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

Parmigiano Reggiano (PR) is a long ripened raw milk product designation of origin (PDO) 18 cheese manufactured according to traditional technology in a defined geographical area. This 19 review focuses on the recent developments in the study of PR microbial ecology and 20 dynamics. Cheesemaking process was studied, starting from the raw milk, followed by the 21 22 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 23 throughout the cheesemaking, and this connected role is related to microbial composition 24 dynamics and proteolytic profile. The cheese microbiota is shaped by the cheesemaking 25 process, that in spite of small variations among dairies ensures the reproducibility of the final 26 product. The cyclic nature of the alternating microbial population in subsequent production 27 batches and the connection with the territory and farming systems lie at the basis of PR 28 29 sensory attributes.

31 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

39 Introduction

Parmigiano Reggiano (PR) is a Product Designation of Origin (PDO) long ripened cheese 40 produced according to a traditional and well-defined technology in a restricted area of the Po 41 river's valley: the provinces of Parma, Reggio Emilia, Modena, Bologna to the left of the 42 Reno River and Mantova to the right of the Po river (single document of the PDO Parmigiano 43 Reggiano, EU No. PDO-IT-02202). PR is made with raw cow's milk and natural whey starter 44 (NWS), a complex undefined culture of lactic acid bacteria (LAB) prepared daily by each 45 dairy by incubating at selected temperatures the whey left from the previous cheesemaking 46 [1,2]. Manufacturing of PR involves several steps (Fig. 1) happening: i) in a copper vat, 47 48 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 49 least 12 months. These phases will lead to the formation of the peculiar organoleptic 50 51 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 57 depends on complex microbial mixtures which act in concert to produce the desired 58 characteristics. Therefore, the colonization of cheese by different microorganism may be 59 studied in terms both of ecological strategy and community development [3]. The bacteria 60 involved in the fermentative process of PR cheese, and more in general of raw milk cheeses 61 62 [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 63 64 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 65 from the beginning of PR cheesemaking, releasing their intracellular content [2,6]. NSLAB 66 are capable to persist until late ripening steps, contributing mainly to secondary proteolysis 67 68 [7,8].

69 This review focuses on the recent advances in the study of PR microbial ecology and 70 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
- 72 at different aging times.

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

Various molecular approaches were employed to describe the complex interplay of microbial 75 species during PR cheesemaking in the last decades (Tab. 1). Starting from classical plate 76 enumeration, application choice has shifted towards molecular based culture-independent 77 methods, became more popular in the study of food microbiome [4,9,10]. The culture-78 dependent approach has been extensively applied to the study of PR until the early 2000s, 79 80 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 81 to recovery minority microorganisms or species with demanding nutritional requirements 82 83 [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]. 84 Nevertheless, the isolation of microorganisms is a crucial step to define the diversity of the 85 microbiota, by subsequent identification and typing of the isolates, generally by molecular 86 techniques. Identification of LAB isolated from PR can be attained by sequencing of the 16S 87 88 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) 89 90 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 91 92 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 93 techniques have shown that strains isolated at different production sites, but also within the 94 same processing plant at different ripening time, harbour genotypic variability, that might be 95 responsible for their adaptation to different steps of the cheesemaking. 96

97 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 98 99 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 100 101 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-102 103 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 104 population composition, and are applied to get a comprehensive view of microbial dynamics, 105 often leading to qualitative or semi-quantitative description of the microbiome. Targeted 106

107 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 108 techniques (Table 1). NWS and fresh curd are the most frequently considered sampling 109 points, possibly due to the ease of sample collection and DNA isolation. For these matrices, 110 indeed, untargeted techniques such as Length Heterogeneity (LH) PCR and HTS have been 111 applied, as well as quantitative, targeted approaches. On the contrary, raw milk used in the 112 production of PR was the less investigated substrate, and only two studies are available that 113 report the microbial composition using culture-independent methods [14,23]. Bottari et al. [6] 114 applied HTS to the study of PR from fresh curd to 24 months ripened cheese, and results can 115 be compared with those obtained through DGGE and LH-PCR by other authors [14,24,25]. In 116 the next paragraphs microbial diversity and dynamics during PR cheesemaking are described, 117 considering the steps that are more relevant in defining the structure of the bacterial 118 community. 119

121

122 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 123 high nutritional value, water content and favourable pH support the growth of many 124 microorganisms, including bacteria of technological relevance such as LAB, among which 125 the species belonging to the genus Lactobacillus, Lacticaseibacillus, and Lactiplantibacillus 126 are frequently recovered [27-30]. In compliance with a novel taxonomic report on the genus 127 Lactobacillus, all the species names and abbreviations reported in this review are in 128 129 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 130 131 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 132 133 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 134 135 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 136 well. Regarding microbial composition (Fig. 3), studies report a concentration of 137 thermophilic LAB in raw milk for PR production around 2 to 3 Log cfu/mL, while 138 mesophilic LAB can vary among 2 to 5 Log cfu/mL, approximatively [11,13,14,32]. By 139 means of LH-PCR performed on DNA extracted from whole bacterial cells of raw milk, Gatti 140 et al. [14] identified low intensity peaks related to various LAB (i.e: Lactobacillus 141 delbrueckii ssp, Lactobacillus helveticus, Lacticaseibacillus spp., Enterococcus spp.). Milani 142 et al. [23] investigated the microbial ecology of raw milk from five different animal 143 husbandries, and found a prevalence of the genera Lactobacillus, Bifidobacterium, 144 Corvnebacterium, Staphylococcus and Streptococcus (Tab. 1). 145

146 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 147 the fat/casein ratio before curdling, but it also leads to changes in the composition of raw 148 milk microbiota, giving a selective advantage to psychrotrophic bacterial groups, including 149 150 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 151 152 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. 153

154 Fugacity versus key role: The case of NWS

Starter culture is an invisible 'ingredient', which blends with autochthonous biota of raw milk 155 to define PR microbiological characteristics. Despite its fugacity, starter culture can be 156 considered one of the main actors in the production of fermented foods [34]. The starter 157 cultures used in PR cheesemaking are exclusively natural, artisanal cultures based on the 158 technique of back-slopping and made by a defined guideline. In detail, the cooked non-159 acidified whey, obtained after the curd cooking, is incubated overnight at a decreasing 160 temperature ranging from 50 °C to 35 °C, giving rise to NWS [2]. This artisanal technique 161 guarantees a microbiological *liaison* among batches of production in the same dairy farms 162 and at the same time allows, in a broad sense, the preservation and the link to the territory of 163 origin. The selective pressures, such as chemical and physical drivers, that define this 164 ecosystem, lead to the selection of a microbiota widely composed by thermophilic, acidic, 165 and moderately heat-resistant LAB [35]. This particular preparation allows the survival of 166 167 different biotypes, needful for the NWS ecosystem equilibrium [3,20].

NWS LAB community was extensively described in many studies through culture-dependent 168 techniques (Tab. 1) [11,13,35–39]. Cultivable thermophilic LAB from NWS, ranging from 7 169 to 10 Log cfu/mL (Fig. 3), showed a certain degree of variation linked to dairy or season 170 conditions, as well as a large variability at strain level rather than species level, showing a 171 high degree of phenotypic and genotypic variability (Table 1) [40]. The coexistence of 172 different biotypes is essential for the functionality of NWS, and at the same time ensures the 173 development/evolution of the ecosystem itself [2]. The use of culture-independent techniques, 174 such as LH-PCR, FISH and HTS turned out to be very useful to identify, track the viability 175 and estimate the microbial dynamics of NWS. All these techniques demonstrated that L. 176 helveticus and L. delbrueckii ssp. lactis are the dominant species recovered in PR NWS, 177 while Streptococcus thermophilus and Limosilactobacillus fermentum are in sub dominance. 178 Other authors have reported the isolation of Pediococcus acidilactici, Lacticaseibacillus 179 rhamnosus and the yeast Kluyveromyces marxianus, although at variable levels [13,41]. 180 Investigation of microbial dynamics trough LH-PCR revealed major peaks in the NWS 181 corresponding to the starter species, and microscope observation trough FISH confirmed that 182 all round-shaped cells present in NWS were hybridized by S. thermophilus-specific probe 183 184 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 185 RealTime- PCR (mRT-PCR) was performed to detect both majority and minority species of 186

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 189 community [22,42]. confirming the data previously obtained through classical 190 microbiological and revealing the presence of unidentified *Lactobacillus* spp. at less than 3% 191 [22]. This unidentified microorganisms' group could be represented by mesophilic 192 microorganisms, belonging to genus Lacticaseibacillus. Indeed, due to the cyclical nature and 193 particular preparation of NWS, species usually present in raw milk are occasionally isolated 194 from NWS, thanks to their capability to resist under low pH, reached overnight by NWS 195 196 [11,13].

Through untargeted culture-independent techniques, a general frame of NWS microbial 197 198 community could be depicted, but to describe fluctuations, dynamics and different microbial behaviours under technological and selective pressures typical of PR cheesemaking a 199 quantitative approach is necessary. For this reason, in a recent study quantitative PCR 200 (qPCR) was applied to obtain a complete overview of NWS microbial ecosystem. L. 201 helveticus and L. delbrueckii ssp. lactis were the dominant species detected in PR NWS, 202 reaching values of 6.41 and 6.98 Log copy n/mL, respectively. On the other hand, L. 203 fermentum and S. thermophilus were found in low percentages reaching values of 3.33 and 204 205 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.

212

213 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, alsoaffected 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.

- 225 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, 226 227 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 228 229 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 230 LH-PCR and metagenomics, revealed that L. delbrueckii and L. helveticus are the main 231 players during acidification and curd fermentation, while S. thermophilus and L. fermentum 232 represent a minority [6,14,22,23]. S. thermophilus species was detected at very low 233 concentration, proving to have limited contribution to acidification [22,23]. During molding, 234 the convective cooling of the cheese curd establishes a thermal gradient which affects the 235 microbiota of the fresh curd [45]. Together with decreasing pH, these parameters represent a 236 selective pressure that shape the microbial community favouring the dominance of the 237 species from the starter. 238
- 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].
- 249 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].

262 Cheese ripening: where slow microbiological changes occur

According to the existing literature, after the brining step, the biodiversity of PR microbiota 263 slightly decreases (1-month old samples), increasing thereafter in 2-month-old samples [6]. 264 This trend is consistent with microbial dynamics observed by culturing methods, showing the 265 growth of NSLAB during the first months of ripening [6,11,13,14,37]. The dominance of 266 SLAB, namely the species L. delbrueckii ssp. lactis and L. helveticus starts to diminish over 267 the development of the NSLAB, which use alternative substrates to grow, such as the 268 269 products released from SLAB autolysis, becoming dominant in the first ripening stages (Fig. 270 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 271 272 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 273 274 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 275 276 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 277 278 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 279 increase of the species L. helveticus and L. delbrueckii ssp. lactis in the lysed cells fraction 280 DNA after the brining step. On the other end, also the lysis of Lacticaseibacillus has been 281

observerd from 6th month of ripening, but to a lesser extent [6,14]. Another species that is 282 frequently associated with ripened cheese is L. fermentum, that is detected in the whole cell 283 fraction of PR microbiota, and can reach high relative abundance during ripening, being 284 correlated with intermediate casein proteolysis products [6]. Other species that are frequently 285 detected by HTS at later ripening steps are Lc. lactis, Lactobacillus crispatus and S. 286 thermophilus, but no data are available on the extent of autolysis [6,11,25]. Transformation of 287 the curd into cheese is therefore an essentially enzymatic process mainly due to the 288 degradation of the major components of the curd into simpler substances. Viable 289 microorganisms, from the growth phase until death, play a central role in promoting both 290 structural and organoleptic changes in the curd and cheese paste. After cell lysis also their 291 released cytoplasmic enzymes could be involved in different biochemical modification. 292

Bottari et al. [6] have described that there is a strong relationship between the microbial 293 dynamics observed during PR ripening and the development of the peculiar proteolytic 294 profile of mature PR. Evolution of the oligopeptide fraction in PR cheese during ripening is 295 characterised by the degradation of casein-derived peptides into oligopeptides, together with 296 the accumulation of non-proteolytic aminoacyl derivatives (NPADS) [49,50]. The evolution 297 of the characteristic sensory profile of PR during its cheesemaking depends on a delicate 298 299 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 300 301 ripening time, are capable to counteract small differences in terms of microbial composition [6]. Indeed, the technological interventions occurring during cheesemaking shape the 302 303 dynamics of the cheese ecosystem leading to a final product with a distinctive sensory 304 profile.

305 Conclusions

A growing number of studies has investigated the role of the microbiota in defining the 306 properties of PR cheese, a world-wide appreciated PDO product. Food microbial ecosystems 307 such as that of PR can be described as a continuous evolving microbial network able to adapt 308 to technological process and seasonal variability thanks to its complex community. Overall, 309 the existing literature suggests that there is a certain degree of variability in the composition 310 of microbial community among different dairy farms, at least in the early stages of PR 311 312 manufacturing. Despite the flux of minority species, the presence and alternation of core microbiome species during cheesemaking ensures a successful fermentation process. 313

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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- The authors have studied the microbial composition and dynamics of 91 whey samples 491 from the PR manufacturing process. A combined approach, making use of qPCR and 492 HTS, was chosen to study two different lines of production, one Conventional and one 493 Organic, over a 10 weeks period. The results highlighted that PR NWS is a dynamic 494 microbial community, able to adapt to the different technological parameters of the 495 manufacturing, while retaining a high level of resilience of the main thermophilic LAB 496 species. Combination of qualitative and quantitative results showed the different 497 adaptive features of the main thermophilic species of NWS, which are subjected to a 498 499 cyclical production process based on back-slopping. Regardless of its initial different composition, NWS resulted to be shaped by technological treatment. 500
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527 Figures captions

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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 ofPR 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:

537 Coppola et al., 2000; Gatti et al., 2008; Neviani et al., 2009; CRPA, 2011; Gatti et al., 2003;

- 538 De Dea Lindner et al., 2008; Bottari et al., 2010; Santarelli et al., 2013 b; Tosi et al., 2006;
- 539 Bottari et al. 2020.

Table

Table 1: Principal microbial genera and species detected along PR manufacturing steps using culture-dependent and culture independent methods. <u>Bacterial names</u> 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 <u>bacterial names</u> 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
	<u>Lactocaseibacillus paracasei</u>	Gatti et al, 2008	Gatti et al, 2008 (RAPD)	Gatti et al, 2008 (LH-PCR)	Milani et al., 2019 (PCR)
	<u>Latilactobacillus curvatus</u>	Neviani et al., 2009		Milani et al., 2019 (HTS)	
	<u>Lactobacillus helveticus</u>	CRPA, 2011			
	<u>Lactobacillus delbrueckii sp.</u>	Coppola et al., 2000			
	<u>Levilactobacillus brevis</u>				
	<u>Lentilactobacillus kefiri</u>				
	<u>Limosilactobacillus fermentum</u>				
X	<u>Lactocaseibacillus rhamnosus</u>				
Mil	<u>Kocuria kristinae</u>				
ław	Alistipes				
<u> </u>	Bacteroides				
	Streptococcus				
	Actinobacteria				
	<u>Lactobacillus</u> spp.				
	<u>Bifidobacterium</u>				
	<u>Corynebacterium</u>				
	<u>Clostridiaceae</u>				
	Lachnospiraceae				

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

	Escherichia			
	Acinetohacter			
	Lactococcus lactis			
	Lactobacillus spn			
	Strentococcus thermonhilus			
	Streptococcus nermophilus			
	Baetovoides			
	Lachnospiraceae			
	<u>Laconospiraceae</u>			
	Propionibacterium			
	Lactobacillus crispatus			
	<u>Streptococcus suis</u>			
	<u>Levilactobacillus brevis</u>	Coppola et al., 2000		
ning	<u>Lactocaseibacillus paracasei</u>			
Brii	<u>Lactobacillus helveticus</u>			
	<u>Lactobacillus delbrueckii ssp.</u>			
	Lactocaseibacillus casei	D		Bottari et al., 2020 (LH-PCR,
		Bottari et al. 2020	Bove et al., 2011 (RAPD, REP)	HIS)
(•0	Lactocaseibacinus rhamnosus	Gatti et al., 2008	Succi et al., 2005 (RAPD)	Gatti et al, 2008 (LH-PCR)
24 m	<u>Limosilactobacillus fermentum</u>	Coppola et al., 2000	REP)	Ercolini et al., 2002 (DGGE)
(1-2	<u>Pediococcus acidilactici</u>	Neviani et al., 2009	Gatti et al., 2008 (RAPD)	Gala et al., 2008 (DGGE)
ing	Levilactobacillus brevis	De Dea Lindner et al.,		
brin		2008	Gala et al., 2008 (RFLP, DGGE)	
ost-l	Lactocaseibacillus paracasei			
e bc	<u>Lactobacillus helveticus</u>			
lees	<u>Lactobacillus delbrueckii ssp. lactis</u>			
Ċ	<u>Lactocaseibacillus casei</u>			
	<u>Lentilactobacillus buchneri</u>			
	Schleiferilactobacillus harbiniensis			

<u>Lentilae</u>	uctobacillus kefiri	
Lactoco	occus lactis	
<u>Streptor</u>	ococcus spp.	
<u>Streptor</u>	ococcus suis	
<u>Streptoe</u>	pcoccus thermophilus	
Lentilad	uctobacillus 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 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.

