How new molecular approaches have contributed to shedding light on microbial dynamics in Parmigiano Reggiano cheese

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Abstract

Parmigiano Reggiano (PR) is a long ripened raw milk product designation of origin (PDO) cheese manufactured according to traditional technology in a defined geographical area. This review focuses on the recent developments in the study of PR microbial ecology and dynamics. Cheesemaking process was studied, starting from the raw milk, followed by the natural whey starter (NWS), the curd and finally the cheese at different aging times. Results reported in different studies highlight how distinct bacterial populations are intertwined throughout the cheesemaking, and this connected role is related to microbial composition dynamics and proteolytic profile. The cheese microbiota is shaped by the cheesemaking process, that in spite of small variations among dairies ensures the reproducibility of the final product. The cyclic nature of the alternating microbial population in subsequent production batches and the connection with the territory and farming systems lie at the basis of PR sensory attributes.

Highlights

- Parmigiano Reggiano manufacturing involves a complex interplay of microbial species
- Raw cow's milk and whey starter define the characteristics of microbial community
- The microbial populations are characterised by wide strain-level biodiversity
- Novel approaches have shed light on microbial dynamics during fermentation
- Cheesemaking process shapes microbiota composition

Introduction

Parmigiano Reggiano (PR) is a Product Designation of Origin (PDO) long ripened cheese produced according to a traditional and well-defined technology in a restricted area of the Po river's valley: the provinces of Parma, Reggio Emilia, Modena, Bologna to the left of the Reno River and Mantova to the right of the Po river (single document of the PDO Parmigiano Reggiano, EU No. PDO-IT-02202). PR is made with raw cow's milk and natural whey starter (NWS), a complex undefined culture of lactic acid bacteria (LAB) prepared daily by each dairy by incubating at selected temperatures the whey left from the previous cheesemaking [1,2]. Manufacturing of PR involves several steps (Fig. 1) happening: i) in a copper vat, where milk, NWS and calf rennet are mixed, cooked and left until the curd is formed and extracted, and ii) outside the vat where the curd is molded, salted in brine and ripened for at least 12 months. These phases will lead to the formation of the peculiar organoleptic properties of the cheese [2,3].

The characteristics of PR cheese are influenced by the microbial dynamics occurring along the entire cheesemaking process. The development of LAB from the starter as well as adventitious secondary LAB modify the biochemical features of the cheese matrix, as a consequence of microbial adaptation to the substrate, which defines PR cheese attributes [3,4].

Cheese fermentation is the result of the activities of a group of microorganisms, and often depends on complex microbial mixtures which act in concert to produce the desired characteristics. Therefore, the colonization of cheese by different microorganism may be studied in terms both of ecological strategy and community development [3]. The bacteria involved in the fermentative process of PR cheese, and more in general of raw milk cheeses [5], can be grouped in two categories: starter LAB (SLAB), namely the species deriving from NWS and non-starter LAB (NSLAB), mostly consisting of facultative heterofermentative lactobacilli originating from raw milk or the manufacturing environment. SLAB are responsible for fast acidification of the curd and undergo extensive autolysis within 1 month from the beginning of PR cheesemaking, releasing their intracellular content [2,6]. NSLAB are capable to persist until late ripening steps, contributing mainly to secondary proteolysis [7,8].

This review focuses on the recent advances in the study of PR microbial ecology and dynamics that were made possible applying various molecular approaches. Results from

various authors are presented, tracing the cheesemaking steps from the raw milk to the cheese at different aging times.

A wide toolbox for the study of PR microbial dynamics and diversity

Various molecular approaches were employed to describe the complex interplay of microbial species during PR cheesemaking in the last decades (Tab. 1). Starting from classical plate enumeration, application choice has shifted towards molecular based culture-independent methods, became more popular in the study of food microbiome [4,9,10]. The culture-dependent approach has been extensively applied to the study of PR until the early 2000s, using culture media suitable for the general recovery of LAB, such as De Man, Rogosa, Sharpe medium (MRS), or specifically developed whey-, curd- or cheese- based media able to recovery minority microorganisms or species with demanding nutritional requirements [11–14]. The main limitation of the culture-dependent approach is the underestimation of microbial populations and, as a consequence, of the whole microbial community [4]. Nevertheless, the isolation of microorganisms is a crucial step to define the diversity of the microbiota, by subsequent identification and typing of the isolates, generally by molecular techniques. Identification of LAB isolated from PR can be attained by sequencing of the 16S rRNA gene [15], species-specific PCR on genes with taxonomic potential [16], or applying multiplex PCR (mPCR) [1] or post-PCR analysis such as high-resolution melting (HRM) analysis [17-19]. Microbial ecosystems such as those involved in the fermentation of dairy products are known to harbour a high degree of intraspecific diversity [20-22]. Indeed, in the case of PR, genotyping of bacteria and yeasts isolated at different points of the cheesemaking has revealed the existence of different biotypes among the prevalent species (Tab. 1). These techniques have shown that strains isolated at different production sites, but also within the same processing plant at different ripening time, harbour genotypic variability, that might be responsible for their adaptation to different steps of the cheesemaking.

Complementary approaches to the description of microbial dynamics in PR have involved culture-independent techniques, that are sensitive and capable to give a “picture” of the microbiota, allowing the detection of minority species, microorganisms that are difficult to cultivate or non-culturable. With regard to a previous review on the microbial ecology of PR cheese [4], the existing knowledge needs to be updated with more recent results, in the light of the introduction of high-throughput sequencing (HTS) in food microbiology [9]. Culture-independent techniques can be subdivided in two categories: untargeted and targeted methods (Fig. 2). Untargeted techniques do not require a previous knowledge of the microbial population composition, and are applied to get a comprehensive view of microbial dynamics, often leading to qualitative or semi-quantitative description of the microbiome. Targeted

techniques, such as qPCR and FISH, allow for absolute quantification of one or few selected microbial species. Different steps of PR cheesemaking have been studied by means of these techniques (Table 1). NWS and fresh curd are the most frequently considered sampling points, possibly due to the ease of sample collection and DNA isolation. For these matrices, indeed, untargeted techniques such as Length Heterogeneity (LH) PCR and HTS have been applied, as well as quantitative, targeted approaches. On the contrary, raw milk used in the production of PR was the less investigated substrate, and only two studies are available that report the microbial composition using culture-independent methods [14,23]. Bottari et al. [6] applied HTS to the study of PR from fresh curd to 24 months ripened cheese, and results can be compared with those obtained through DGGE and LH-PCR by other authors [14,24,25]. In the next paragraphs microbial diversity and dynamics during PR cheesemaking are described, considering the steps that are more relevant in defining the structure of the bacterial community.

Raw cow's milk: the prelude of cheese microbiota

Raw cow's milk is known to be characterized by a complex microbial community [26]. The high nutritional value, water content and favourable pH support the growth of many microorganisms, including bacteria of technological relevance such as LAB, among which the species belonging to the genus *Lactobacillus, Lacticaseibacillus*, and *Lactiplantibacillus* are frequently recovered [27-30]. In compliance with a novel taxonomic report on the genus *Lactobacillus*, all the species names and abbreviations reported in this review are in compliance with the most recent nomenclature [28]. Microbial contamination of the raw milk can occur by different routes: the cow, air, feedstuffs, milk handling equipment and the milker, forming a native microbiota that can subsequently develop in the cheese [5]. Besides, it is known that microbial composition of raw milk can be affected by factors such as stage of lactation, seasonal variation, feeding system and diet [30]. As an example, a metabolomics approach is capable to discriminate raw milk for the production of PR, due to the feeding regimen that does not allow for the use of corn silage for cow's feeding [31]. It can be postulated that slight differences in milk composition might affect raw milk microbiome as well. Regarding microbial composition (Fig. 3), studies report a concentration of thermophilic LAB in raw milk for PR production around 2 to 3 Log cfu/mL, while mesophilic LAB can vary among 2 to 5 Log cfu/mL, approximatively [11,13,14,32]. By means of LH-PCR performed on DNA extracted from whole bacterial cells of raw milk, Gatti et al. [14] identified low intensity peaks related to various LAB (i.e: *Lactobacillus delbrueckii* ssp, *Lactobacillus helveticus*, *Lacticaseibacillus* spp., *Enterococcus* spp.). Milani et al. [23] investigated the microbial ecology of raw milk from five different animal husbandries, and found a prevalence of the genera *Lactobacillus*, *Bifidobacterium*, *Corynebacterium*, *Staphylococcus* and *Streptococcus* (Tab. 1).

The production of PR starts with the blending of the half-skimmed raw milk of the night and the whole raw milk of the morning [2]. The overnight creaming step is performed to balance the fat/casein ratio before curdling, but it also leads to changes in the composition of raw milk microbiota, giving a selective advantage to psychrotrophic bacterial groups, including some LAB [33]. This observation is corroborated by a study from CRPA [32], that investigated the cultivable microbiota of 400 samples of PR raw milk, reporting a small increase of mesophilic LAB during the creaming step, starting from a concentration of 3.45 Log cfu/mL in raw milk and reaching 3.81 Log cfu/mL in the vat.

Fugacity versus key role: The case of NWS

Starter culture is an invisible 'ingredient', which blends with autochthonous biota of raw milk to define PR microbiological characteristics. Despite its fugacity, starter culture can be considered one of the main actors in the production of fermented foods [34]. The starter cultures used in PR cheesemaking are exclusively natural, artisanal cultures based on the technique of back-slopping and made by a defined guideline. In detail, the cooked non-acidified whey, obtained after the curd cooking, is incubated overnight at a decreasing temperature ranging from 50 °C to 35 °C, giving rise to NWS [2]. This artisanal technique guarantees a microbiological *liaison* among batches of production in the same dairy farms and at the same time allows, in a broad sense, the preservation and the link to the territory of origin. The selective pressures, such as chemical and physical drivers, that define this ecosystem, lead to the selection of a microbiota widely composed by thermophilic, acidic, and moderately heat-resistant LAB [35]. This particular preparation allows the survival of different biotypes, needful for the NWS ecosystem equilibrium [3,20].

NWS LAB community was extensively described in many studies through culture-dependent techniques (Tab. 1) [11,13,35–39]. Cultivable thermophilic LAB from NWS, ranging from 7 to 10 Log cfu/mL (Fig. 3), showed a certain degree of variation linked to dairy or season conditions, as well as a large variability at strain level rather than species level, showing a high degree of phenotypic and genotypic variability (Table 1) [40]. The coexistence of different biotypes is essential for the functionality of NWS, and at the same time ensures the development/evolution of the ecosystem itself [2]. The use of culture-independent techniques, such as LH-PCR, FISH and HTS turned out to be very useful to identify, track the viability and estimate the microbial dynamics of NWS. All these techniques demonstrated that *L. helveticus* and *L. delbrueckii* ssp. *lactis* are the dominant species recovered in PR NWS, while *Streptococcus thermophilus* and *Limosilactobacillus fermentum* are in sub dominance. Other authors have reported the isolation of *Pediococcus acidilactici*, *Lacticaseibacillus rhamnosus* and the yeast *Kluyveromyces marxianus*, although at variable levels [13,41]. Investigation of microbial dynamics trough LH-PCR revealed major peaks in the NWS corresponding to the starter species, and microscope observation trough FISH confirmed that all round-shaped cells present in NWS were hybridized by *S. thermophilus*-specific probe excluding the presence of enterococci [36]. To overcome the limits of LH-PCR, mostly due to low detection limit (around 10^4–10^5 cfu/mL) and uncertain peak detection, a Multiplex RealTime- PCR (mRT-PCR) was performed to detect both majority and minority species of

thermophilic SLAB, providing a fast and sensitive detection for the species *L. helveticus*, *L. delbrueckii*, *S. thermophilus* and *L. fermentum* [1].

HTS has taken over as a sensitive technique to describe relative abundances of SLAB community [22,42]. confirming the data previously obtained through classical microbiological and revealing the presence of unidentified *Lactobacillus* spp. at less than 3% [22]. This unidentified microorganisms' group could be represented by mesophilic microorganisms, belonging to genus *Lacticaseibacillus*. Indeed, due to the cyclical nature and particular preparation of NWS, species usually present in raw milk are occasionally isolated from NWS, thanks to their capability to resist under low pH, reached overnight by NWS [11,13].

Through untargeted culture-independent techniques, a general frame of NWS microbial community could be depicted, but to describe fluctuations, dynamics and different microbial behaviours under technological and selective pressures typical of PR cheesemaking a quantitative approach is necessary. For this reason, in a recent study quantitative PCR (qPCR) was applied to obtain a complete overview of NWS microbial ecosystem. *L. helveticus* and *L. delbrueckii* ssp. *lactis* were the dominant species detected in PR NWS, reaching values of 6.41 and 6.98 Log copy n/mL, respectively. On the other hand, *L. fermentum* and *S. thermophilus* were found in low percentages reaching values of 3.33 and 5.55 Log copy n/mL, respectively [42].

Quantitative analysis revealed that the equilibrium established among the main starter species, *L. helveticus* and *L. delbrueckii* ssp. *lactis,* changes shortly after NWS is added to the vat milk, when the cooking step occurs. *L. helveticus* decreases slightly, while *L. delbrueckii* ssp. *lactis* adapts faster and increases in number. During overnight incubation, whey acidity increases, favouring *L. helveticus* growth and re-establishing the original and the consequent stability of NWS during production cycles.

Curd acidification: technological pressure drives the microbial dynamics

The fermentation process begins after the addition of NWS into the vat milk. SLAB species quickly adapt to the vat environment [42], but their development and growth occurs mainly during acidification and after the curd extraction [2,4]. After milk stirring and coagulation, a uniform but at the same time stochastic distribution and immobilization of microbes takes

place in the curd, leading to the creation of different microscopic ecological niches, also affected to diverse fluctuations over time and space [43,44].

Curd acidification is the result of microbial and chemical modification of the milk that leads to a correct whey drainage. Curd microbiota is mainly characterized by thermophilic LAB, primarily resulting from NWS. The conversion of lactose into lactic acid and the decrease of pH, are undoubtedly the first biochemical changes that take place within 24h from cheese molding, ensuring the stability of the product.

During this step, different environmental changes occurs in the matrix and drive the microbial ecology within the curd mass. Despite a possible underestimation of viable cells number, plate counts show that during the molding step thermophilic LAB remain stable between 6 and 8 Log cfu/g [38]. On the other hand, this is the first manufacturing step where mesophilic LAB start to increase, reaching values up to 5 Log cfu/g (Fig. 3), and a wide variability in term of cultivable microbial species is observed (Tab. 1). Culture-independent approach, like LH-PCR and metagenomics, revealed that *L. delbrueckii* and *L. helveticus* are the main players during acidification and curd fermentation, while *S. thermophilus* and *L. fermentum* represent a minority [6,14,22,23]. *S. thermophilus* species was detected at very low concentration, proving to have limited contribution to acidification [22,23]. During molding, the convective cooling of the cheese curd establishes a thermal gradient which affects the microbiota of the fresh curd [45]. Together with decreasing pH, these parameters represent a selective pressure that shape the microbial community favouring the dominance of the species from the starter.

Next to the species discussed so far, isolates belonging to the species *L. rhamnosus*, *Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *P. acidilactici*, *Limosilactobacillus reuteri* and *Kocuria cristinae* were identified in fresh curd [13,14,46].

Metagenomic analysis confirmed the presence of these species, revealing also a cluster of microorganisms identified as former *Lactobacillus* spp. [22,23]. According to novel taxonomy [28], probably these sequences can be attributed to NSLAB species, such as genus *Lacticaseibacillus*, that do not contribute to acidification in the early stages of PR cheesemaking, but acquire a key role during cheese ripening [2,7]. Other genera, like *Acinetobacter* spp., *Propionibacterium* spp., *Bifidobacterium*, *Bacteroides* and members of *Lachnospiraceae* family were also found at very low relative abundances, below 1% [23].

Curd brining: a technological step driving microbial dynamics

A crucial technological parameter that influences the cheese microbiota is represented by the brining step. Salt acts as an abiotic stressor on the microbial community causing the decrease of thermophilic SLAB in the matrix, until a sizable fraction of the starter cells undergoes autolysis. Few data are available of microbial modifications occurring during submerged brining, but Coppola et al. [11] reported a decrease of about 0.5 Log cfu/g of thermophilic LAB, and a simultaneous increase of about 1 order of magnitude of mesophilic species (Fig. 3).

After 16 to 24 days of brining, culture-dependent approaches showed a significant decrease of SLAB concentration, at about 5 to 7 Log cfu/g, probably due to the depletion of lactose and autolytic activities (Fig. 3) [6,37]. On the other hand, the concentration of mesophilic LAB continues to increase, as shown by community profiling and counts around 5 to 6 Log cfu/g (Fig. 3) [6,37].

Cheese ripening: where slow microbiological changes occur

According to the existing literature, after the brining step, the biodiversity of PR microbiota slightly decreases (1-month old samples), increasing thereafter in 2-month-old samples [6]. This trend is consistent with microbial dynamics observed by culturing methods, showing the growth of NSLAB during the first months of ripening [6,11,13,14,37]. The dominance of SLAB, namely the species *L. delbrueckii* ssp. *lactis* and *L. helveticus* starts to diminish over the development of the NSLAB, which use alternative substrates to grow, such as the products released from SLAB autolysis, becoming dominant in the first ripening stages (Fig. 3) [6,14,47]. The species most frequently isolated from PR after brining belong to the genus *Lacticaseibacillus*, in particular *L. rhamnosus*, followed by *Lacticaseibacillus paracasei* and *L. casei* [13,48]. Application of various fingerprinting techniques allowed to describe the diversity existing among the isolates from the *Lacticaseibacillus* group. Isolates from the species *L. rhamnosus* and *L. paracasei* are frequently recovered, with some being present only in the early curd cheesemaking stages, and others persisting throughout the ripening process. A reduction in the number of biotypes of these species was observed after 14 months of ripening, probably due to reduction of bacteria viability or cultivability [46,48]. This reduction in terms of species richness and diversity is strongly supported by results obtained on the whole and lysed fraction of total bacterial DNA by means of LH-PCR, that showed an increase of the species *L. helveticus* and *L. delbrueckii* ssp. *lactis* in the lysed cells fraction DNA after the brining step. On the other end, also the lysis of *Lacticaseibacillus* has been

observerd from 6th month of ripening, but to a lesser extent [6,14]. Another species that is frequently associated with ripened cheese is *L. fermentum*, that is detected in the whole cell fraction of PR microbiota, and can reach high relative abundance during ripening, being correlated with intermediate casein proteolysis products [6]. Other species that are frequently detected by HTS at later ripening steps are *Lc. lactis*, *Lactobacillus crispatus* and *S. thermophilus*, but no data are available on the extent of autolysis [6,11,25]. Transformation of the curd into cheese is therefore an essentially enzymatic process mainly due to the degradation of the major components of the curd into simpler substances. Viable microorganisms, from the growth phase until death, play a central role in promoting both structural and organoleptic changes in the curd and cheese paste. After cell lysis also their released cytoplasmic enzymes could be involved in different biochemical modification.

Bottari et al. [6] have described that there is a strong relationship between the microbial dynamics observed during PR ripening and the development of the peculiar proteolytic profile of mature PR. Evolution of the oligopeptide fraction in PR cheese during ripening is characterised by the degradation of casein-derived peptides into oligopeptides, together with the accumulation of non-proteolytic aminoacyl derivatives (NPADS) [49,50]. The evolution of the characteristic sensory profile of PR during its cheesemaking depends on a delicate balance between the enzymatic production and degradation of substrates and the development of the cheese microbiota. Yet, the entire PR manufacturing process, but mostly the long ripening time, are capable to counteract small differences in terms of microbial composition [6]. Indeed, the technological interventions occurring during cheesemaking shape the dynamics of the cheese ecosystem leading to a final product with a distinctive sensory profile.

Conclusions

A growing number of studies has investigated the role of the microbiota in defining the properties of PR cheese, a world-wide appreciated PDO product. Food microbial ecosystems such as that of PR can be described as a continuous evolving microbial network able to adapt to technological process and seasonal variability thanks to its complex community. Overall, the existing literature suggests that there is a certain degree of variability in the composition of microbial community among different dairy farms, at least in the early stages of PR manufacturing. Despite the flux of minority species, the presence and alternation of core microbiome species during cheesemaking ensures a successful fermentation process.

We conclude the review by outlining open questions on the technological role of cultivable *versus* non-cultivable microorganisms. Also, it is noteworthy to highlight that limited data are available regarding the microbiota of raw cow's milk for PR production and during submerged cheese brining. Future research efforts should combine comprehensive sampling schemes with available high-throughput technologies to fill the existing gap in knowledge.

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   In this paper the authors describe how microbiota of PR contributes significantly to the final characteristics of cheeses due to the growth and interaction between cheese microorganisms during processing and ripening. The cheese making process and time of ripening shape this complex ecosystem, determining the diversity in the final peptide and amino acid composition of the product. The study of microbial and peptide profiles of 6 dairies highlighted the presence of differences between samples coming from different manufacturers, but ripening time proved to be the main factor determining cheese composition.

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This review analyzes the microbial community of milk from various hosts, including humans, and tries to define a shared microbiota definition. By analysing the microbial composition of milk in humans and ruminants a core milk microbiota is postulated.

This core microbiota is affected in its composition by various parameters, such as host factors like health status or genetics, or environmental factors like geographical influence and diet. The review reports as well a series of interesting questions on viability, origin, and factors driving the composition of milk microbiota, and the role on infant's and mother's health.

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The authors have evaluated the taxonomy of *Lactobacillaceae* and *Leuconostocaceae* on the basis of whole genome sequences. In particular, a polyphasic approach based on various phylogeneti parameters led to the reclassification of genus *Lactobacillus* into 25 genera. This reclassification reflects the phylogenetic position of the microorganisms, and groups lactobacilli into robust clades with shared ecological and metabolic properties. It is necessary that future publications regarding lactobacilli update the nomenclature of the strains according to this novel classification.

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The authors have studied the microbial composition and dynamics of 91 whey samples from the PR manufacturing process. A combined approach, making use of qPCR and HTS , was chosen to study two different lines of production, one Conventional and one Organic, over a 10 weeks period. The results highlighted that PR NWS is a dynamic microbial community, able to adapt to the different technological parameters of the manufacturing, while retaining a high level of resilience of the main thermophilic LAB species. Combination of qualitative and quantitative results showed the different adaptive features of the main thermophilic species of NWS, which are subjected to a cyclical production process based on back-slopping. Regardless of its initial different composition, NWS resulted to be shaped by technological treatment.

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Figures captions

Figure 1: Schematic representation of the PR cheesemaking process. The figure is divided in two blocks: vat operations, that concern initial milk transformation phases, while ripening process regards curd maturation until the obtaining of mature cheese.

Figure 2: Combination of approaches used to describe microbial dynamics and diversity of PR cheese.

Figure 3 : Microbial evolution of Mesophilic and Thermophilic LAB during PR cheesemaking. Microbial counts were performed in MRS culture media, except for mesophilc counts from Bottari et al., 2020, done trough cheese agar media (CAM) at 37°C. Data from: Coppola et al., 2000; Gatti et al., 2008; Neviani et al., 2009; CRPA, 2011; Gatti et al., 2003; De Dea Lindner et al., 2008; Bottari et al., 2010; Santarelli et al., 2013 b; Tosi et al., 2006; Bottari et al. 2020.

Table

Table 1: Principal microbial genera and species detected along PR manufacturing steps using culture-dependent and culture independent methods. **Bacterial names** in bold and double underlined were detected both by culture dependent and independent methods; **bacterial names** in bold were identified by at least two culture independent methods, underlined bacterial names not in bold were identified only by HTS, *bacterial names* which are just in italics were identified only by means of one method, other than HTS.

		Culture-dependent		Culture-Independent	
Substrate	**Microrganisms**	**Plate count**	**Genotyping**	**Untargeted**	**Targeted**
Raw Milk	***Lactocaseibacillus paracasei***	Gatti et al, 2008	Gatti et al, 2008 (RAPD)	Gatti et al, 2008 (LH-PCR)	Milani et al., 2019 (PCR)
	Latilactobacillus curvatus	Neviani et al., 2009		Milani et al., 2019 (HTS)	
	Lactobacillus helveticus	CRPA, 2011			
	Lactobacillus delbrueckii sp.	Coppola et al., 2000			
	Levilactobacillus brevis				
	Lentilactobacillus kefiri				
	Limosilactobacillus fermentum				
	Lactocaseibacillus rhamnosus				
	Kocuria kristinae				
	Alistipes				
	Bacteroides				
	Streptococcus				
	Actinobacteria				
	Lactobacillus spp.				
	Bifidobacterium				
	Corynebacterium				
	Clostridiaceae				
	Lachnospiraceae				

	Peptostreptococcaceae *Ruminococcaceae* *Lactoplantibacillus plantarum* *Enterococcus* spp.				
NWS	***Lactobacillus helveticus*** ***Lactobacillus delbrueckii* ssp. *lactis*** ***Limosilactobacillus fermentum*** ***Pediococcus acidilactici*** ***Lactocaseibacillus rhamnosus*** ***Kluyveromyces marxianus*** ***Streptococcus thermophilus*** *Lactobacillus spp.*	Gatti et al., 2008 Coppola et al., 2000 Gatti et al., 2003 De Dea Lindner et al., 2008 Bottari et al., 2010 Santarelli et al., 2013 b Tosi et al., 2006 Cocconcelli et al., 1997	Giraffa et al., 2003 (RFLP) Gatti et al., 2003 (RAPD, RFLP) Gatti et al., 2004 (RFLP) Giraffa et al., 2004 (RAPD, PFGE) Gatti et al., 2008 (RAPD) Lombardi et al., 2002 (RAPD) Coloretti et al., 2017 (RFLP, RAPD) Cocconcelli et al., 1997 (RAPD)	Gatti et al, 2008 (LH-PCR) Bottari et al., 2010 (LH-PCR) Santarelli et al., 2013 (LH-PCR) De Filippis et al., 2014 (HTS) Bertani et al., 2019 (HTS)	Bottari et al., 2010 (FISH) Santarelli et al., 2013 (FISH) Bertani et al., 2019 (qPCR) Bottari et al., 2013 (mPCR)
Curd (0 - 48 h)	***Lactobacillus delbrueckii ssp. lactis*** ***Lactobacillus helveticus*** ***Lactocaseibacillus casei*** ***Lactiplantibacillus plantarum*** ***Limosilactobacillus fermentum*** ***Lactocaseibacillus rhamnosus*** ***Limosilactobacillus reuteri*** ***Levilactobacillus brevis*** ***Lactocaseibacillus paracasei*** ***Kocuria kristinae*** ***Pediococcus acidilactici*** ***Bifidobacterium spp.*** *Bifidobacterium longum*	Gatti et al., 2008 Coppola et al., 2000 Santarelli et al., 2013 Bottari et al., 2020 Neviani et al., 2009 De Dea Lindner et al., 2008	Bove et al., 2011 (RAPD, REP) Gatti et al., 2008 (RAPD)	Gatti et al, 2008 (LH-PCR) Bottari et al., 2020 (LH-PCR, HTS) Santarelli et al., 2013 (LH-PCR) De Filippis et al., 2014 (HTS) Milani et al., 2019 (HTS)	Santarelli et al., 2013 (FISH)

	Escherichia *Acinetobacter* *Lactococcus lactis* *Lactobacillus spp.* *Streptococcus thermophilus* *Streptococcus parauberis* *Bacteroides* *Lachnospiraceae* *Propionibacterium* *Lactobacillus crispatus* *Streptococcus suis*			
Brining	***Levilactobacillus brevis*** ***Lactocaseibacillus paracasei*** ***Lactobacillus helveticus*** ***Lactobacillus delbrueckii ssp.***	Coppola et al., 2000		
Cheese post-brining (1-24 mo.)	***Lactocaseibacillus casei*** ***Lactocaseibacillus rhamnosus*** ***Limosilactobacillus fermentum*** ***Pediococcus acidilactici*** ***Levilactobacillus brevis*** ***Lactocaseibacillus paracasei*** ***Lactobacillus helveticus*** ***Lactobacillus delbrueckii ssp. lactis*** ***Lactocaseibacillus casei*** ***Lentilactobacillus buchneri*** *Schleiferilactobacillus harbiniensis*	Bottari et al. 2020 Gatti et al., 2008 Coppola et al., 2000 Neviani et al., 2009 De Dea Lindner et al., 2008	Bove et al., 2011 (RAPD, REP) Succi et al., 2005 (RAPD) Solieri et al., 2012 (16S-ARDRA, REP) Gatti et al., 2008 (RAPD) Gala et al., 2008 (RFLP, DGGE)	Bottari et al., 2020 (LH-PCR, HTS) Gatti et al, 2008 (LH-PCR) Ercolini et al., 2002 (DGGE) Gala et al., 2008 (DGGE)

	Lentilactobacillus kefiri *Lactococcus lactis* *Streptococcus spp.* *Streptococcus suis* *Streptococcus thermophilus* *Lentilactobacillus parabuchneri*		

Figure 1: Schematic representation of the PR cheesemaking process. The figure is divided in two blocks: vat operations, that concern initial milk transformation phases, while ripening process regards curd maturation until the obtaining of mature cheese.

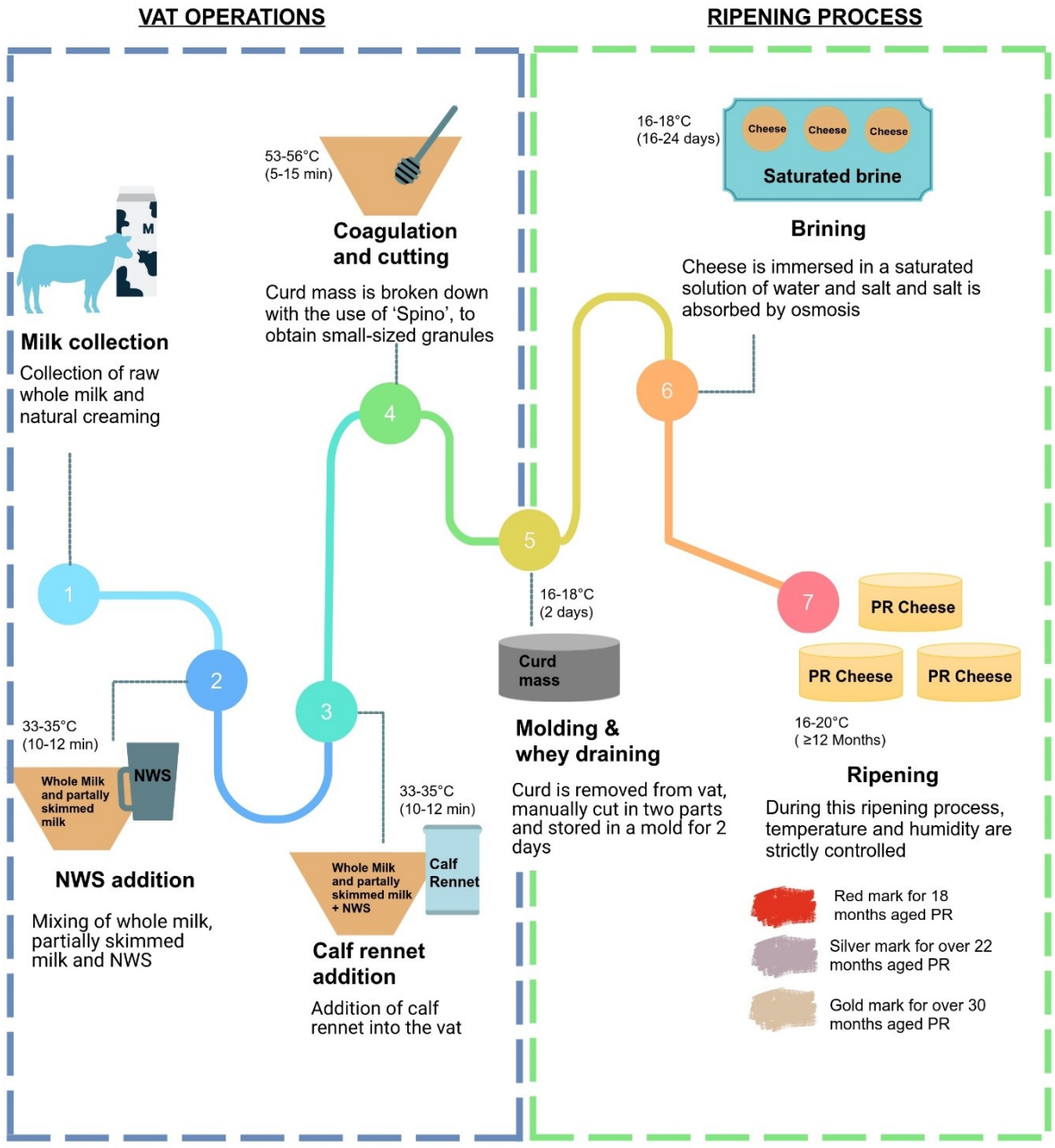

Figure 2: Combination of approaches used to describe microbial dynamics and diversity of PR cheese. VBNC: Viable But Not Culturable.

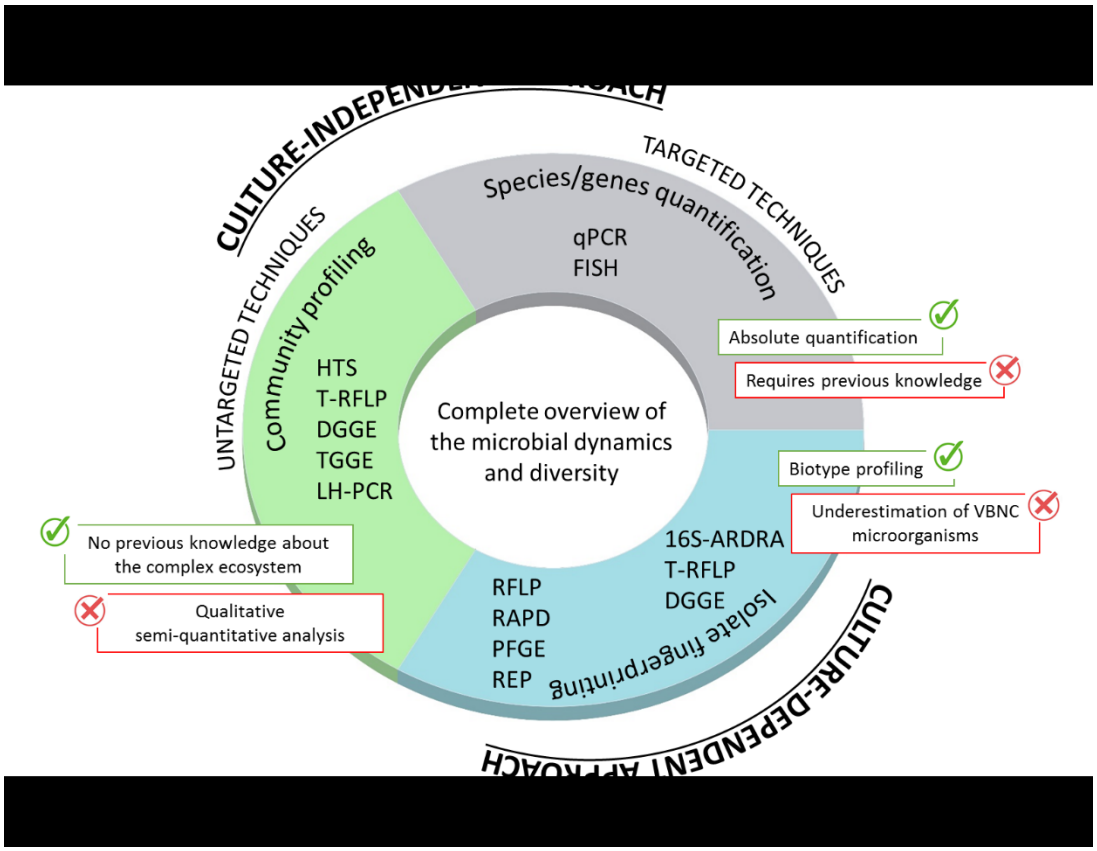

Figure 3 : Microbial evolution of Mesophilic and Thermophilic LAB during PR cheesemaking. Microbial counts were performed in MRS culture media, except for mesophilc counts from Bottari et al., 2020, done trough cheese agar media (CAM) at 37°C. Mo. : months; h: hours. Data from: Coppola et al., 2000; Gatti et al., 2008; Neviani et al., 2009; CRPA, 2011; Gatti et al., 2003; De Dea Lindner et al., 2008; Bottari et al., 2010; Santarelli et al., 2013 b; Tosi et al., 2006; Bottari et al. 2020.