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

# Manuscript Details

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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.

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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**

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- 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.

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- 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.

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- 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.
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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 Gobbetti

*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 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.

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

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STRUCTURED ABSTRACT

*Background*

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**Keywords**

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

## 1. Introduction

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

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

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

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

## 2. The methodology approach

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

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

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

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

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

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

## 3. The players

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

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

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

Speaking about players in cheese manufacture, bacteriophages have a fundamental role because of their selective pressure within the bacterial community. Milk, whey and bacteria theme-selves, containing temperate phage, are the primary entry routes for bacteriophages into cheese environments. Overall, numbers from $10^2$ PFU/$m^3$ to $10^8$ PFU/$m^3$ are detectable in the air of cheese manufacturing plants. Efforts to prevent phage infections in dairy plants include factory design, sanitation, ventilation, process changes, modified media for SLAB cultivation, and especially SLAB culture rotation. SLAB from natural cultures are more tolerant to phage infection because their cultivation in an environment heavily contaminated with bacteriophages favors the dominance of resistant biotypes (Marcò, Moineau, & Quiberoni, 2012). Overall, mixed cultures have the highest degree of resistance against bacteriophage infections. Bacteriophages become integral members of the microbial community and may drive the generation of a phenotypic diversity, which enhances performance and robustness. The "kill the winner" hypothesis is an attempt, also for cheese ecosystems, to state that bacteriophages prevent, together with other biotic and abiotic factors, a winner from emerging, thus assembling coexistent and diverse species. Consequently, each bacterial species/biotype shows consecutive cycles of exponential growth, followed by abrupt declines (Xue & Goldenfeld, 2017). If this is true, the level of surviving species is inversely related to modifications in their growth rates but increases with the occurrence and extent of phage-induced collapses. The understanding of phage diversity at cheese processing sites may shed light on population dynamics. Novel emerging bacteriophages have been discovered continuously as in the case of *Str. thermophilus* 987 phages (McDonnell et al., 2016), which show several common genetic features with lactococcal phage (P335 group) and other *Str. thermophilus* phages. This relatedness suggests genetic exchanges, thus resulting in emerging phage group. The usual practice of using mixture of SLAB, as in the

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

## 4. Raw *vs*. pasteurized milk

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

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

Refrigeration modifies the microbial balance in milk. The population shifts from Gram-positive to Gram-negative bacteria. As the most common psychrotrophs, *Pseudomonas* spp. dominate in raw milk, together with *Acinetobacter* ssp. and *Enterobacteriaceae* (e.g., *Hafnia alvei*) (Fricker, Skanseng, Rudi, Stessl, & Ehling-Schulz, 2011). Psychrotrophic bacteria largely grew in milk, increasing ca. 3.0 log cfu/mL during 3 days at 8°C or 7 days at 4°C (Rasolofo, St-Gelais, LaPointe, & Roy, 2010). Small variations in storage temperature and duration led to a different balance of species/biotype. Vat raw milk contains thiocyanate ($SCN^-$), oxidation of which by the lactoperoxidase (LP)-$H_2O_2$ system determines bactericidal or bacteriostatic effects depending on Gram-negative or Gram-positive bacteria (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014).

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

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

## 6. Cheese manufacture

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

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

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

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

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

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

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

## 7. Cheese ripening

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

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

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

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

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

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

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

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

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

## 9. 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, intended as a consortium of SLAB and NSLAB. 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 methodology (e.g., omics techniques and integrated system biology) has 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.

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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** | |
| *Leuconostoc mesenteroides* subsp. *cremoris* | DSS, MSS, NS | LA, D, P, L, AR, C |
| *Lactococcus lactis* subsp. *lactis* ($cit^-$) | DSS, MSS, NS | LA, D, P, L, AR, C, SM |
| *Lc. lactis* subsp. *lactis* ($cit^+$) | DSS, MSS | LA, D, P, L, C |
| *Lactococcus lactis* subsp. *cremoris* | DSS, MSS, NS | LA, D, P, L, AR, C, SM |
| | **Thermophilic species** | |
| *Lactobacillus delbruecki* subsp. *lactis* | DSS, MSS, NS | LA, AR, C, P, PG |
| *Lactobacillus delbruecki* subsp. *bulgaricus* | DSS, MSS, NS | LA, P, L |
| *Lactobacillus delbruecki* subsp. *bulgaricus* | DSS, MSS, NS | LA, P, L |
| *Lactobacillus helveticus* | DSS, MSS, NS | LA, AR, C, P, PG, |
| *Streptococcus thermophilus* | 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, $CO_2$; 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** |
| *Lb. parabuchneri* | Caciocavallo Pugliese, Malga | - | + | - |
| *Lb. diolivorans* | Fontina, Malga | + | - | - |
| *Lb. reuteri* | Kasar | - | + | - |
| *Lb. curvatus* | Caciocavallo Silano, Istrian, Gruyère,Quesalla Arochena, Canestrato Pugliese, Bellie, Dulses, Gouda type, Castelmagno, Pecorino Sardo, Pecorino Marchigiano | + | + | + |
| *Lb. hilgardii* | Malga | + | - | - |
| *Lb. fermentum* | Ragusano, Caciocavallo Silano, Parmigiano Reggiano, Bitto, Bryndza, Bellie, Dulses, Gouda type, Pecorino Sardo, Pecorino Marchigiano | + | + | + |
| *Lb. brevis* | Caciocavallo Pugliese, Malga, Gruyère, Bryndza, Pico, Zlatar, 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** |
| *Lb. coryniformis* | Malga, Kasar, Torta Arochena | + | + | - |
| *Lb. harbinensis* | Parmigiano Reggiano | + | - | - |
| *Lb. rhamnosus* | Caciocavallo Palermitano, Provolone del Monaco, Parmigiano Reggiano, Bitto, Grana Padano, Grana Trentino, Bryndza, Bellie, Dulses, Gouda type, Pecorino Sardo, Pecorino Marchigiano | + | + | + |
| *Lb. casei* | Ragusano, Caciocavallo Pugliese, Fontina, Caciocavallo Palermitano, Kasar, Parmigiano Reggiano, Grana Padano, Bryndza, Pico, Saint-Nectaire, Canestrato Pugliese, Castelmagno, Pecorino Sardo, Pecorino Marchigiano | + | + | + |
| *Lb. paracasei* | 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** |
| *Lb. plantarum* | Ragusano, Caciocavallo Pugliese, Caciocavallo Silano, Fontina, Malga, Kasar, Bitto, Casin, Poro, Zlatar, Darfiyeh, Feta, Quesallia Arachena, Raschera, Bellie, Dulses, Gouda type, Canestrato Pugliese, Genestoso, Torta Arochena, Salers, Castelmagno, Ossau-Iratry, Pecorino di Filiano, Pecorino del Reatino, Alberquillia, Pecorino Sardo, Pecorino Marchigiano, Calenzana | + | + | + |
| *Lb. paraplantarum* | Darfiyeh, Quesallia Arachena | - | - | - |
| *Lb. pentosus* | Pecorino Sardo, Pecorino Marchigiano, Darfiyeh | - | - | - |
| *Lb. fabifermentans* | Darfiyeh | - | - | - |
| *Lb. buchneri* | Feta | - | - | - |
| *Lb. perolens* | Bellie, Dulses, 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 | *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus paracasei* | Lactate dehydrogenase (*ldh*) and alcohol dehydrogenase (*adh*) genes | *ldh* gene was upregulated in *Lc. lactis* at the end of cheese ripening under energy starvation conditions while was downregulated in *L. paracasei* 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 *Lb. paracasei* | |
| Metabolic cooperation | *Lc. lactis* ssp. and *Leuconostoc mesenteroides* | Caseinolytic activity | Essential free amino acids and small peptides provided by *Lc. lactis* substained growth of *L. mesenteroides* | (Smid & Kleerebezem, 2014) |
| | *Lactobacillus casei* and *Lc. lactis* | Proteolysis | The induction of peptidase genes in *Lb. casei* followed the initial proteolysis by *Lc. lactis* | (Desfossés-Foucault et al., 2014) |
| | Combinations of SLAB | High peptidolytic activities | Tools to accelerate and diversify proteolysis during cheese ripening | (Courtin et al., 2002) |
| | *Lc. lactis* and *Lb. paracasei* | Cystathionine-β-synthetase gene | The use of methionine and cysteine was allows for the lactococcal gene upregulation | (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 | Free amino acids | Increased liberation of free amino acids | (Kieronczyk et al., 2003) |
| | *Lactobacillus helveticus* and *Lactobacillus. rhamnosus* PR1019 | Starvation | Release of ribonucleosides when the starter undergoes lysis and growth advantage on NSLAB during cheese ripening | (Lazzi et al., 2014) |
| | *Lactobacillus delbrueckii* NRRL-B445 and *Lb. helveticus* 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 $PrtP^+$*Lc. Lactis* 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 | *Lc. lactis* and *Lb. paracasei* | *clpC*, *dnaJ*, and *groES* genes | Genes were upregulated in *Lb. paracasei* during co-colture but were down-regulated during ripening as consequence of decreased viable cells of *Lc. lactis* | (Desfossés-Foucault et al., 2014) |

**Table 3**. Continue

| Type of interaction | Organisms involved | Compounds/mechanisms involved | Findings | References |
|---|---|---|---|---|
| Proto-cooperation | *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* | 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 | *Lb. plantarum* and lactic acid bacteria or *PlnA* | Plantaricin A | Induction of proteins involved in ABC transport and peptide uptake | (Calasso et al., 2013) |
| | *Lb. helveticus* and NSLAB | Two 2[5H]-furanones | Involvement in the autolysis phenomenon and release of enzymes | (Ndagijimana et al., 2006) |
| | *Lb. plantarum NC8* and gram-positive bacteria | Plantaricin and plantaricin-like peptides | Induction of plantaricin production in *Lb. plantarum* by plantaricin-like peptides produced by other gram-positive bacteria | (Maldonado et al., 2004) |

**Figure 1.**

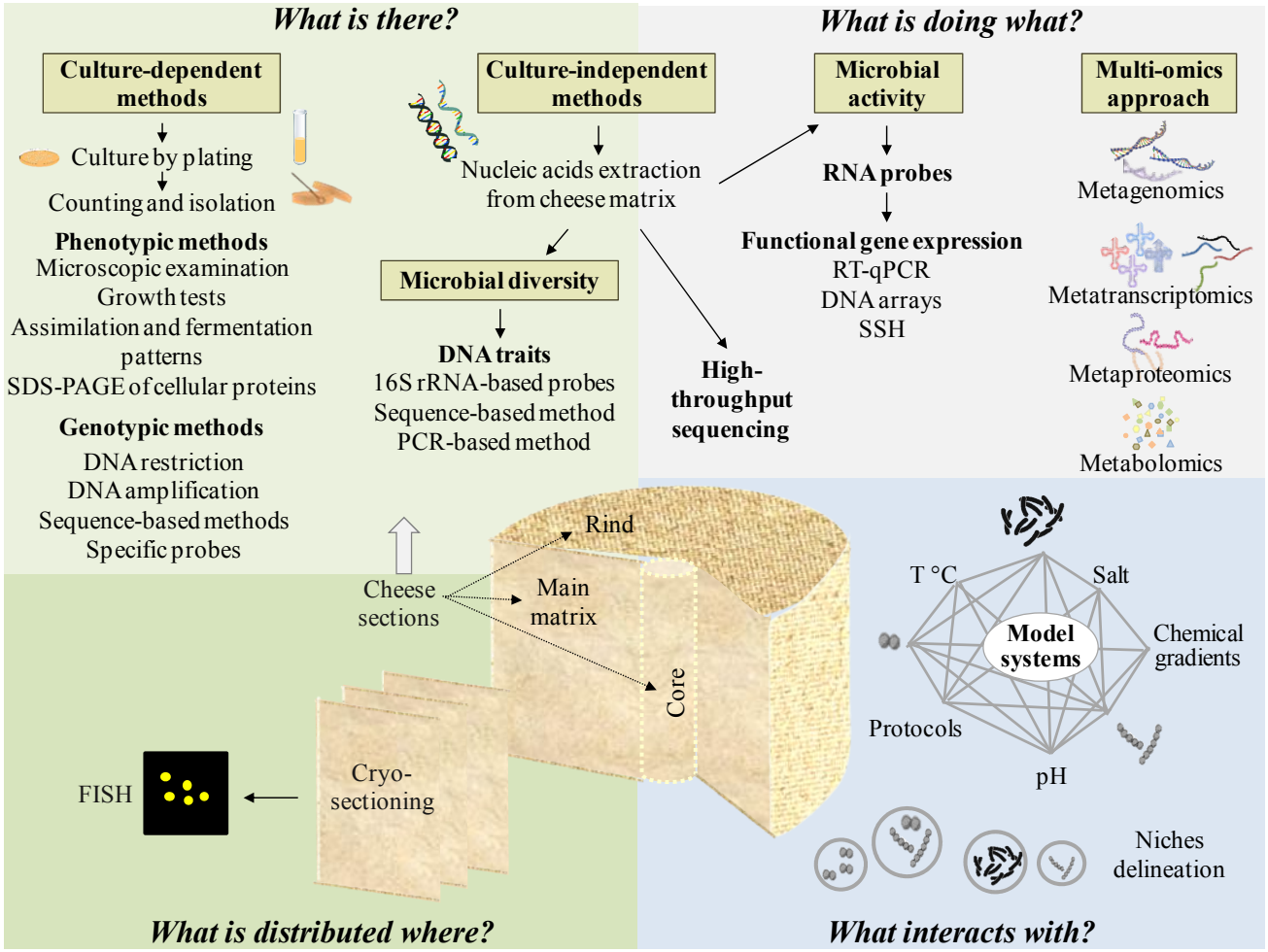
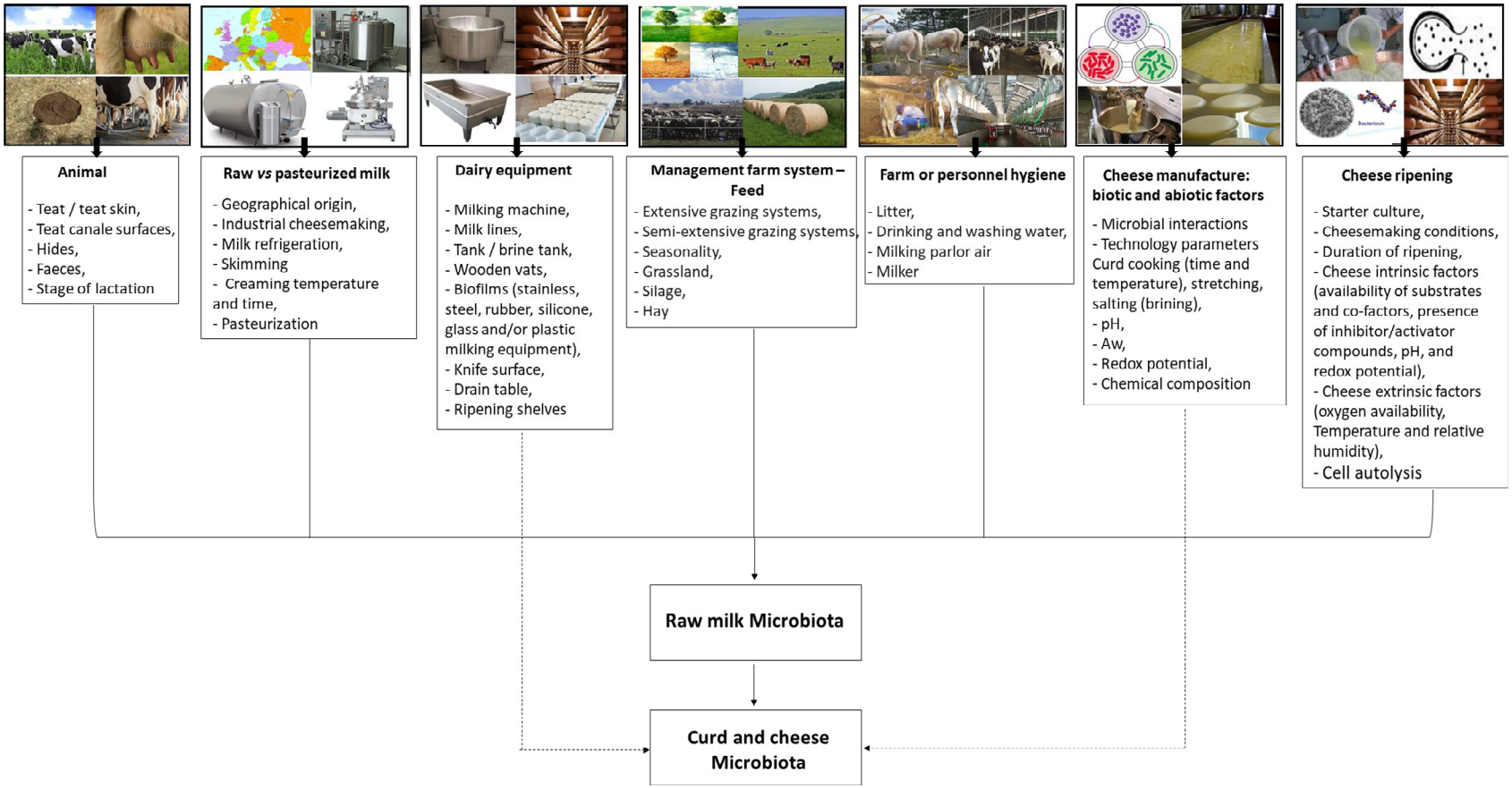

What is there?
What is doing what?
Culture-dependent methods
Culture by plating
Counting and isolation
Phenotypic methods
Microscopic examination
Growth tests
Assimilation and fermentation patterns
SDS-PAGE of cellular proteins
Genotypic methods
DNA restriction
DNA amplification
Sequence-based methods
Specific probes
Culture-independent methods
Nucleic acids extraction from cheese matrix
Microbial diversity
DNA traits
16S rRNA-based probes
Sequence-based method
PCR-based method
High-throughput sequencing
Microbial activity
RNA probes
Functional gene expression
RT-qPCR
DNA arrays
SSH
Multi-omics approach
Metagenomics
Metatranscriptomics
Metaproteomics
Metabolomics
Cheese sections
Rind
Main matrix
Core
Cryo-sectioning
FISH
T °C
Salt
Model systems
Chemical gradients
Protocols
pH
Niches delineation
What is distributed where?
What interacts with?

**Figure 2.**


Animal
- Teat / teat skin,
- Teat canale surfaces,
- Hides,
- Faeces,
- Stage of lactation
Raw vs pasteurized milk
- Geographical origin,
- Industrial cheesemaking,
- Milk refrigeration,
- Skimming
- Creaming temperature and time,
- Pasteurization
Dairy equipment
- Milking machine,
- Milk lines,
- Tank / brine tank,
- Wooden vats,
- Biofilms (stainless, steel, rubber, silicone, glass and/or plastic milking equipment),
- Knife surface,
- Drain table,
- Ripening shelves
Management farm system – Feed
- Extensive grazing systems,
- Semi-extensive grazing systems,
- Seasonality,
- Grassland,
- Silage,
- Hay
Farm or personnel hygiene
- Litter,
- Drinking and washing water,
- Milking parlor air
- Milker
Cheese manufacture: biotic and abiotic factors
- Microbial interactions
- Technology parameters Curd cooking (time and temperature), stretching, salting (brining),
- pH,
- Aw,
- Redox potential,
- Chemical composition
Cheese ripening
- Starter culture,
- Cheesemaking conditions,
- Duration of ripening,
- Cheese intrinsic factors (availability of substrates and co-factors, presence of inhibitor/activator compounds, pH, and redox potential),
- Cheese extrinsic factors (oxygen availability, Temperature and relative humidity),
- Cell autolysis
Raw milk Microbiota
Curd and cheese Microbiota