAB and NSLAB. If such
535 drivers are efficient to guarantee microbial and cheese diversities, on the other hand, their control
536 is the fundamental pre-requisite to synchronize and balance microbiological events. The
537 methodology (e.g., omics techniques and integrated system biology) has markedly improved to
538 concretize this ambitious goal. Facing and improving the knowledge on the main drivers, the
539 current step should focus on a unique puzzle of coexisting species/biotypes likely a super-
540 organism, whose guide has to consider all casehardened microbial elements.

541

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544

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792 **Legends to figures**

793 **Figure 1.** The methodology approach to study the community structure and activity of cheese
794 microbiota (Adapted from Ndoye et al., 2011; Sheik et al., 2014).

795 FISH, Fluorescence in situ hybridization; RT-qPCR, Reverse transcription - quantitative PCR;
796 SDS-PAGE, Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis; SSH, Suppression
797 subtractive hybridization.

798 **Figure 2.** Potential microbial sources that determine the cheese lactic acid bacteria biota.

800 **Table 1.** Examples of mesophilic and thermophilic primary starters, starter type and their
 801 function.

Species	Starter type	Starter function
Mesophilic species		
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	DSS, MSS, NS	LA, D, P, L, AR, C
<i>Lactococcus lactis</i> subsp. <i>lactis</i> (cit)	DSS, MSS, NS	LA, D, P, L, AR, C, SM
<i>Lc. lactis</i> subsp. <i>lactis</i> (cit ⁺)	DSS, MSS	LA, D, P, L, C
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	DSS, MSS, NS	LA, D, P, L, AR, C, SM
Thermophilic species		
<i>Lactobacillus delbruecki</i> subsp. <i>lactis</i>	DSS, MSS, NS	LA, AR, C, P, PG
<i>Lactobacillus delbruecki</i> subsp. <i>bulgaricus</i>	DSS, MSS, NS	LA, P, L
<i>Lactobacillus delbruecki</i> subsp. <i>bulgaricus</i>	DSS, MSS, NS	LA, P, L
<i>Lactobacillus helveticus</i>	DSS, MSS, NS	LA, AR, C, P, PG,
<i>Streptococcus thermophilus</i>	DSS, MSS, NS	LA, AR, C, P, PG, L, SM

802 DSS, defined strain starter; MSS, mixed strain starter; NS, natural starter; LA, lactic acid; D,
 803 diacetyl; P, proteolysis; L, lipolysis; AR, aroma; C, CO₂; SM, surface smear; PG, propionic acid
 804 and gas.

Table 2. Mesophilic obligate and facultative hetero-fermentative lactobacilli variously identified as contaminants in thirty-eight cheese varieties differentiated for some technology treatments

Obligate hetero-fermentative				
Species	Cheese	Curd cooking	Curd stretching	Curd pressing
<i>Lb. parabuchneri</i>	Caciocavallo Pugliese, Malga	-	+	-
<i>Lb. diolivorans</i>	Fontina, Malga	+	-	-
<i>Lb. reuteri</i>	Kasar	-	+	-
<i>Lb. curvatus</i>	Caciocavallo Silano, Istrian, Gruyère, Quesalla Arochena, Canestrato Pugliese, Bellie, Dulses, Gouda type, Castelmagno, Pecorino Sardo, Pecorino Marchigiano	+	+	+
<i>Lb. hilgardii</i>	Malga	+	-	-
<i>Lb. fermentum</i>	Ragusano, Caciocavallo Silano, Parmigiano Reggiano, Bitto, Bryndza, Bellie, Dulses, Gouda type, Pecorino Sardo, Pecorino Marchigiano	+	+	+
<i>Lb. brevis</i>	Caciocavallo Pugliese, Malga, Gruyère, Bryndza, Pico, Zlata, Canestrato Pugliese, Bellie, Dulses, Gouda type, Ossau-Iratry, Pecorino Sardo, Pecorino Marchigiano, Alberquilla	+	+	+

Table 2. Continued

Facultative hetero-fermentative				
Species	Cheese	Curd cooking	Curd stretching	Curd pressing
<i>Lb. coryniformis</i>	Malga, Kasar, Torta Arochena	+	+	-
<i>Lb. harbinensis</i>	Parmigiano Reggiano	+	-	-
<i>Lb. rhamnosus</i>	Caciocavallo Palermitano, Provolone del Monaco, Parmigiano Reggiano, Bitto, Grana Padano, Grana Trentino, Bryndza, Bellie, Dulses, Gouda type, Pecorino Sardo, Pecorino Marchigiano	+	+	+
<i>Lb. casei</i>	Ragusano, Caciocavallo Pugliese, Fontina, Caciocavallo Palermitano, Kasar, Parmigiano Reggiano, Grana Padano, Bryndza, Pico, Saint-Nectaire, Canestrato Pugliese, Castelmagno, Pecorino Sardo, Pecorino Marchigiano	+	+	+
<i>Lb. paracasei</i>	Caciocavallo Pugliese, Caciocavallo Silano, Malga, Provolone del Monaco, Parmigiano Reggiano, Bitto, Grana Padano, Grana Trentino, Zlatar, Feta, Quesallia Arachena, Raschera, Bellie, Dulses, Gouda type, Pecorino Sardo, Genestoso, Torta Arochena, Salers, Ossau-Iratry, Pecorino di Filiano, Pecorino del Reatino, Alberquillia	+	+	+

Table 2. Continued

Facultative hetero-fermentative				
Species	Cheese	Curd cooking	Curd stretching	Curd pressing
<i>Lb. plantarum</i>	Ragusano, Caciocavallo Pugliese, Caciocavallo Silano, Fontina, Malga, Kasar, Bitto, Casin, Poro, Zlatar, Darfiyeh, Feta, Quesallia Arachena, Raschera, Bellie, Dulces, Gouda type, Canestrato Pugliese, Genestoso, Torta Arochena, Salers, Castelmagno, Ossau-Iratry, Pecorino di Filiano, Pecorino del Reatino, Alberquillia, Pecorino Sardo, Pecorino Marchigiano, Calenzana	+	+	+
<i>Lb. paraplantarum</i>	Darfiyeh, Quesallia Arachena	-	-	-
<i>Lb. pentosus</i>	Pecorino Sardo, Pecorino Marchigiano, Darfiyeh	-	-	-
<i>Lb. fabifermentans</i>	Darfiyeh	-	-	-
<i>Lb. buchneri</i>	Feta	-	-	-
<i>Lb. perolens</i>	Bellie, Dulces, Gouda type	-	-	+

Table 3. Examples of interactions among starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) in cheeses.

Type of interaction	Organisms involved	Compounds/mechanisms involved	Findings	References
Competition	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> and <i>Lactobacillus paracasei</i>	Lactate dehydrogenase (<i>ldh</i>) and alcohol dehydrogenase (<i>adh</i>) genes	<i>ldh</i> gene was upregulated in <i>Lc. lactis</i> at the end of cheese ripening under energy starvation conditions while was downregulated in <i>L. paracasei</i> for a switch to mixed acid fermentation	(Desfossés-Foucault et al., 2014)
		Deoxyribose-phosphate aldolase and phosphofructokinase 1 genes	Genes were upregulated under heat and salt stresses in <i>Lb. paracasei</i>	
Metabolic cooperation	<i>Lc. lactis</i> ssp. and <i>Leuconostoc mesenteroides</i>	Caseinolytic activity	Essential free amino acids and small peptides provided by <i>Lc. lactis</i> substained growth of <i>L. mesenteroides</i>	(Smid & Kleerebezem, 2014)
	<i>Lactobacillus casei</i> and <i>Lc. lactis</i>	Proteolysis	The induction of peptidase genes in <i>Lb. casei</i> followed the initial proteolysis by <i>Lc. lactis</i>	(Desfossés-Foucault et al., 2014)
	Combinations of SLAB <i>Lc. lactis</i> and <i>Lb. paracasei</i>	High peptidolytic activities Cystathionine- β -synthetase gene	Tools to accelerate and diversify proteolysis during cheese ripening The use of methionine and cysteine was allows for the lactococcal gene upregulation	(Courtin et al., 2002) (Desfossés-Foucault et al., 2014)

Table 3. Continue

Type of interaction	Organisms involved	Compounds/mechanisms involved	Findings	References
Metabolic cooperation	Mesophilic SLABand GDH positive NSLAB <i>Lactobacillus helveticus</i> and <i>Lactobacillus rhamnosus</i> PR1019	Free amino acids Starvation	Increased liberation of free amino acids Release of ribonucleosides when the starter undergoes lysis and growth advantage on NSLAB during cheese ripening	(Kieronczyk et al., 2003) (Lazzi et al., 2014)
	<i>Lactobacillus delbrueckii</i> NRRL-B445 and <i>Lb. helveticus</i> NRRLB1937	Lactic acid	Improved production of lactic acid from glucose by the mixed culture of which the first is a good lactate producer and stimulated by the latter	(Lee et al., 2001)
Commensalism	PrtP ⁻ and PrtP ⁺ <i>Lc. Lactis</i> strains	Peptides	PrtP ⁻ strains benefit from the peptides released from milk protein through the action of extracellular proteases produced by PrtP ⁺ strains, which do not seem directly affected	(Hugenholtz et al., 1987)
Stress response	<i>Lc. lactis</i> and <i>Lb. paracasei</i>	<i>clpC</i> , <i>dnaJ</i> , and <i>groES</i> genes	Genes were upregulated in <i>Lb. paracasei</i> during co-culture but were down-regulated during ripening as consequence of decreased viable cells of <i>Lc. lactis</i>	(Desfossés-Foucault et al., 2014)

Table 3. Continue

Type of interaction	Organisms involved	Compounds/mechanisms involved	Findings	References
Proto-cooperation	<i>Streptococcus thermophilus</i> and <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i>	Enzymes	Changes in the level of synthesis of enzymes related to amino acid biosynthesis, carbon and purine-pyrimidine metabolisms, and response regulator RR05	(Herve-Jimenez et al., 2008)
Quorum sensing	<i>Lb. plantarum</i> and lactic acid bacteria or <i>PlnA</i>	Plantaricin A	Induction of proteins involved in ABC transport and peptide uptake	(Calasso et al., 2013)
	<i>Lb. helveticus</i> and NSLAB	Two 2[5H]-furanones	Involvement in the autolysis phenomenon and release of enzymes	(Ndagijimana et al., 2006)
	<i>Lb. plantarum</i> NC8 and gram-positive bacteria	Plantaricin and plantaricin-like peptides	Induction of plantaricin production in <i>Lb. plantarum</i> by plantaricin-like peptides produced by other gram-positive bacteria	(Maldonado et al., 2004)

Figure 1.

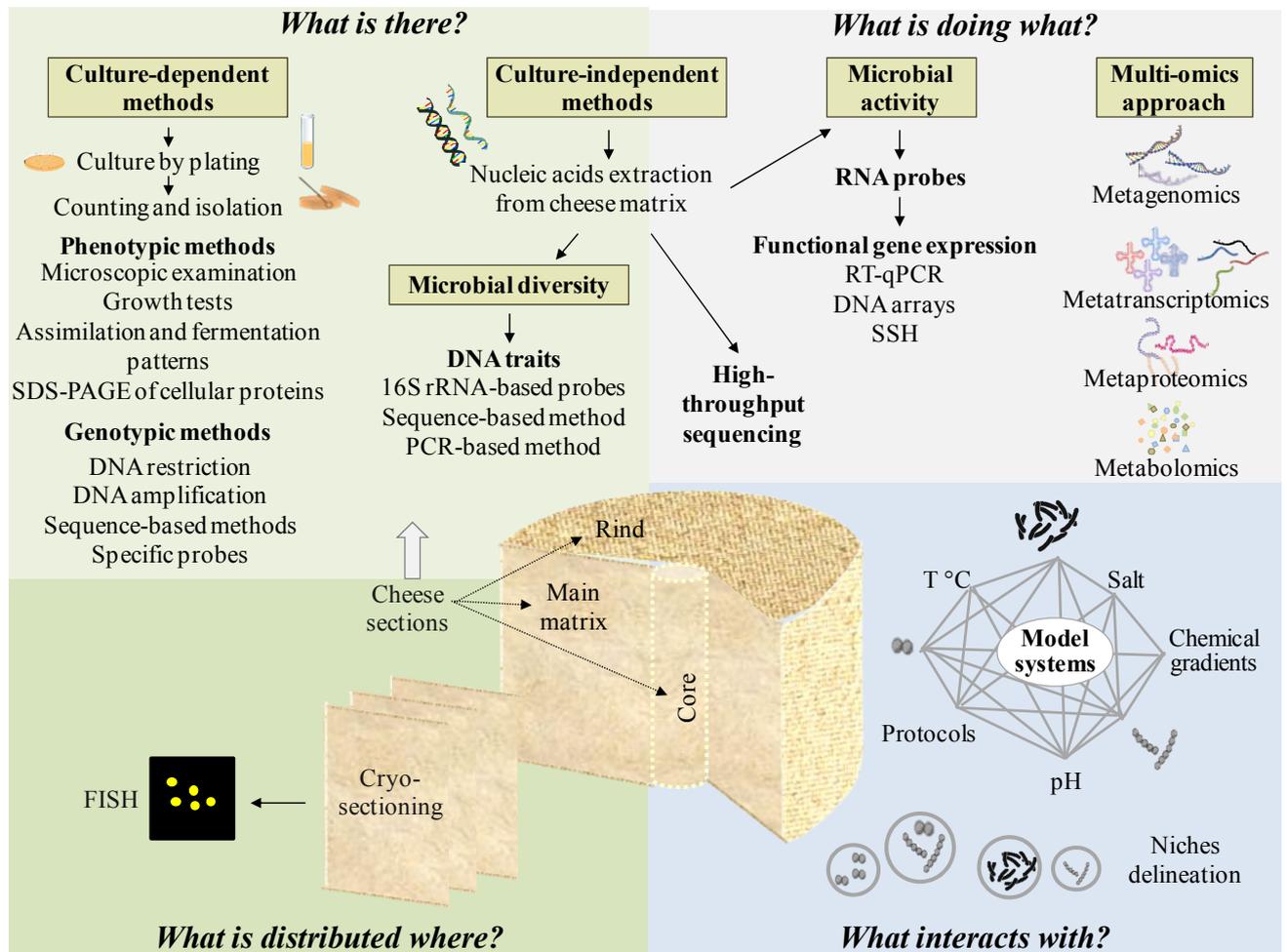


Figure 2.

