Dynamics of a natural bacterial community under technological and environ-mental pressures: the case of natural whey starter for Parmigiano Reggiano cheese

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Gaia Bertani[a$#], Alessia Levante[a$#], Camilla Lazzi[a], Benedetta Bottari[a], Monica Gatti[a], Erasmo Neviani[a]

[a] Department of Food and Drug, University of Parma, Parco Area delle Scienze 49/A, 43124, Parma, Italy

[$]Both authors contributed equally to this manuscript

[#] **Address correspondence to:**

Dr. Gaia Bertani

Department of Food and Drug, University of Parma, Parco Area delle Scienze 49/A, 43124, Parma, Italy

E-mail: gaia.bertani@studenti.unipr.it

Phone: +39 0521 906524

Dr. Alessia Levante

Department of Food and Drug, University of Parma, Parco Area delle Scienze 49/A, 43124, Parma, Italy

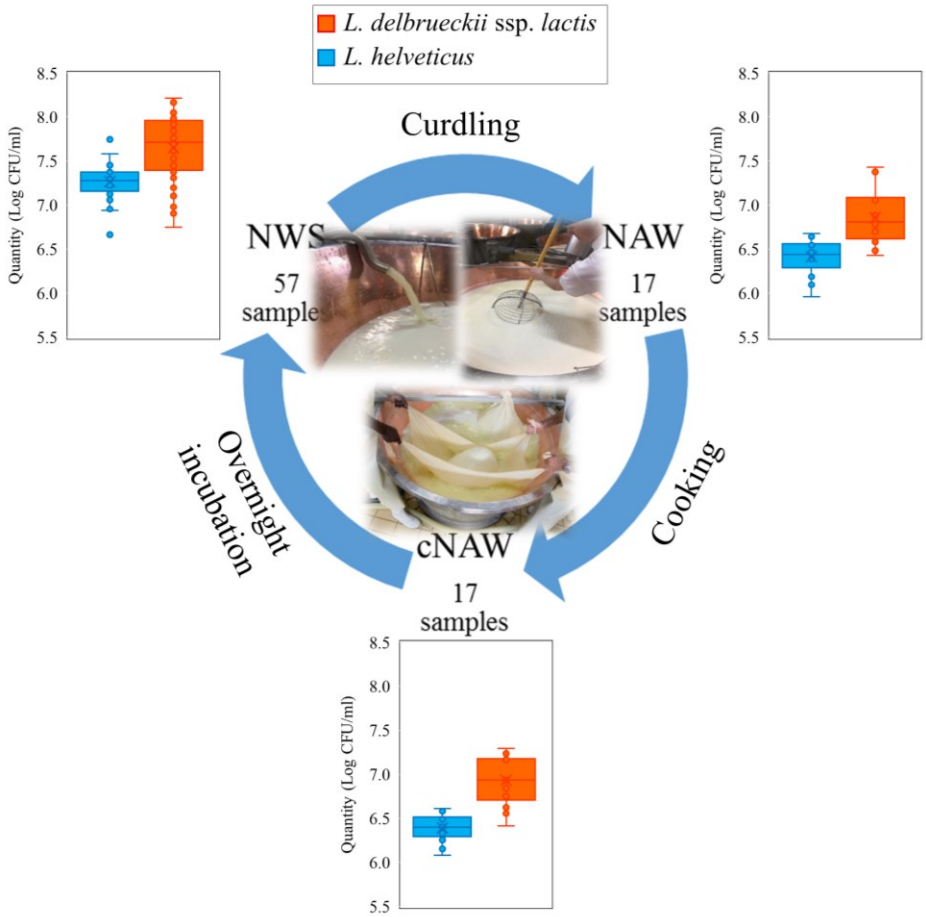
E-mail: alessia.levante@unipr.it

Phone: +39 0521906524

Highlights

1. qPCR and metataxonomics were applied to describe the dynamics of NWS microbiota
2. Species present in the whey respond differently to cheese-making technology
3. The microbiota of NWS, regardless of initial composition, is shaped by technology.

Graphical abstract



Abstract

Natural starter cultures are undefined multiple-strains culture communities of mostly thermophilic lactic acid bacteria (LAB), in association with minor amounts of mesophilic bacteria, which structure could be affected by small changes in the parameters of the cheese/whey-making process. This study aims to investigate the complex microbiota of natural whey starter (NWS) used in Parmigiano Reggiano (PR) cheese-making, focusing on both the absolute and relative abundance of bacterial species and on the modification of the bacterial community under environmental and technological pressures.

To reach this purpose a combined approach, using quantitative PCR (qPCR) and High-Throughput Sequencing (HTS), was used to investigate the bacterial dynamics of 91 whey samples collected during different steps of PR cheese-making, in one dairy, through two different lines of production, one Conventional and one Organic, over a 10 weeks period. Our results highlighted that NWS used for the production of PR cheese is a dynamic microbial community, which adapts to the different technological parameters encountered in the cheese/NWS manufacturing process, while retaining a high level of resilience of the thermophilic LAB species mainly involved in the steps of curd acidification and the early maturation process. Differences were also observed in bacterial species diversity between samples from Conventional and Organic line but, in conclusion, NWS resulted to be shaped by technological treatments, regardless of its initial different composition.

Keywords: Natural whey starter, thermophilic lactic acid bacteria, qPCR, High-Throughput Sequencing, undefined starter cultures.

1. Introduction

Parmigiano Reggiano (PR) is a Protected Designated Origin (PDO) cooked, long ripened and hard-textured cheese exclusively produced in a defined geographical area (Gatti, Bottari, Lazzi, Neviani, & Mucchetti, 2014).

PR is manufactured employing raw cow's milk, using a mixture of partially skimmed and whole milk. In detail, the evening milk is partially skimmed after overnight spontaneous creaming, and subsequently mixed in a 1:1 ratio with the whole milk from the following morning milking. The vat milk is heated at 32 °-34 °C and added with natural whey starter culture (NWS) and then calf rennet to achieve milk coagulation. As soon as the curd reaches the proper firmness, it is broken down with the use of 'Spino', a dedicated tool employed to obtain small-sized granules (approximately the size of rice grains), with a size distribution that is not too large (Iezzi et al., 2012). At this manufacturing step whey syneresis occurs and this whey is not yet acidified(non-acidified whey, NAW), compared to NWS. Afterwards the curd is heated up to 54 °C (53 °–56 °C) for 5-15 minutes under stirring. Following cooking, the granules previously obtained aggregate at the bottom of the vat. Curd is removed from vat, manually cut in two parts and stored in a mold for 2 days, entering the subsequent manufacturing steps that will lead to the final product, as carefully reviewed elsewhere (Gatti et al., 2014; Gobbetti, Neviani, & Fox, 2018). The remaining whey, that is cooked and not acid (cooked non-acidified whey, cNAW; Bottari et al., 2010), is recovered and incubated at a decreasing temperature to produce NWS to be used for the cheese-making of the following day. This back-slopping procedure establishes a microbiological *liaison* among subsequent batches of production. During the first few hours of cheese-making, the thermophilic microorganisms present in NWS grow in the curd and play a key role in the acidification step (Johnson, 2013). The thermophilic microbiota able to resist to the curd cooking temperature is also crucial for the cheese ripening (De Dea Lindner et al., 2008; Gatti et al., 2008). NWS cultures are undefined multiple-strain cultures of thermophilic lactic acid bacteria (LAB) (G. Giraffa et al., 2004; G. Giraffa & Neviani, 1999; Giorgio Giraffa, Gatti, Rossetti, Senini, & Neviani, 2000; Lombardi et al., 2002),

78 which have a greater dynamic survival under different selective pressures if compared to selected or
79 single strain cultures (Erkus et al., 2013). Previous studies concerning PR NWS attributed
80 predominant microbiota to *Lactobacillus helveticus* (Cocconcelli, Parisi, Senini, & Bottazzi, 1997;
81 Coppola et al., 2000; Gatti, Lazzi, Rossetti, Mucchetti, & Neviani, 2003; Gatti, Trivisano, Fabrizi,
82 Neviani, & Gardini, 2004). Further knowledge on microbial structure of PR NWS was provided by
83 Bottari et al. (2010) by using culture-independent techniques. The use of Length Heterogeneity
84 (LH)-PCR and Fluorescence In Situ Hybridization (FISH), demonstrated that the majority of tested
85 PR NWS were composed by comparable percentages of *Lactobacillus helveticus* and *Lactobacillus*
86 *delbrueckii,* while *Lactobacillus fermentum* and *Streptococcus thermophilus* were present in lower
87 amounts. It has been demonstrated that small changes in the technological parameters like
88 temperature of curd cooking, temperature and modality of cNAW cooling and differences in the
89 acidity and pH, could affect the bacterial consortium present in NWS (Rossetti et al., 2008). Despite
90 the findings provided by these studies, the composition of these microbial cultures adapted to
91 selective pressure of food processing is not fully understood yet. Therefore, in this study 91 PR
92 whey samples were collected from one PR dairy over 3 months. Fifty-seven NWS were collected
93 just before addition to the vat milk, while 17 NAW samples were collected after the use of 'Spino'
94 and further 17 cNAW samples after the curd cooking treatment. Samples were taken from two
95 different production lines: one Conventional and one Organic, where the Organic line employs milk
96 from cows fed only with organic feed The two production lines are independent, and are compliant
97 to the disciplinary regulations provided for PR manufacturing (Single document of the PDO
98 Parmigiano Reggiano). This study was performed in order to understand the dynamics of bacterial
99 structure of whey over time, according to the different production line and in response to the
100 different technological steps. The aim of this work was: (i) to characterise the bacterial community
101 of the two type of whey collected to increase the existing knowledge, and (ii) to investigate how
102 selective and environmental pressures shape the bacterial community typical of PR NWS.

103 **2. Materials and Methods**

104 **2.1. Selection of natural whey starter samples**

105 Ninety-one whey samples used in PR manufacturing were collected from one dairy located in the
106 province of Parma and belonging to the PR PDO cheese area of production. In the cheese factory,
107 the Organic and Conventional lines are kept separated and each makes use of different milk and
108 uses its own NWS, which is propagated separately. Both production lines use milk that respects the
109 disciplinary regulations provided for PR manufacturing (Single document of the PDO Parmigiano
110 Reggiano), which differ in the fact that the organic line employs milk from breeders certifying that
111 cows only eat organic feed. These samples were collected during different steps of PR
112 cheese/NWS-making (Fig. 1): in particular, 57 NWS samples were collected just before the addition
113 to the vat milk, 17 samples after the use of 'Spino', i.e. right after the curdling step, and 17 samples
114 after the curd cooking step. The latter are non-acidified whey (NAW) and cooked non-acidified
115 whey (cNAW) samples, respectively. The 57 NWS samples were collected three times a week,
116 while the NAW and cNAW were sampled from the same vat once a week, from August to October
117 2017. NWS acidity was measured and expressed as Soxhlet-Henkel degrees (°SH; Mucchetti and
118 Neviani, 2006). Samples were shipped to the laboratory under refrigerated conditions, and stored at
119 -80°C before the analysis.

120 **2.2. DNA extraction and quantification**

121 Microbial DNA extraction was performed using DNeasy Blood and Tissue Kit (Qiagen, Hilden,
122 Germany) with slight modification. The following steps were performed to remove fat and milk
123 impurities: 1 mL of NWS was mixed with 2% (wt/vol) of sodium citrate solution at 50 °C in 10 mL
124 final volume for 30'. Ten mL of NAW and cNAW were mixed with 2% (wt/vol) of sodium citrate
125 solution at 50 °C in 20 mL final volume for 30'. The mixtures were centrifuged at 10000 rpm for 10
126 min at 4 °C. The supernatant and fat layers were removed, and the cells were re-suspended in 1 mL
127 of sodium citrate, repeating the washing step for 2 times until all impurities were removed. The cell
128 suspensions were centrifuged at 10000 rpm for 10 min at 4 °C. Subsequently, the manufacturer's

protocol for DNA extraction from Gram positive bacteria was followed, doubling the reagents volumes. Briefly, the cells were lysed in 360 µL of lysis buffer containing 25 mg/mL of lysozyme for 30' at 37 °C. The lysed cell suspension was protease treated for 30' at 56 °C. At the end of the spin-column protocol, the DNA was eluted with 60 µL of nuclease-free water, and the concentration and purity of the extracted nucleic acids were determined by Nanodrop (NanoDrop™ 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.3. Absolute quantification through qPCR

The absolute quantification of *L. helveticus*, *L. delbrueckii* ssp. *lactis, S. thermophilus* and *L. fermentum* species, was performed on the 91 collected samples, using specific primers (Table 1) designed on *phe*S gene sequences (Bottari et al., 2013). Master mix for each primer pair contained: 10 µl of 2× PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Milan, Italy), forward and reverse primers at a concentration of 250 nM and nuclease-free water to a total of 20 µL per well. All the reactions were performed in duplicate, and no template controls (NTC) were included in each experiment. Total DNA, previously extracted, was 10-fold diluted with nuclease free water and added to the reaction in a 5 µL volume. The plate, after a short centrifugation, was placed in the QuantStudio® 3 instrument (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and thermal cycle run as follows: a first hold stage of 2 min at 50 °C followed by 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, during which fluorescence acquisition took place, and a final melting curve stage from 60 ° to 95 °C with a temperature gradient of 0.1 °C/s.

For the absolute quantification, standard curves were constructed using purified genomic DNA of type strains of *L. helveticus* LMG 6413, *L. delbrueckii* ssp. *Lactis* LMG 7942, *S. thermophilus* LMG 6896 and *L. fermentum* LMG 6902*,* calculating *pheS* copy number as described in Bottari et al. (2013). The standard curves were constructed from serially 10-fold diluted reference strains DNA at known copy number, covering a dilution range of 6 orders (for *L. helveticus*, *L. delbrueckii* ssp. *lactis* and *S. thermophilus*) and 8 orders of magnitude as regards *L. fermentum*, then plotting

154 the resulting threshold cycles (C_q), against the logarithm of the *pheS* copy number. The copy
155 number of *pheS* gene of each species was calculated in the 91 whey samples by comparing the C_q of
156 the sample with that of the respective standard curve.

157 **2.4. Bacterial community profiling**

158 Bacterial diversity was evaluated in 34 samples: 17 NWS, of which 10 from the Conventional and 7
159 from the Organic line, and the corresponding 17 cNAW. The V3-V4 region of the 16S rRNA gene
160 was amplified and sequenced using primers and PCR conditions previously described in Takahashi
161 et al. (2014). Library preparation and sequencing were carried out by BMR Genomics (Padova,
162 Italy), on a MiSeq platform (Illumina Italy s.r.l., Milan, Italy), leading to 300 bp, paired-ends
163 approach. The quality of the 16S rRNA amplicon raw reads was checked through FastQC
164 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Using PrinSEq
165 (http://prinseq.sourceforge.net/), raw reads shorter than 380 bp were discarded, and the bases with a
166 Phred score below 20 were trimmed. Through FLASH (Magoč & Salzberg, 2011), paired-ends
167 reads were joined. Merged sequences were then analysed using QIIME 1.9.1 software (Caporaso et
168 al., 2010). The OTUs were picked at 99% of similarity using RDP classifier and the most
169 representative sequences of each cluster were selected in order to assign taxonomy, using the
170 Greengenes database (DeSantis et al., 2006). All samples were rarefied to 13500 reads per sample
171 for subsequent analysis, to prevent any biases, due to different depths of sequencing. Alpha
172 diversity indices (Observed species, Good's coverage, Chao1 richness and Shannon diversity index)
173 were calculated using QIIME 1.9.1 version. In order to avoid biases due to different sequencing
174 depths, all samples were rarefied at 13500 reads.

175 **2.5. Statistical analysis**

176 All data obtained as absolute quantification of bacterial species were statistically elaborated using
177 SPSS Statistics 21.0 software (SPSS Inc., Chicago, IL). Student's *t*-test was used to assess statistical
178 difference between quantities (significance level, $p < 0.05$). Correlation coefficient among NWS

179 acidity and species abundance was evaluated by bivariate Pearson's correlation. Statistical
180 significance between measured alpha-diversity indexes was evaluated through Student's *t*-test using
181 QIIME.

182 **3. Results**

183 **3.1. Absolute quantification**

184 The absolute abundances of the dominant species, *L. helveticus* and *L. delbrueckii* ssp. *lactis*, and
185 the minor species, *S. thermophilus* and *L. fermentum,* were evaluated through a culture-independent
186 approach, by qPCR assay. Calibration curves were used to assess the absolute abundances of these
187 species in 91 samples. These belonged to two different production lines, Conventional and Organic,
188 and were collected at different stages of PR cheese/NWS-making. qPCR data allowed to describe
189 the mean values of the species detected in Conventional and Organic samples (Fig. 2). In detail, the
190 NWS showed a predominance of *L. delbrueckii* ssp. *lactis* in both production lines ($p < 0.001$), with
191 a mean concentration of about 7.35 ± 0.53 log copy n/mL in the Conventional line and 7.39 ± 0.44
192 log copy n/mL in the Organic one. *L. helveticus* showed a significant difference in terms of
193 abundance ($p < 0.05$) between Conventional and Organic NWS, with mean values of 7.20 ± 0.18
194 log copy n/mL and 7.32 ± 0.16 log copy n/mL respectively. The different concentration of the
195 bacterial species between the two NWS, despite statistical significance, is small and is not expected
196 to affect the technological properties of NWS. The absolute amount of *S. thermophilus* was also
197 statistically different ($p < 0.001$) between the Conventional and the Organic NWS, with mean
198 values of 6.58 ± 0.27 log copy n/mL and 5.98 ± 1.09 log copy n/mL respectively. Also in this case,
199 the different measured amounts between the two NWS is not expected to have a biologically
200 relevant effect. Regarding *L. fermentum*, different amounts were observed ($p < 0.05$), in the
201 Conventional and the Organic NWS, with mean values of 1.73 ± 0.73 log copy n/mL and 4.34 ±
202 0.37 log copy n/mL, respectively. In the NAW samples, corresponding to the samples taken after
203 the curdling, a decrease of the abundances of all the four analysed species was observed (Fig. 2).
204 Considering both production lines, *L. helveticus* and *L. delbrueckii* ssp. *lactis* mean values were

6.41 ± 0.20 and 6.98 ± 0.42 log copy n/mL respectively, while *S. thermophilus* and *L. fermentum* abundances were 5.55 ± 0.98 and 3.33 ± 0.98 log copy n/mL respectively. After curd cooking, in cNAW, mean values of 6.39 ± 0.15, 7.74 ± 0.28, 5.53 ± 0.97 and 3.33 ± 0.95 log copy n/mL were observed for *L. helveticus*, *L. delbrueckii* ssp. *lactis, S. thermophilus* and *L. fermentum,* respectively, for both production lines. Any statistical difference found between the Conventional and Organic lines for NWS samples was lost during the cheese-making process, with the exception of *L. fermentum*. This species shows significantly higher amounts ($p < 0.05$) through the Organic production line.

To reveal the species behaviour under the acid stress occurring during the overnight incubation of the cNAW, the species abundances were then plotted against the measured NWS sample acidity. As shown in Fig. 3, *L. helveticus* seems to better adapt to the increasing acidity reached in some samples. Indeed, there is a positive correlation ($p<0.001$) between the NWS acidity and the copy number reached from this species (Fig. 3). The species *L. fermentum* shows a similar correlation trend (Fig. 3, $p < 0.001$). Conversely, *L. delbrueckii* ssp. *lactis* shows a significantly negative correlation with increased acidity values, as well as *S. thermophilus* ($p < 0.001$). It is noteworthy that, on average, Organic NWS samples showed higher acidity values when compared to Conventional ones, with average values of 28.95 ± 1.46 and 26.29 ± 1.99 °SH ($p < 0.001$) respectively.

The relative abundance of the four studied species in the 57 NWS was evaluated during the 10 weeks sampling period (Fig. 4). *L. delbrueckii* ssp. *lactis* is the predominant species, but all the four LAB species showed variability across the period, especially in the Organic production line. It is noteworthy that, in the Conventional line, *L. delbrueckii* ssp. *lactis* was the predominant species during all 10 weeks sampling time. (Fig. 4). On the other hand, in the Organic production line, *L. delbrueckii* ssp. *lactis* represented the most abundant species in all the tested NWS in the first few weeks, while later on *L. helveticus* became the predominant species (Fig. 4). Considering the minority species, *S. thermophilus* was found in both the production lines at low concentrations, with

231 a lower variability over time in the Conventional line than in the Organic one. *L. fermentum* species
232 was found to be less 1% in both production lines.

233 **3.2. 16S rRNA Gene High Troughput Sequencing**

234 A total of 3,727,122 raw reads were obtained after the sequencing step, of which 884,189 passed the
235 filtering steps performed using QIIME software pipeline, with an average value of 26,006 reads per
236 sample, and an average read length of 468 bp. For each sample, indices describing the alpha-
237 diversity of the bacterial community were obtained and reported in Table 2.
238 A total of 102 OTUs were identified, of which only five have a relative abundance higher than 0.1
239 % in at least five samples, as reported in Fig. 5 for Conventional and Organic samples. *L. helveticus*
240 and *L. delbrueckii* were the major OTUs found in all analysed samples, with mean values of 50.5%
241 and 34.2 %, respectively. Regarding Conventional samples, *L. delbrueckii* relative abundance
242 ranged from 16.7% to 61.7%, while *L. helveticus* abundance ranged from 26.1% to 62.7% (Fig. 5).
243 On the other hand, in samples from the Organic line, *L. delbrueckii* ranged from 3.9% to 58.2%
244 while *L. helveticus* from 27.1% to 94.3%. The variability of *L. helveticus* was thus significantly
245 higher in samples from the Organic line than in samples from the Conventional one (T-test, $p <$
246 0.05). *Streptococcus* ssp. was found in all samples, with values between 3.6% and 18.7% in the
247 Conventional and 0.1% and 18.9% in the Organic one. As for other species, unidentified
248 *Streptococcaceae* were below 3.8%, while unidentified *Lactobacillus* ssp. was found in all samples
249 at less than 3%.
250 Interestingly, in most cNAW samples there is an increase in the relative abundance of *L. delbrueckii*
251 compared to NWS. After cooking, *L. delbrueckii* average abundance shifts from 18.9 ± 16.1 % to
252 40.0 ± 15.9% (T-test, $p < 0.05$) in the Organic samples, and from 31.5 ± 13.0% to 43.6 ± 12.2% in
253 the Conventional ones. Instead, *L. helveticus* abundance is significantly lowered after the curd
254 cooking step in the Conventional line samples, decreasing from 51.5 ± 11.6% to 38.5 ± 9.9% (T-

255 test, $p<0.05$). In the Organic production line, *L. helveticus* decreases from 66.8 ± 23.5% to 49.8 ±
256 20.5 %, after cooking.
257 The rarefaction analysis and the calculated diversity indexes show that there was a satisfactory
258 coverage for all analysed samples (Table 2), and allowed to describe the diversity among the
259 bacterial communities of the analysed samples. Samples belonging to the Conventional line show a
260 significantly higher number of observed species ($p < 0.001$), a significantly higher richness ($p <$
261 0.05), expressed by Chao1 index, as well as a higher diversity among the samples (Shannon index,
262 $p < 0.001$), when compared to samples belonging to the Organic line.
263 The diversity indexes vary according to the exposure to the cooking step, indeed cNAW samples
264 show significantly higher observed species and an increased species richness ($p < 0.001$) compared
265 to NWS samples. Conversely, the species diversity (Shannon index) is not affected by the cooking
266 step.

267 **4. Discussion**

268 Nowadays it is increasingly common to study microbial communities using high-throughput
269 sequencing technology which, giving the relative abundance at taxon level in an approach referred
270 to as metataxonomics (Marchesi & Ravel, 2015), allows to achieve an overview of the community
271 composition (De Filippis et al., 2018; De Filippis et al., 2017; Widder et al., 2016). Still, in order to
272 estimate the community dynamics in complex bacterial cultures under technological selective
273 pressures, more detailed information are necessary, such as the absolute quantification (Berry &
274 Widder, 2014; Philippot et al., 2009). To our knowledge, this is the first study aiming to investigate
275 the complexity of NWS used in PR cheese-making, focusing on both the absolute and relative
276 abundance of bacterial species and on the modification of this bacterial community under
277 environmental and technological pressures. A combined approach, using qPCR assay and
278 metataxonomic analysis, was carried out to investigate the bacterial dynamics of 91 whey samples
279 collected during different steps of PR cheese-making, in one dairy plant, through two different lines
280 of production, one Conventional and one Organic, over a 10 weeks period. Through qPCR, the 57

281 collected NWS showed a higher abundance of *L. delbrueckii* ssp. *lactis* over *L. helveticus*,
282 differently from what consistently reported in the literature regarding PR NWS, that observes an
283 opposite distribution of the two thermophilic species (Cocconcelli et al., 1997; Coppola et al., 2000;
284 Gatti et al., 2003, 2004). On the other hand, our data are consistent with Bottari et al. (2010) who,
285 through culture-independent approaches, found a high variability among the considered NWS, with
286 several samples showing comparable percentages of *L. helveticus* and *L. delbrueckii* ssp. *lactis*.
287 Nevertheless, the presently observed "inverted ratio", could be a peculiarity of the chosen dairy
288 plant, since the intrinsically artisanal nature of the NWS leads to a great variability among the
289 samples of different PR manufacturers (Bottari et al., 2010; Gatti et al., 2004). qPCR assay revealed
290 also the presence of minor species, particularly *S. thermophilus* and *L. fermentum*, in all the 91
291 samples, in agreement with observation of other authors (Alessandria et al., 2016; Benedetta Bottari
292 et al., 2013; De Filippis et al., 2014). In the present study, *S. thermophilus* amount was higher than
293 previously observed in PR NWS (Bottari et al., 2010), but this could be due to the limit of detection
294 of the culture-independent approaches formerly used. *L. fermentum* species was found at low
295 concentration (<1%) in all the samples. Recently, it has been demonstrated that this species is able
296 to survive throughout the ripening in a dairy product with a similar cheese-making technology,
297 representing a fraction of the metabolically active microbial population in the advanced stages of
298 ripening (Levante et al., 2017).
299 Nowadays, consumers have oriented their purchase preferences toward organic products, with the
300 attitude that organic dairy farming is kinder to the environment, animals, and people (Schwendel et
301 al., 2014a). Despite the existence of compositional difference between organic and conventional
302 milk is reported (Schwendel et al., 2014a; Schwendel et al., 2014b), only one study has assessed if
303 this affected microbial composition, by measuring differences in the aerobic spore-forming bacteria
304 (Coorevits et al., 2008). To our knowledge, there are no indications on how the different cow
305 feeding might affect the LAB microbiota of Conventional or Organic milk that daily interacts with
306 the NWS. In the present study, the absolute abundances of the target species in NWS were found to

be significantly different comparing samples from Conventional or Organic production lines, but the observed differences are small and probably not relevant on the technological processes. These differences are subsequently flattened in response to the technological treatments, which are mainly represented by the curdling process, followed by the breaking step and the cooking step. The only species that seems to be unaffected is *L. fermentum*, that represents a minority one. These data could suggest that NWS samples, regardless of their initial different composition, respond stably following the treatments. The technological process, in particular the addition of NWS to milk, the curdling, breaking and cooking steps are probably the selective pressures that shape the structure of this natural starter culture. It is well known that weak changes due to seasonal variations or technological parameters modifications, such as differences in the acidity and pH, could affect the bacterial consortium present in NWS (Gatti et al., 2014; Rossetti et al., 2008; Santarelli et al., 2008). In good agreement with literature, our results showed a high degree of variability of *L. helveticus* and *L. delbrueckii* ssp. *lactis* in both the production lines, over the ten weeks. The minority species, such as *S. thermophilus* and *L. fermentum* were detected in low concentrations both in Conventional and Organic production lines, appearing to be more stable over the analysed period. Despite their low abundance in the NWS and in the first cheese-making steps, these species could be involved in the following stages of production and ripening (Bottari et al., 2010; Gatti et al., 2014).

Studies performed on NWS used for the manufacturing of other cheeses have shown how these undefined cultures, characterized by a low complexity LAB community, can still harbour a certain degree of strain-level diversity. This observation was recently described in various types of NWS for the species *Lactococcus lactis* (Frantzen, Kleppen, & Holo, 2018), *Leuconostoc mesenteroides* (Frantzen et al., 2017), and *L. helveticus* (Schmid et al., 2018; Somerville et al., 2019).

Previous studies have highlighted that NWS species and biotypes show different ability to adapt to the curd ecosystem and to evolve during cheese ripening (Giraffa & Neviani, 1999). Recently, Moser et al. (2018) have shown how NWS used for the production of Gruyère-type cheese were

characterized by a multi-strain population of *L. helveticus*, and that different strains are capable to coexist and persist dynamically through a prolonged ripening time. The growing efforts on characterization of NWS underline the importance of a deeper understanding of composition of the bacterial community to maintain culture diversity and performance.

Regarding the relative composition of the bacterial community, the calculated alpha indices show that the two production lines have different communities, although dominated by the main acidifying thermophilic species, in good agreement with other studies (Bottari et al., 2010; De Filippis et al., 2014; Giraffa et al., 1996). Indeed, exposure to thermal stress does not lead to a significant reduction of the Shannon index, since the NWS is already dominated by few thermophilic species that are only slightly affected by the cooking temperature. Interestingly, the variations observed in terms of absolute abundances of *L. helveticus* and *L. delbrueckii* ssp. *lactis* in response to thermal and acid stress, show some differences with the relative abundance results obtained from metataxonomic analysis. However, the sole metataxonomic analysis fails to depict if the observed change in the relative abundance of *L. helveticus* is due to an increased number of *L. delbrueckii* ssp. *lactis*, or to a progressive loss of viability of the *L. helveticus* itself. The analysis of quantitative data obtained through qPCR about *L. helveticus* and *L. delbrueckii* ssp. *lactis* in NAW suggests that both the species quickly adapt to the vat environment. *L. helveticus* quantity decreases in NAW compared to NWS, due to the dilution of the NWS into the vat milk, and remains mostly unchanged after the cooking step. On the other hand, *L. delbrueckii* ssp. *lactis* abundance is less affected by dilution in vat milk, and increases after the cooking step. This increase suggests that this species better resists to thermal treatment. cNAW samples composition is different, in terms of species abundance, from the starting NWS. After curd extraction, the cNAW left in the vat, that has undergone the thermal stress imposed by the cooking temperature, is cooled down overnight, passing through temperatures that represent the optimum range (37 °-42 °C) for the thermophilic LAB. By this process the cNAW becomes the NWS ready for the cheese-making of the following day. The data obtained, in agreement with other works (Chamba, 2000; Cocconcelli et al., 1997;

Neviani, Divizia, Abbiati, & Gatti, 1995), suggest that the most abundant species present in the whey appear to respond differently to temperature gradients and to the different composition of the environment (i.e. vat milk under heating or cNAW under acidification) during cheese/NWS-making. Indeed, *L. helveticus* is regarded as a more acid-tolerant species, and this might explain why this species could increase in number after the overnight incubation of the cNAW, when acidity quickly increases. Indeed, there is a positive correlation between NWS acidity and the abundance of *L. helveticus* measured in the samples. Conversely, the inoculated vat milk has higher pH values (of about 6.0) that, in combination with a possibly higher tolerance to the thermal stress of *L. delbrueckii* ssp. *lactis*, might explain the numerical increase of this species in NAW and cNAW.

5. Conclusions

This work provides a description of the natural starter cultures microbiota involved in Parmigiano Reggiano PDO Italian cheese-making over time and in response to different technological pressures during its production. Whey samples from one PR dairy plant, during 10 weeks, in two different production lines, Conventional and Organic, were studied throughout cheese/NWS-making. The combination of quantitative results obtained with qPCR and HTS highlighted that NWS used for the production of PR cheese is a dynamic microbial community. The microbiota adapts to the different technological parameters encountered in the cheese/NWS manufacturing process, while retaining a high level of resilience of the thermophilic LAB species mainly involved in the steps of curd acidification and the early maturation process. The peculiar adaptive features of the main thermophilic species of NWS allows this undefined culture to retain its technological function, by adapting to a cyclical production process based on back-slopping. The flattening of the observed differences in bacterial species diversity between Organic and Conventional NWS after the cooking step, confirms that the microbiota of NWS, regardless of its initial different composition, is shaped by technological treatments.

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Declaration of interest

The authors declare they have no conflicts of interest.

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543

544 **Figure captions**

545 **Figure 1.** Schematic representation of the cheese/NWS-making process and the whey sampling
546 points. NWS: Natural whey starter, NAW: non-acidified whey, cNAW: cooked non-acidified whey.

547 **Figure 2.** Boxplot of the absolute quantification of the species *L. helveticus*, *L. delbrueckii* ssp.
548 *lactis, S. thermophilus* and *L. fermentum* in the different samples collected: natural whey starters
549 (NWS), non-acidified whey (NAW) and cooked non-acidified whey (cNAW). ***** Statistically
550 significant difference, $p< 0.05$; ****** Statistically significant difference, $p< 0.001$

551 **Figure 3.** Linear correlation between the bacterial counts measured in NWS samples and the
552 corresponding acidity, measured in Soxhlet-Henkel degrees (°SH). The panels represent the trend of
553 the species *L. helveticus*, *L. delbrueckii* ssp. *lactis, S. thermophilus* and *L. fermentum* in the
554 different samples collected. Pearson's correlation coefficient (ρ) is reported at the bottom of each
555 graph, statistical significance is shown (** $p < 0.001$).

556 **Figure 4.** Average distribution of the relative abundance (expressed as percentage) of the species *L.*
557 *helveticus*, *L. delbrueckii* ssp. *lactis, S. thermophilus* and *L. fermentum* during the 10 weeks
558 sampling.

559 **Figure 5.** Incidence of OTUs based on 16S rRNA gene of acid and cooked whey samples belonging
560 to Conventional or Organic production lines. The NWS and cNAW (CON- for Conventional and
561 ORG- for Organic line, respectively) samples from the same date are shown next to each other. The
562 bar graph describes the relative abundance of each species. Only the OTUs whose abundance is
563 higher than 0.1% in at least 90% of the samples are reported, the remaining OTUs are grouped in
564 the category "Others".

565

566 **Tables**

567 **Table 1:** Primer pairs used in this study; the sequences are the same as reported in Bottari et al.,

568 2010.

Primer	Primer sequence (5'→3')	Length (bp)	Size (bp)	GC%
LlpheSF	ACGTTGACGCTGACCACC	18	51	60
LlpheSR	GGCTTGAACTGGTGAAGTCTG	21		
StpheSF	GAAGAAATCTTGCTTCGCACTC	22	50	46
StpheSR	AGTGTACGAGCTTGGACAGGA	21		
LfpheSF	CTACGTGACCCCGTCTGTTT	20	76	58
LfpheSR	GAAGTCGTGTTGTTCCAGCA	20		
LhpheSF	TTGATGGTGAAGACTTGCTTAGAA	24	51	45
LhpheSR	CTCTGGCTTGGTCACCTGAA	20		

569

570

571 **Table 2:** Observed species, Chao 1 and Shannon indexes and estimated sample coverage for 16S
572 rRNA amplicons from NWS (Natural whey starter) and cNAW (cooked non-acidified whey)
573 samples. All data are reported as mean ± S.D.

Sample Type	Description	Observed species	Chao1	Shannon	Good's coverage
NWS	Conv-7/9	19.7 ± 2.58	43.85 ± 16.96	1.49 ± 0.01	0.999 ± 0
	Conv-27/9	23 ± 3.27	54.63 ± 27.6	1.86 ± 0.01	0.999 ± 0
	Conv-18/9	23.3 ± 2.45	58.13 ± 27.99	1.78 ± 0.01	0.999 ± 0
	Conv-31/8	20.8 ± 3.01	55.45 ± 31.54	1.84 ± 0.01	0.999 ± 0
	Conv-4/8	27.6 ± 2.88	68.35 ± 33.8	1.79 ± 0.01	0.999 ± 0
	Conv-7/8	19 ± 2.58	38.88 ± 19.53	1.82 ± 0.01	0.999 ± 0
	Conv-16/8	22.8 ± 3.65	53.72 ± 28.9	1.76 ± 0.01	0.999 ± 0
	Conv-23/8	17.2 ± 1.81	29.45 ± 10.2	1.87 ± 0.01	1 ± 0
	Conv-13/9	23.6 ± 3.86	58.09 ± 33.29	1.85 ± 0.01	0.999 ± 0
	Conv-6/10	28 ± 2.4	60.18 ± 13.35	1.88 ± 0.01	0.999 ± 0
	Org-7/9	23.6 ± 2.12	49.48 ± 16.82	1.08 ± 0.01	0.999 ± 0
	Org-29/9	19.1 ± 1.2	31.99 ± 9.22	0.74 ± 0.01	0.999 ± 0
	Org-1/9	20.5 ± 1.65	31.33 ± 10.59	1.99 ± 0.01	0.999 ± 0
	Org-8/8	17.1 ± 1.73	31.45 ± 11.66	1.64 ± 0.01	1 ± 0
	Org-16/8	16.1 ± 1.6	23.05 ± 6.32	2 ± 0.01	1 ± 0
	Org-23/8	20 ± 0	34 ± 0	1.87 ± 0	0.999 ± 0
	Org-10/10	15.1 ± 2.33	33.1 ± 20.14	0.47 ± 0.01	0.999 ± 0
cNAW	Conv-7/9	44.3 ± 4.45	101.17 ± 39.71	1.7 ± 0.01	0.998 ± 0
	Conv-27/9	47.1 ± 3.14	92.59 ± 29.88	1.87 ± 0.02	0.998 ± 0
	Conv-18/9	31.3 ± 2.75	51.49 ± 16.29	1.68 ± 0.01	0.999 ± 0
	Conv-31/8	67.4 ± 3.57	112.9 ± 30.94	2.19 ± 0.02	0.998 ± 0
	Conv-4/8	66 ± 4.71	115.99 ± 17.85	2.53 ± 0.01	0.998 ± 0
	Conv-7/8	63.4 ± 5.93	126.34 ± 39.09	2.04 ± 0.01	0.998 ± 0
	Conv-16/8	65.9 ± 4.07	104.96 ± 17.7	1.93 ± 0.01	0.998 ± 0
	Conv-23/8	38.5 ± 6.57	108.66 ± 37.1	1.81 ± 0.01	0.998 ± 0
	Conv-13/9	57.1 ± 3.31	108.37 ± 36.78	1.96 ± 0.01	0.998 ± 0
	Conv-6/10	79 ± 3.8	179.25 ± 54.5	2.19 ± 0.01	0.997 ± 0
	Org-7/9	24.5 ± 4.77	63.14 ± 38.77	1.47 ± 0.01	0.999 ± 0
	Org-29/9	34.9 ± 5.24	90.42 ± 76.19	1.16 ± 0.01	0.998 ± 0
	Org-1/9	25.5 ± 3.44	71.52 ± 54.04	1.7 ± 0.01	0.999 ± 0
	Org-8/8	26.7 ± 3.02	62.99 ± 27.72	1.86 ± 0.01	0.999 ± 0
	Org-16/8	27.6 ± 1.84	65.17 ± 17.48	1.86 ± 0.01	0.999 ± 0
	Org-23/8	26.1 ± 3.54	89.95 ± 62.83	1.76 ± 0.01	0.999 ± 0
	Org-10/10	30.6 ± 4.09	80.99 ± 28.54	1.01 ± 0.01	0.999 ± 0

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