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Study of *Lactococcus lactis* during advanced ripening stages of model cheeses characterized by GC-MS.

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ABSTRACT

Lactococcus lactis, extensively used as dairy starter, has recently been detected in cheese, as metabolically active cells, in advanced ripening stages, by culture-independent methods. In this study, we assessed the viability of *L. lactis* subsp*. lactis* in model cheeses, up to 180 days of ripening. In addition, we studied the expression of *metC* and *als* genes involved in the production of aroma compounds by Gas Chromatography-Mass Spectrometry (GC-MS). Three *L. lactis* subsp. *lactis* commercial starters were inoculated in pasteurized milk and model cheeses were manufactured and ripened for six months. Samples were analyzed at manufacturing and ripening steps, in terms of viability of *L. lactis* by both traditional plating and direct analysis of RNA by reverse transcription quantitative PCR (RT-qPCR) and in terms of aroma profile by GC-MS. Relatively to RT-qPCR analysis, *L. lactis* was found viable throughout the whole process of cheesemaking and aging, with final average loads of 3-4 Log CFU/g at 180 days. On the contrary, the microorganism was not detected, in ripened samples, by traditional plating on M17 medium, suggesting its entering in a viable but not cultivable (VBNC) state. The aroma profiles of the cheeses highlighted the presence of volatile compounds related to cheese flavor as acetoin, diacetyl, 2,3-butanediol and dimethyl disulfide, whose presence was partially correlated to *metC* and *als* genes expression. These results add new insights on the capability of *L. lactis* to persist during late cheese ripening and suggest a potential contribution of the microorganism to cheese flavor formation.

Keywords: *Lactococcus lactis;* ripening; volatile aroma compounds; RT-qPCR.

Chemical compounds studied in this article:

Lactose (PubChem CID: 84571); Glucose (PubChem CID: 53782692); Galactose (PubChem CID: 439357); Lactic acid (PubChem CID: 61503); Citric acid (PubChem CID: 311); Butyric acid (PubChem CID: 264); Acetic acid (PubChem CID: 176); Propionic acid (PubChem CID: 1032); Pyruvic acid (PubChem CID: 1060); 2-Butanone (PubChem CID: 6569); 2-Heptanone (PubChem CID: 8051); 2-Nonanone (PubChem CID: 13187); 2-Propanone, 1-hydroxy- (PubChem CID: 8299);

Acetoin (PubChem CID: 179); Acetone (PubChem CID: 180); Diacetyl (PubChem CID: 650); 1-Butanol (PubChem CID: 263); 1-Hexanol (PubChem CID: 8103); 1-Hexanol, 2-ethyl- (PubChem CID: 7720); 1-Octanol (PubChem CID: 957); 1-Octen 3-ol (PubChem CID: 18827); 2,3-Butanediol (PubChem CID: 262); 2-Buten-1-ol, 3-methyl- (PubChem CID: 11173); 3-Buten-1-ol, 3-methyl- (PubChem CID: 12988); Diethylene glycol (PubChem CID: 8117); Ethanol (PubChem CID: 702); Ethanol, 2-(2-ethoxyethoxy)- (PubChem CID: 8146); Ethanol, 2-butoxy- (PubChem CID: 8133); Isobutanol (PubChem CID: 6560); Isopentyl alcohol (PubChem CID: 31260); alpha.-Pinene (PubChem CID: 6654); delta 3-carene (PubChem CID: 26049); Limonene (PubChem CID: 22311); Menthol (PubChem CID: 1254); Disulfide, dimethyl (PubChem CID: 12232); Dimethyl sulfone (PubChem CID: 6213); 2,3-Butanediol diacetate (PubChem CID: 66193); Butyl acetate (PubChem CID: 31272); Ethyl acetate (PubChem CID: 8857); Ethyl decanoate (PubChem CID: 8048); Ethyl hexanoate (PubChem CID: 31265); Ethyl octanoate (PubChem CID: 7799); Methyl 2-hydroxy 2-methyl- propionate (PubChem CID: 75024); Hexanal (PubChem CID: 6184); Nonanal (PubChem CID: 31289); Butanoic acid (PubChem CID: 264); Decanoic acid (PubChem CID: 2969); Heptanoic acid (PubChem CID: 8094); Hexanoic acid (PubChem CID: 8892); Isobutyric acid (PubChem CID: 6590); Isovaleric acid (PubChem CID: 10430); Nonanoic acid (PubChem CID: 8158); Octanoic Acid (PubChem CID: 379); Pentanoic acid (PubChem CID: 7991); Propanoic acid (PubChem CID: 1032); Benzaldehyde (PubChem CID: 240); Benzenecarboxylic acid (PubChem CID: 243); Benzyl Alcohol (PubChem CID: 244); beta-Phenoxy ethyl alcohol (PubChem CID: 31226); Phenol (PubChem CID: 996); Phenyl ethyl Alcohol (PubChem CID: 6054); delta-Decalactone (PubChem CID: 12810); delta-Octanolactone (PubChem CID: 12777); Undecane (PubChem CID: 14257).

1. INTRODUCTION

Flavour development in dairy fermentations, most notably cheeses, results from a series of (bio) chemical processes in which starter and secondary cultures provide enzymes. *Lactococcus lactis* is the predominant microorganism in starter cultures used for cheese making and its enzymes, which act during cheese manufacture and early ripening, affect the organoleptic properties of the products in terms of texture, flavour and aroma development (Kunji et al., 1993; Ercan et al., 2015; García-cayuela et al., 2012; Rijnen et al., 2003). As the result of its technological ability, *L. lactis* has been the subject of numerous studies to determine the main relevant characteristics useful for the selection of the best strains for dairy applications (Taïbi et al., 2011; Van Hylckama Vlieg et al., 2006). Although *L. lactis* is a key microorganism in cheese making and early ripening (when it is found with high count values by traditional plating), in late ripening the microorganism is outcome by secondary microbiota, mainly lactobacilli, and its presence has been highlighted by some authors (Desfossés-Foucault et al., 2013; Dolci et al., 2014; Flórez and Mayo, 2006; Masoud et al., 2011) exclusively by culture-independent methods. Thus, it was hypothesized that *L. lactis* was present, in ripened cheeses, in a viable but not cultivable (VBNC) state and trials to resuscitate it were performed in previous studies (Ruggirello et al., 2016). In the present study, we investigated the persistence of *L. lactis* subsp*. lactis* in model cheeses in order to further confirm the evidences obtained in our previous researches (Ruggirello et al., 2016, 2014). In addition, we studied the expression, in *L. lactis*, of genes involved in the production of aroma compounds to evaluate if any possible correlation could be hypothesized between their expression and volatile aroma compounds detected in cheese samples. Precisely, we chose *metC* gene encoding cystathionine β-lyase (CβL) and *als* gene coding for α-acetolactate synthase (α-*ALS*) (Platteeuw et al., 1995; Toonen et al., 1994). CβL is responsible to catalyse the deamination of methionine to methanethiol (Smit et al., 2005; Weimer, 2007) toward the production of sulphur aroma compounds (dimethyl sulphide, DMDS and dimethyl trisulphide, DMTS) while α-*ALS* represents the first enzyme of the diacetyl/acetoin biosynthetic route (Hugenholtz and Starrenburg, 1992; Platteeuw et al., 1995; Snoep et al., 1992; Verhue and Tjan, 1991). Finally, we studied the aroma profiles of model cheeses by Gas Chromatography-Mass Spectrometry (GC-MS) and we evaluated if any possible

correlation could be hypothesized between *als* and *metC* gene expression in *L. lactis* and volatile aroma compounds detected in cheese samples in order to define a potential technological role of the microorganism also in late ripening.

2. MATERIALS AND METHODS

2.1 Cheese making and sampling.

Commercial starter cultures, named in this paper K, M and B, were purchased as lyophilized cells of *L. lactis* subsp. *lactis* and used as inoculum for cheese-making of Toma-like miniature cheeses according to the protocol described by Bertolino et al. (2008) with some modifications as reported by Ruggirello et al. (2016). Overall, six productions (2 for each starter) were carried out in aseptic conditions. Milk, curd and cheese samples were collected, for each production, in duplicate, as follow: milk before inoculum of the starter (BLANK), milk after inoculum of the starter (MILK), curd after draining (DRY), cheese immediately after salting (SALT) and cheese at 15, 30, 60, 90, 120, 150 and 180 days of ripening. Pasteurized milk used for cheese making was checked, before starter inoculum, for the absence of *L. lactis* cells by qPCR as reported by Ruggirello et al., (2014). Values of pH were measured in milk samples immediately after inoculum, curd after salting and cheese at the end of ripening.

2.2 Microbial analyses and *L. lactis* detection by qPCR.

Ten millilitres (milk) or grams (curd and cheese) of each sample were homogenised in 90 mL of sterile Ringer solution (Oxoid, Milan, Italy) in a Stomacher (Interscience, Rockland, MA, USA) for 5 min. The resulting suspension was serially diluted and included in M17 agar (Biolife, Milan, Italy) supplemented with lactose (5 g/L). In addition, the 10^{-1} dilution was plated for bulk formation as described by Ercolini et al. (2001). The plates were incubated, aerobically, at 30 °C for 48h and colony-forming units (CFU) were subsequently counted. Bulk formations were covered with 1 mL of Ringer, scraped off with a sterile L-shape spreader and harvested with a pipette in tubes, which were stored at - 20 °C before DNA extraction. DNA extracted according to the method reported by Cocolin et al. (2001) was then submitted to *L. lactis* selective qPCR analysis with the protocol

optimized and described by Ruggirello et al. (2014), in order to investigate the presence of *L. lactis* cultivable cells.

2.3 Analysis of *tuf*, *metC* and *als* gene expression by RT-qPCR.

2.3.1 RNA extraction. Ten millilitres of milk and ten grams of curd and cheese samples were flash frozen in liquid nitrogen to inhibit further microbial biochemical activities (in particular DNA transcription) and, then, stored at -80 °C until RNA extraction. Just before extraction, samples were slowly thawed at 4 ^{o}C. RNA extraction was performed by using TRIzol© reagent (FMB, Trevose, USA) according to the manufacturer's instructions with some modifications. Briefly, once thawed, each matrix was resuspended in 25 mL of cold TRIzol© reagent and homogenised for 5 min at 260 rpm with a Stomacher® 400 Circulator (Seward Ltd, Worthing, UK). Then, 1 mL of homogenised slurries was moved in a 2 mL tube containing 0.3 g of 1 mm diameter glass beads and three homogenization steps of 50, 45 and 30 sec were performed at high speed, using a Tissue Lyser LT (Qiagen, Germany). Subsequently, samples were centrifuged at 12,000 x g for 10 min at 4 ^{o}C and the supernatants collected and transferred in a new tube after removing fat top layer by a sterile loop. Two hundred millilitres of chloroform were added to the supernatants and the tubes shaken, vigorously, by hand, for 15 sec and incubated for 2–3 min at room temperature. After centrifugation at 12,000 x g for 15 min at 4 °C, the aqueous phase was moved in a new tube and added of 0.5 mL 100% isopropanol for RNA precipitation. In order to remove contaminants, the solution was purified using RNeasy columns (QIAGEN, Hilden, Germany), following the manufacturer's recommendations with an additional final lithium chloride (LiCl) precipitation. Briefly, LiCl (Life technologies, Dublin, Ireland) was added to the final volume of 30 µl of the sample with a final concentration of 3 M and all the samples were chilled 1 h at -20 ^{o}C. After centrifugation at 16,000 x g for 20 min at 4 °C, supernatant was discarded and RNA pellet resuspended in sterile water. The addition of LiCl allows to remove inhibitors of RNA translation and cDNA synthesis from RNA preparations (Cathala et al., 1983). Remaining DNA was digested using TURBO-DNase (Ambion, Milan, Italy) according to the manufacturer's protocol. After treatment, 15 mM EDTA was used to deactivate DNase enzyme and 1U RiboGuard RNase Inhibitor (Epicentre, Madison, WI, USA) was

added to the samples subsequently incubated at 75 ^{o}C for 10 min and, finally, stored at -80 °C. RNA extractions were performed in duplicate for each dairy sample. The amount and integrity of total RNA extracted were analysed by using Experion™ Automated Electrophoresis System with Experion HighSens chips (Bio-Rad, Milan, Italy). The absence of residual DNA was verified by qPCR using the protocol described by Ruggirello et al. (2014).

2.3.2 *Tuf* gene analysis. In order to study the expression, in *L. lactis*, of the housekeeping gene *tuf*, encoding elongation factor Tu, RNA samples were submitted to retrotranscription by M-MLV Reverse Transcriptase kit (Promega, Milan, Italy). Briefly, RT reactions were performed as follows: 0.5 µL of RNA were mixed with 1 µL of TUF2r (100 mM) (Ruggirello et al., 2014) in a reaction volume of 10 µL by addition of ultrapure water. The mixtures were maintained at 75 °C for 5 min for RNA denaturation and immediately placed on ice for 10 min. Five microliters of M-MLV RT Buffer (1X), 5 µL of dNTPs (10mM each) and 1 µL of M-MLV Reverse Transcriptase (8 U/µL) were added to the mixtures in a final volume of 25 µL by addition of ultrapure water. RT reactions were carried out at 42 °C for 1 h in a Biorad DNA Engine thermal cycler (Bio-Rad) and the cDNA submitted to qPCR by using primers TUF2f and TUF2r, according to the protocol optimized by Ruggirello et al. (2014).

***2.3.3 metC* and *als* gene analysis**

Milk, curd and cheese samples were then investigated for the expression, in *L. lactis*, of *metC* and *als* genes involved in aroma production pathways. The *metC* and *als* genes expression was compared with the expression of *tuf* gene which was used as reference gene.

2.3.3.1 Primer efficiency. *L. lactis* selective primers were used (Table 1) and, before performing in RT-qPCR, their efficiency (E) was determined on DNA extracted from *L. lactis* starter B used as template. The E values were calculated for each gene according to Pfaffl (2001). Samples of DNA were diluted (100, 50, 25, 5, 1 and 0.25 ng/ml) and used to construct the standard curves. Amplifications were performed in a Biorad DNA Engine thermal cycler (Bio-Rad), in 10 µl reaction mixture containing 1 µl of DNA, 5 µl of SsoAdvancedTM SYBR@ Green Supermix (Bio-Rad, Hercules, CA, USA), 400 nM and 50 nM of Tuf2f and Tuf2r primers or 900 nM of metCr-metCf and

alsr-alsf primer pair. A negative control with no DNA was also included in each run, as well as a positive control with genomic *L. lactis* DNA. Thermal cycle consisted of a 20 sec denaturation step at 95 °C, followed by 40 two-step cycles of 5 sec at 95 °C and 20 sec at 54.2 °C. In addition, melting curve analysis was performed to check the presence of a single amplification product for each primer pair. Dilutions were replicated three times and used singly.

2.3.3.2 RT-qPCR. Retrotranscription was performed by M-MLV Reverse Transcriptase kit (Promega, Milan, Italy) as reported above (2.3.2) by using primers metCr and alsr for *metC* and *als* gene analysis, respectively. Complementary DNA (cDNA) of MILK, SALT and 15, 30, 60, 90, 150, 180 ripening day samples was then amplified by qPCR in a Biorad DNA Engine thermal cycler (Bio-Rad) using 96-well plates and following the protocol reported above (2.3.3.1). In addition, melting curve analysis was performed to check the presence of a single amplification product for each primer pair.

2.4 Analysis of volatile compounds.

Volatile organic compounds (VOCs) were studied in curd and cheese samples. Two grams of each grated and homogenised sample were placed in a 20 mL vial, added of 10 µl of internal standard solution (2-octanol) at 10 ppm, fitted with a PTFE silicone septum (Supelco, Bellefonte, PA, USA) through which the headspace solid phase micro extraction (HS-SPME) syringe needle, fitted with a Stable Flex 1 cm–50/30 µm divinylbenzene-carboxen-polydimethylsiloxane (DVB-CAR-PDMS) fibre (Supelco, Bellefonte, PA, USA), was introduced. The vials were placed in a heat/stir plate at 40 °C for 20 min for the extraction phase. After exposure to the headspace (HS), the fibre was retracted and transferred to the GC/qMS injector operated in the splitless mode at 260 °C for 2 min. GC/qMS analysis was performed with a Shimadzu GC-2010 gas chromatograph equipped with a Shimadzu QP-2010 Plus quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a DB-WAXETR capillary column (30 m × 0.25 mm, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, USA). The temperature program started at 40 °C for 5 min, increased at a rate of 10 °C min^{-1} to 80 °C, then at rate of 5 °C min^{-1} to 240 °C. The carrier gas (He) flow-rate was 1 mL min^{-1}. The injection port temperature was 260 °C; the ion source and the interface

temperature were 240 °C. The detection was carried out by electron impact mass spectrometry in total ion current (TIC) mode, using an ionization energy of 70 eV. The mass acquisition range was m/z 33–300. Peak identification of each volatile metabolite was performed by comparison of the retention time and mass spectra of eluting compounds to those of the pure standard. Compounds were semi quantified by calculating the area under the ion quantifier peak of each compound (Yarlagadda et al., 2014). All analyses were performed in duplicate for each samples.

2.5 Organic acid and sugar profiles.

The organic acids (citric, pyruvic, lactic, acetic, butyric and propionic) and sugars (lactose, glucose and galactose) were determined for all dairy samples by high performance liquid chromatography (HPLC), according to the method described by Marchiani et al. (2016). Briefly, samples (5 g) were added to 25 mL of 0.013 NH2SO4 (mobile phase) and mixed for 10 min with a Stomacher® 400 Circulator (Seward Ltd, Worthing, UK). The slurries were subsequently centrifuged at 5,000 x g for 30 min at 10 °C, and the supernatant was filtered through 0.45 µm polypropylene membrane filter (VWR, Milan, Italy). The HPLC system (Thermo Finnigan Spectra System, San Jose, USA) was equipped with an isocratic pump (P4000), a multiple autosampler (AS3000) fitted with a 20 µL loop, a UV detector (UV100) set at 210 and a refractive index detector RI-150. The analyses were performed isocratically at 0.8 mL min-1 at 65 °C, with a 300 × 7.8 mm i.e. cation exchange column (Aminex HPX-87H) equipped with a Cation H+ Microguard cartridge (Bio-Rad Laboratories, Hercules, USA). Three replicates were analysed for each sample. Data processing was carried out using Chrom QuestTM chromatography data system (ThermoQuest Corporation, San Jose, USA). Analytical grade reagents were used as standards (Sigma-Aldrich, St. Louis, USA). All analyses were performed in duplicate for each samples.

2.6 Statistical analysis.

Statistical analysis was performed using the Microsoft Excel 2010, SPSS v23.0 (SPSS, Inc., Chicago, Ill., USA) R package (www.r-project.org) and STATISTICA (StatSoft Inc., Tulsa, USA) programs.

285 Threshold cycle (Ct) values from RT-qPCR were exported to Excel for relative quantification. In
286 order to use parametric tests for gene expression analysis (ANOVA), off-scale data were replaced
287 with fictive Ct values (Kubista and Sindelka, 2007). Fictive Ct values are set to the highest Ct
288 observed for a truly positive sample, which is assumed the level of detection (LOD), plus one. In
289 the current study, the cut off was set at 36 cycles. As reported by some authors (Kubista and
290 Sindelka, 2007; Mataragas et al., 2015), this corresponds to assigning a concentration that is half
291 of the LOD to the off-scale samples. This is no more erroneous than assigning a zero
292 concentration to these samples, because there is no evidence that they are blank. It is only known
293 that the amount of the target in these samples is lower than the detection limit.
294 Data were converted to relative expression and log-values (fold change) (Kubista and Sindelka,
295 2007; Livak and Schmittgen, 2001). In order to measure gene expression as a function of time and
296 the three *L. lactis* starters, relative quantification was carried out. Thus, target gene expression in
297 curd and cheese samples was compared with the expression detected in MILK samples (after
298 inoculum of the starters) by one-way ANOVA. Tuckey's test for means comparison was carried out
299 at a P value of < 0.05. Mixed repeated measures ANOVA was performed to investigate whether
300 *metC* and *als* gene expression was dependent on different ripening times (factor A) and/or different
301 starters (factor B).
302 VOCs production was visualized by heatmap designed by R package and Ward's criterion was
303 used for clustering. Principal component analysis (PCA) was performed specifically on DMDS,
304 acetoin, diacetyl and 2,3-bunanediol compounds.
305 Mixed repeated measures ANOVA and Tuckey's test for means comparison were carried out for
306 HPLC values, in order to explore differences related to cheeses manufactured by different starters
307 and sampling time. For all the analyses significant results were associated to $P < 0.05$ or $P < 0.001$
308 or $P < 0.001$.
309

310 **3. Results**

311 **3.1 *L. lactis* detection throughout manufacturing and ripening**

312 **3.1.1 Culture-dependent approach.** By plating on M17 agar, the three starters were found, after
313 inoculum in MILK, with loads of about 7-8 Log CFU/mL and they reached maximum peaks of about
314 8-9 Log CFU/mL in curd after drying (DRY) and salting step (SALT); then, at 15 and 30 ripening
315 days, they showed a downward trend, reaching the lowest counts of 3-4 Log CFU/g at 90 ripening
316 days (B and M) and of 5 Log CFU/g at 60 ripening days (K). After that, the starters did not grow
317 anymore on M17 (Table 2). The results of bulk formation analysis confirmed microbial count data
318 (Table 2), since no *L. lactis* cultivable cells were detected by qPCR in those samples where no
319 colonies grew on M17. A decrease of pH from 6.5 ± 0.2 (MILK) to 5.1 ± 0.3 (SALT) and to 4.2 ± 0.2
320 (180) was observed.
321 **3.1.2 Culture-independent approach.** The presence of metabolically active cells of *L. lactis*
322 starters was investigated by RT-qPCR directly on milk, curd and cheese samples. Their presence
323 was quantified by interpolating the Ct values obtained in the standard curve equation optimised
324 and described in a previous work (Ruggirello et al., 2014). The results are presented in Table 2
325 and compared with the data obtained from culture-dependent approach. Starters were found in
326 MILK with minimal loads (about 1-2 Log CFU/mL), probably due to the fact that, after the inoculum
327 of the starter, *L. lactis* cells had not yet metabolically reactivated (sampling time was 5 minutes
328 subsequent the inoculum). Then, the three starters reached values of about 7-8 Log CFU/g during
329 manufacturing and their performance, throughout ripening, seemed not to be affected from the
330 initial low loads in milk. They reached values of about 6 Log CFU/g at 120 days of ripening and at
331 150 days, starter B was still found at 6 Log CFU/g while K and M loads dropped to 4 Log CFU/g
332 (Table 2). Finally, they were all present after 180 ripening days with 3-4 Log CFU/g.
333
334 **3.2 Relative expression of *L. lactis metC* and *als* genes in miniature cheeses.**
335 The expression of *metC* and *als* genes in *L. lactis* starters was studied by RT-qPCR throughout
336 manufacturing and ripening of miniature Toma-like cheeses. Efficiency and R^2 values are shown in
337 Table 1. No primer-dimers were generated during the 40 cycles.
338 Figure 1 shows the changes of the two gene expression from manufacturing to 180 ripening days,
339 compared with their expression in the samples taken as control (MILK). Because no C_t values were

340 registered for the *als* gene in cheeses made with the starter M, these samples were excluded from
341 the graph.
342 One-way ANOVA highlighted a significant over expression of *metC* gene in cheese sample SALT
343 produced with K starter and in samples at 150 and 180 ripening days produced with starter B. No
344 significant differences were found for *als* gene expression along ripening time.
345 Mixed repeated measures ANOVA underlined a significant influence on *metC* expression due to
346 ripening time and starter strain ($F= 7.46$, $P < 0.05$), as well as a significant interaction between the
347 two variables (factor A and B) ($F= 20.694$, $P < 0.05$), while *als* gene expression was not statistically
348 affected neither by factor A nor factor B. The *metC* gene was over-expressed by starters K and B
349 after salting time ($F= 25.349$, $P < 0,05$) whereas it was down regulated in starter M. K was over
350 expressed at 15 and 30 days ($F= 27.311$ and $F= 11.99$ respectively, $P < 0,05$) unlike B and M that
351 were down regulated. At 60 days all the starters up regulated the gene. Down regulation started
352 from 90 days in K and from 150 days in M. The *metC* gene expression increased in starter B from
353 60 to 180 days of ripening with a significance difference compared to starter M at 150 days ($F=$
354 $78,97$, $P<0,05$) and starters M and K at 180 days ($F= 133.73$, $P < 0,05$).
355 The *als* gene was down regulated during both manufacturing and ripening by B and K starters
356 (values for K not detected at 60, 90 and 150 days), while it was over expressed in 180-old cheeses
357 made with B ($F= 65.21$, $P < 0.05$). As reported above, no C_t values were detected in starter M
358
359 **3.3 Volatile compounds analysis**.
360 HS-SPME and GC-MS allowed the identification of 56 different VOCs: 11 acids, 7 esters, 7
361 ketones, 2 aldehydes, 14 alcohols, 2 lactones, 4 monoterpenes, 2 sulphur compounds, 6 aromatic
362 compounds and 1 undecane compound not ascribable to these chemical classes. Mixed repeated
363 measures ANOVA was carried out to investigate whether VOCs production in cheeses was
364 different depending on the different ripening time (factor A) and/or on the different *L. lactis* starter
365 strain (factor B). The results showed that 82.1% ($F=32.69$, $P< 0.05$) and 53.5% ($F=18.21$, $P< 0.05$)
366 of the VOCs were significantly influenced by factor A and factor B, respectively. Moreover, a
367 significant interaction was highlighted between the two factors ($F= 8.63$, $P < 0.05$) for 57.1% of the

368 VOCs, showing that ripening time had a different effect on VOCs production, depending on *L. lactis*
369 starter strain used in miniature cheeses. Ketones were the chemical class mainly represented
370 during manufacturing (48.2% w/w of VOCs) and ripening (53.6% w/w of VOCs). Alcohols were
371 found at percentage of 36.4% and 24.9% and acids at 9.8% w/w and 20.2% w/w in manufacturing
372 and ripening, respectively. Regarding VOCs production, an interesting separation between milk,
373 manufacturing and ripening time was showed by heatmap in figure 2. Early ripened samples were
374 characterized by high concentration of ketones as 1-hydroxy- 2-propanone, acetoin, acetone and
375 diacetyl; among alcohols, 2-ethyl-1-hexanol, 2,3-butanediol, 3- methyl-2-buten-1-ol and ethanol
376 were the most abundant followed by monoterpenes (limonene and menthol), 2,3-butanediol
377 diacetate ester and hexanal aldehyde. During cheese ripening, an increment of acids was
378 observed. The highest amounts were found for acetic, butanoic and hexanoic acids with higher
379 levels of butanoic acid in cheeses produced with starter B more than the ones obtained with K and
380 M ($F= 36.75$, $P < 0.05$). It was interesting to note (figure 2) that starter K moved earlier to the
381 cluster on the left (after just 30 days) compared to B and M due to its high production of acetate,
382 ethanol, acetoin, diacetyl and 2,3-butanediol compounds.
383 Aroma compounds derived from cysteine and methionine catabolism (DMDS) and citrate (acetoin,
384 diacetyl and 2,3-butanediol) pathways were specifically analysed by PCA analysis (Figure 3).
385 Results underlined a certain degree of separation among the three *L. lactis* starters. DMDS
386 originated from cysteine and methionine catabolism, represented 2.3% of total VOCs during
387 manufacturing and ripening. The compound was significantly affected by both factor A ($F= 30.16$,
388 $P< 0.05$) and B ($F=130.73$, $P < 0.05$), being produced with higher concentrations by starters K and
389 B than in cheese produced with starter M. Also acetoin was significantly affected by both factors
390 ($F= 27.69$ and $F= 24.01$ respectively for starter and time factors, $P< 0.05$). 2-Butanone varied
391 significantly among the starters being more produced by K ($F= 18.08$, $P< 0.05$) whereas 2,3-
392 butanediol and diacetyl amounts varied only along ripening time ($F= 3.56$ and $F= 6.86$ respectively,
393 $P< =0.05$) without significant differences among the starter. Among ketones, acetoin, which forms
394 from diacetyl together with 2-butanone and 2, 3-butanediol, was the most abundant compound
395 detected in ripened samples. Its highest amount was found in samples at 15 days and decreased,

396 during ripening, in cheeses produced with starters B and M while remained high in cheeses made
397 with starter K ($P < 0.05$). Diacetyl was found starting from MILK samples after inoculum and
398 increased in ripened cheeses. Lastly, 2,3-butanediol, was detected in manufacturing (DRY
399 samples) and showed constant values until 180 days of ripening.
400
401 **3.4 Organic acid and sugar profiles.** Organic acid and sugar contents are reported in Table 3.
402 Mixed repeated measures ANOVA was carried out to investigate whether organic acids and sugars
403 production in cheeses was different depending on the different ripening time (factor A) and/or on
404 the different *L. lactis* starter strain (factor B). Results showed that lactose was completely
405 converted into lactate by starters metabolism or lost into whey after salting step by starters K and
406 M while after 15 days by B ($F= 3179.22$, $P< 0.001$). Glucose was not detected neither in milk nor in
407 cheese samples, whereas galactose was present at low concentrations from manufacturing (DRY)
408 until the end of ripening and affected more significantly by factor time ($F=183.47$, $P< 0,05$) than
409 factor starter ($F=15.39$, $P< 0,05$). Lactic acid was the most abundant organic acid in all the ripened
410 samples analysed. Its production, significantly affected by factor time, increased until to
411 represent30%, 25% and 25% of the total organic acid content in 180 days ripened cheeses made
412 with starter B, M and K, respectively. Citric acid, already detected in milk samples, was significantly
413 consumed throughout manufacturing and ripening ($F= 651.18$, $P< 0.01$), due to its conversion into
414 acetate, acetoin and diacetyl. Acetic acid, not significantly affected neither by factor A nor factor B,
415 was present at low concentrations and reached the maximum values of 0.62, 1.65 and 1.70 mg/g
416 in cheeses at 180 days, manufactured with starters B, K and M, respectively. Highest
417 concentrations of pyruvic acid were detected during manufacturing (DRY samples); then, a light
418 decrease was observed until 180 days of ripening when cheeses made with starter B showed
419 higher concentration than K and M ($F= 133.33$, $P< 0.001$). Propionic acid concentration, affected
420 by factor time ($F= 9103.23$, $P< 0.01$),increased throughout manufacture and early ripening without
421 significant differences between starters. Then, its trend slightly decreased and at 180 days of
422 ripening, it represented 3.6% of total organic acids. Butyric acid, affected by both factors, time and

starter, formed during manufacturing and increased significantly during late ripening stages (F= 7925.47, P< 0.001) reaching higher value for starter B at 180 days (F= 252, P< 0.001).

4. Discussion

Microbiological analysis showed that *L. lactis* was present with the highest loads of 8-9 Log CFU/g during manufacturing and early ripening and it started to decrease, markedly, at 30 days of ripening until not being anymore detected in late stages. After salting, at 15 ripening days, an initial slight decrement was observed. It is known that salt addition promotes starter permeabilisation and cell autolysis during early stages of ripening (Rulikowska et al., 2013; Wilkinson et al., 1994), which allow intracellular enzymes to access to the substrates within the cheese matrix (Guinee, 2004; Sheehan et al., 2005). Several authors (McCarthy et al., 2015; Møller et al., 2007; Ramírez-Nuñez and Ruth Romero-Medran, 2011; Rulikowska et al., 2013; Yanachkina et al., 2016) studied the effect, on *L. lactis* cell autolysis, of changes in cheese parameter as salt concentration, pH gradient, fat content, ripening time, and they suggested their significant impact on *L. lactis* growth dynamics. They also underlined that the impact is strictly strain dependent since there is a tolerance degree by *L. lactis* in response to cheese changes. Moreover, Taibi et al. (2011) hypothesized that the reduction of viable counts, following osmotic stress, could be attributed to the entering of the cells in a catabolically and/or anabolically inactive state, which made them unable to multiply, more than to a process of autolysis. According with these findings, we observed, in a previous work (Ruggirello et al., 2016), a strain dependent attitude of *L. lactis* to enter in a VBNC state as well as to resuscitate on an enrichment medium.

In agreement with these observations and considering microbial count results, starters B and M behaved more similarly than K, dropping at 60 days from 6-7 to 3 Log CFU/g, whereas K decreased slowly to 5 Log CFU/g and disappeared in 90 day cheeses. This could be due to the different capability of *L. lactis* starters to response to environmental variations which occur during ripening process (Dugat-Bony et al., 2015). Particularly, the high NaCl content may transform a part of the lactococcal population into VBNC cells or intact dead cells, which are only detected by culture-independent methods as PCR-TTGE (Casalta et al., 2009). Nevertheless, some authors

(Broadbent et al., 2013; Muehlenkamp-Ulate and Warthesen, 1999) found *L. lactis* starters, by plating technique, until six months of ripening in Cheddar cheese, with loads of 3-6 Log CFU/g. Their explanation was that microbial population dynamics are altered by perturbations in cheese microenvironment (e.g., acid and salt in moisture (S/M) contents, redox, and water activity).

A RT-qPCR protocol, selective for *L. lactis*, was applied to the total microbial RNA extracted directly from milk, curd and cheese matrices, overcoming the issues related to the cultivation step. No statistical differences were observed between three starters by RT-qPCR. Results showed the presence of *L. lactis* in all miniature cheeses, up to the sixth month of ripening confirming the presence of viable cells of this microorganism during the whole ripening process. No statistical differences were observed between the three starters by RT-qPCR. Data obtained by direct *L. lactis* RNA analysis were compared with data observed by traditional plating and a similar trend was observed in samples taken at manufacturing and early ripening stages. On the contrary, starting from cheeses at 60 days of ripening, counts detected by direct RNA analysis were markedly higher than values obtained on M17 and, finally, *L. lactis* was not more found by traditional plating. This discrepancy highlighted, one more time, a probably entry of *L. lactis* in a VBNC state as hypothesized in our previous work (Ruggirello et al., 2016) and by other authors (Desfossés-Foucault et al., 2013; Dolci et al., 2014, 2010; Flórez and Mayo, 2006; Masoud et al., 2011; Rantsiou et al., 2008). These evidences support the thesis that cultural media cannot be used to enumerate or detect VBNC cells (Colwel, 2009) and underline the usefulness of direct RNA analysis in assessing the viability of microorganisms, which could retain the ability of synthesizing RNA, although losing the capability to form colonies on synthetic medium. However, purity and integrity of RNA are critical elements for the overall success of RNA-based analyses (Fleige and Pfaffl, 2006) and getting good quality nucleic acids can be tough and laborious when the matrix is complex as cheese. A polyphasic approach, combining culture-dependent and -independent methods, should minimize the biases in both methods, showing a more accurate view of the structure of microbial communities (Ampe et al., 1999; Delbès et al., 2007; Ercolini D, Moschetti G, Blaiotta G, 2001; Flórez and Mayo, 2006).

478 Our data were in agreement with results reported by other authors. Desfossés-Foucault et al.
479 (2014, 2013) found, by RT-qPCR, that *L. lactis* retained transcriptional activity for six month of
480 Cheddar ripening while populations rapidly declined to totally disappear, in early ripening stages,
481 on M17 medium; Dolci et al. (2014) found *L. lactis*, by RT-PCR-DGGE and pyrosequencing, in
482 Fontina PDO cheese, up to three months of ripening. Similarly, other authors detected the
483 microorganism, by culture-independent methods, in the following ripened products: Danish
484 cheeses (Masoud et al., 2011), Castelmagno PDO (Dolci et al., 2010), Feta PDO (Rantsiou et al.,
485 2008), Toma PDO, Raschera PDO, Asiago PDO and Pecorino sardo PDO (Ruggirello et al., 2014)
486 and Celenzana (Casalta et al., 2009).
487 Our results partially differed from data found in our previous study (Ruggirello et al., 2016) where
488 the same *L. lactis* starters were used in model cheeses with the only difference that no strict
489 aseptic conditions were used during manufacturing and, probably, for this reason, higher microbial
490 counts were found on M17 during the ripening due to the growth of non- starter lactic acid bacteria
491 (NSLAB). Furthermore, in the present research, the three starters entered in VBNC state earlier
492 compared to the performance showed in the previous study (Ruggirello et al., 2016). This fact
493 could be related to physiological stresses they underwent during the two similar but probably not
494 identical experimental manufacturing conditions. These results support, once again, the hypothesis
495 that *L. lactis* entering in VBNC state is not only strain dependent, but is also affected by the
496 different environmental parameters and changes. Stuart et al. (1999) demonstrated the different
497 ability of *L. lactis* strains to exhaust lactose, which delayed their entry in VBNC state. Sugar
498 starvation has been recognized as a key stress in lactococci, although also others stress factors,
499 as pH or temperature, can contribute to VBNC state, during fermentation process (Ganesan et al.,
500 2007; Weimer, 2011).
501 In order to evaluate if *L. lactis*, in addition to being alive during ripening time, was also
502 metabolically active in terms of technological gene expression, *metC* and *als* genes were chosen
503 to be studied because involved in important aroma production pathways. Particularly, *metC* gene
504 encodes CβL (Alting et al., 1995) that can convert cystathionine to homocysteine but is also able to
505 catalyse an α,γ elimination. Starting from methionine as substrate, it catalyses the production of

506 volatile sulphur compounds (DMDS and DMTS) which are important flavours in cheese
507 (McSweeney and Sousa, 2000; Rutten et al., 2000). DMDS is associated with sensory attribute of
508 garlic, cheesy or sulphur aroma (Rijnen et al., 2003) while DMTS is related to aroma of cooked
509 cabbage, broccoli or cauliflower (McSweeney and Sousa, 2000). Fernández et al. (2011) found
510 that inactivation of CβL does not affect the formation of VOCs and that other genes, homologous to
511 cystathionine lyase and aminotransferases, are present in the genome of *L. lactis,* and they could
512 also contributed to VOCs production (Bolotin et al., 2001). *Als* gene encodes α-*ALS* (Platteeuw et
513 al., 1995; Toonen et al., 1994) involved in the biosynthetic pathway of diacetyl/acetoin and 2,3-
514 butanediol, which are the principal flavour compounds produced from citrate metabolism (Hassan
515 et al., 2013). Diacetyl is an important aroma compound in a number of cheese varieties, including
516 Dutch-type cheeses, Quarg and Cottage cheese (McSweeney and Sousa, 2000). The *metC* and
517 *als* genes expression results were showed in terms of fold change during manufacturing and
518 ripening time respect to time 0, corresponding to milk samples after inoculum of the starter (MILK).
519 The *metC* gene was either down or over expressed during manufacturing and ripening time in
520 respect to the inoculated milk samples. These dynamics reflect the several perturbations which
521 happen during cheese making and ripening processes. The three starters behaved differently. In
522 particular starter K showed an up regulation after salting step and starter B during late ripening.
523 Differently, *als* gene was down regulated in all samples with the exception of cheeses at 180 days
524 of ripening and produced with starter B, where the gene was over expressed. Then, the presence
525 of aroma compounds related with *metC* and *als* gene pathways were evaluated. Notably, DMDS
526 was detected, even if in small amounts, in all cheeses, throughout manufacturing and ripening with
527 higher amounts in starter B and K than in starter M. On the contrary, acetoin and diacetyl ketones
528 and 2,3-butanediol alcohol were among the most abundant molecules found during the whole
529 process. Usually, acetoin is produced in much higher quantities than diacetyl whereas production
530 of 2,3- butanediol has never been studied in detail (Hassan et al., 2013). Also, despite its
531 importance, the exact reactions which result in the formation of diacetyl remain unclear. In addition,
532 aroma production varied according to the starters used for cheesemaking. Thus, higher amounts of
533 acetoin were detected in cheeses produced with starter K, while diacetyl and 2,3-butanediol were

more abundant in cheeses produced with starter K and M. Finally, we evaluated if any correlation could be hypothesized between gene expression and aroma analysis. While *metC* gene expression could be related to the production of DMDS, on the contrary, the presence of acetoin, diacetyl and 2,3-butanediol in cheeses could not be correlated with down regulation of *als* gene during manufacturing and ripening time. It could be hypothesized that the early *als* gene expression, after starter inoculum in milk, led to release of α-acetolactate, which was then converted to aroma compounds during ripening time. Surprisingly, a marked up expression of *als* gene in cheeses produced at 180 days with starter B was detected. These results highlight a transcriptional activity, even if minimal, in *L. lactis* also during late ripening, supposedly related to the production of flavour compounds, according with observations reported by Desfossés-Foucault et al. (2014). Moreover, our results confirmed, especially for *metC*, a starter's effect. This underlines the importance to emphasize, as already known, starter characteristics in strain selection.

Volatile compound analysis by GC-MS showed an interesting separation, in terms of aroma production, between milk, early production and ripening time. Higher amounts of ketones and alcohols were detected during manufacturing and in early ripened cheeses while high contents of acids were observed during late ripening. Ketones are formed by enzymatic oxidation of free fatty acids to β-ketoacids and their consequent decarboxylation to ketones. They are very important compounds for dairy products because they have very particular odours and low perception thresholds (McSweeney, 2004; McSweeney and Sousa, 2000). Their presence during manufacturing could corroborate higher *als* gene expression in MILK than during ripening time. Finally, the trend of the different compounds detected and quantified by HPLC was comparable with the one usually found in commercial cheeses, confirming the experimental reliability of these model cheeses.

5. Conclusion

The results reported in the present study add new insights on the capability of *L. lactis* to persist in cheese, during late ripening and up to six months, as metabolically active cells in a hypothetical

562 VBNC state, suggesting a possible technological contribution of the microorganism also during
563 ripening. The expression of *metC* and *als* genes in *L. lactis*, partially associated with the presence
564 of volatile aroma compounds in ripened cheeses, could support our thesis. In addition, the *L. lactis*
565 starters used in this study for the production of model cheeses behaved differently, in part,
566 highlighting, once again, the importance of a selection at strain level in new starter formulation.
567 Data obtained from this study could be considered a first preliminary contribute to the development
568 of new potential tools for starter selection. Nevertheless, additional efforts will be needed to better
569 interpret *L. lactis* VBNC state in cheese and to investigate its potential contribution to flavour
570 formation in late ripening.
571
572 **Author Contributions**
573 Conceived and designed the experiments: LC, PD, MR. Performed the experiments: MR, MG, MB.
574 Analysed the data: MR, IF, MB. Contributed reagents/materials/analysis tools: LC, PD. Wrote the
575 paper: MR.
576
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