Lactobacillus paracasei 4341 as adjunct culture to enhance flavor in short ripened Caciotta-type cheese

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EB, substantial contributions to conception and design, acquisition of data, analysis and interpretation of all the data, drafting the article. **CM**, substantial contributions to conception and design, organic acid and volatile compounds analysis, interpretation of data, drafting the article. **AL**, RealTime qPCR assay statistical analysis, interpretation of data, drafting the article. **MA**, Color, rheological, sensory and statistical analysis, drafting the article. **EN**, critical revision and final approval of the version to be published. **FG**, substantial contributions to conception, design analysis, interpretation of data and drafting the article. **MG** substantial contributions to conception, interpretation of data, drafting the final version to be published.

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Abstract

Caciotta is the name used to define a type of Italian semi-hard cheese Caciotta-type cheese. Due to the short ripening time, pasteurization is necessary to eliminate the potential pathogenic bacteria, which may be present in raw milk, causing also the reduction of ripened cheese flavor. The purpose of this research was to evaluate the effect of a selected wild *Lactobacillus paracasei* strain experimentally used as adjunct culture to enhance the flavour formation in a short-ripened caciotta-type cheese. An integrated polyphasic approach was used to compare the experimental and control Caciotta produced in a company located in Emilia Romagna region (Italy). It was demonstrated how the *L. paracasei* 4341 was able to develop in curd and cheese interacting with the acidifying commercial starter. The main acidifying starter species, were differently affected by the presence of the adjunct culture. *Streptococcus thermophilus* shown comparable behavior in all cheese-making step of control and experimental Caciotta, while *Lactobacillus delbrueckii* subsp *bulgaricus* , growth was slowed down by the presence of the adjunct culture during the whole ripening time. The higher amount of volatile compounds and organic acids due to the adjunct *L. paracasei* 4341 lead to a clear differentiation of the experimental Caciotta respect to the control, in terms of aromatic profile, color, texture and sensorial perception.

Keywords: Experimental Caciotta cheese, adjunct culture *Lactobacillus paracasei*, cheese flavour, RT-qPCR, sensory characteristics

1. Introduction

The name Caciotta derives from the Italian term “cacio”, which is the familiar term to indicate the cheese (Mucchetti, & Neviani, 2006). The organoleptic properties of this kind of cheese could vary

depending on the tradition of the different geographical zone where it is produced (Gobbetti, Neviani, Fox, & Varanini, 2018). Usually, Caciotta is intended as a semi-soft cheese with a short-medium ripening time, with a weight of around 1 kg produced from pasteurized whole cow`s milk alone or a mixture of cow's and ewe's milk. After pasteurization, the milk is cooled at 37°C and usually inoculated with commercial/selected thermophilic and/or mesophilic lactic acid bacteria (Gobbetti, Neviani, Fox, & Varanini, 2018). The rind has a dark ivory color, and the inner part has a lighter color and a compact texture (Aquilanti et al., 2011). The ripening time, (commonly from 15–20 days to 2–6 months), can make pasteurization necessary to eliminate the potential pathogenic bacteria which may be present in raw milk (Aquilanti et al., 2011). The flavour of Caciotta can vary in function of the production area, time of ripening and the milk used. Differences in the aroma profile of cows', ewes' and goat's milk are known, and, among them cow milk is known as the poorest one in terms of quantity and variety of aroma compounds (Moio, Dekimpe, & Etievant, 1993).

Moreover, by eliminating the majority of microorganisms, the pasteurization not only reduces the potential defects, but also drastically impact on the overall flavor of ripened cheese reducing the indigenous microflora that are known to contribute to the flavor of cheeses made with raw milk (Buchin et al., 1998Chambers, Esteve & Retiveau, 2009;). It has been already reported from different authors that cheeses made with pasteurized milk have a lower overall aroma intensity and somewhat different flavors, than those made from raw milk (Albenzio et al., 2001; Chambers, Corsetti, Minervini & Gobbetti, 2006; Aquilanti et al., 2011; Esteve & Retiveau, 2009; Di Cagno, Quinto,;).

Differences in terms of sensory profile were also found in uncooked and cooked cow's milk cheeses (Cheddar, Gouda, Raclette, Morbier-type, Cantal-type) compared to sheep or goat milk cheeses (Ballesteros, Poveda, Gonza, Cabezas, 2006Rodriguez-Alonso, Centeno, Grabal, 2009; Cornu et al., 2009), due to the diverse raw milk microflora (Callon, Berdague´, Dufour & Montel, 2005).

Moreover, the reason of "lack of flavour" in cheeses produced with pasteurized milk (Chambers, Esteve & Retiveau, 2009; Colonna, Durham & Meunier-Goddik, 2011) can be also due to the denaturation of milk enzymes such as proteases or lipases (Hickey, Kilcawley, Beresford & Wilkinson, 2007; Crow, Curry & Hayes, 2001).

For the cheese industry it is mandatory to produce safe cheeses, but without undervaluing the organoleptic properties, that are crucial in determining consumer's acceptance. For this reason, the producers began to look for new strategies such as the use of "adjunct cultures", that can be defined as selected strains added to milk during cheese-making for different purposes than lactic acid production (El Soda, Madkor & Tong, 2000; Settanni & Moschetti, 2001). This definition almost coincides with that of secondary starters which are involved in the development and improvement of cheese sensory quality or in the speedup of cheese ripening (Settanni & Moschetti, 2001; Smid & Kleerebezem, 2014). The adjuncts cultures are specifically selected and intentionally added during cheese-making process to increment the autochthonous milk non-starter lactic acid bacteria (NSLAB) reduced by pasteurization (Leroy & de Vuyst, 2004). NSLAB mainly consist of mesophilic facultative and obligate hetero-fermentative lactobacilli belonging to *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, *Lactobacillus rhamnosus* and *Lactobacillus casei* species (Levante et al., 2017). NSLAB have the opposite growth kinetic as compared to starter lactic acid bacteria (SLAB) commonly used in cheeses production. In fact, after curd manufacture, their number is low, ranging from 10^2 to 10^3 cfu g/1, and then increases until 10^7-10^9 cfu g/1 after a few to several months of ripening (Gatti, Bottari, Lazzi, Neviani & Mucchetti, 2013; Gobbetti et al., 2018), thanks to their capacity to tolerate the hostile environment of cheese, characterized by low pH, presence of salt, low moisture and nutrient depletion. Their multiplication in these conditions contribute to the development of the typical flavor of many cheeses (Settanni & Moschetti, 2001). In fact, during ripening, the residual lactose is fully depleted and thus a variety of chemical compounds such as peptides and amino acids are considered as potential

energy sources for NSLAB (Sgarbi et al., 2013). Furthermore, they are also able to use compounds such as carbohydrates deriving from glycomacropeptides of caseins and glycoproteins deriving from fat globule membranes (Gobbetti et al., 2018).

The effect of NSLAB on cheese ripening is strain-dependent and the selection of adjunct cultures from NSLAB strains is the most time-consuming but productive way to improve the cheese flavor or accelerate ripening (Gobbetti et al., 2018). As an example, in a previous study, experimental pasteurized milk cheeses were made by inoculating milk with a single or in combination of wild strains of *Lactobacillus*. The manufactured cheese had higher scores for sensory attributes, due to more complex volatile profiles than that produced with commercial strains. This was observed in different cheeses e.g. Cheddar (Williams & Banks, 1997; Rehman et al., 2000), Roncal (Ortigosa, Arizcun, Irigoyen, Oneca, & Torre, 2006), Manchego (Gomez-Ruiz, Cabezas, Martinez- Castro, Gonzalez-Vinas, & Poveda, 2008), Greek Feta (Sarantinopoulos, Kalantzopoulos, & Tsakalidou, 2002), and Pecorino Siciliano (Randazzo, Torriani, Akkermans, De Vos & Vaughan, 2002). With this in mind, the objective of this work was to evaluate the use of a previously studied wild *L. paracasei* strain (Bancalari et al., 2017) as adjunct culture to enhance the flavour formation in short-ripened Caciotta-type cheese, produced with pasteurized cow milk. The strains used to this purpose, was chosen for its ability to produce *in-vitro* acetoin and diacetyl, very important aromatic compounds that are known to be responsible for giving a pleasant buttery and creamy odor in cheese. With this purpose, in a cheese-making company, an experimental Caciotta cheese was made with the addition of *Lactobacillus paracasei* 4341 chosen as adjunct culture for its previously evaluated potential technological properties (Bancalari et al., 2017) and all the cheese-making process was followed until the end of cheese ripening. An integrated polyphasic approach was used to compare the experimental and control cheese to find out if any differences, due to the use of adjunct culture, existed.

2. Materials and methods

2.1 Aromatic adjunctive culture preparation

The strain *Lactobacillus paracasei* 4341, belonging to the culture collection of Food and Drug Department of the University of Parma, maintained as frozen stock culture in MRS (Oxoid, Ltd., Basingstoke, United Kingdom) broth containing 20% (v/v) glycerol at −80°C, was recovered in MRS broth by two overnight sub-culturing (2% v/v) at 30°C. The cells were harvested by centrifugation (1000 rpm for 10 min), washed and re-suspended in sterile water and then used to perform other 2 overnight sub-culturing (2% v/v) in UHT whole milk.

To prepare the adjunct culture (AJ4341) 200 ml of UHT whole milk was inoculated with the last overnight sub-culturing to reach a final concentration of $5.0x10^8$ cfu/ml. The viable cells concentration was verified before and after the final inoculum in milk through plating on MRS agar (Oxoid, Ltd., Basingstoke, United Kingdom) at 30°C for 72 h.

AJ4341 was stored into sterile plastic bags and frozen. Before the use, frozen milk culture was thawed in a thermostatic bath at 39°C for 20 minutes.

2.2 Experimental cheese-making design

Two types of Italian Caciotta cheese were industrially produced in a company located in Emilia Romagna region (Caseificio Mambelli, Bertinoro, Italy): i) a control cheese (CC) was produced only with the commercial starters Lyofast Y080 B (Sacco System, Cadorago, Italy) composed by *Streptococcus thermophilus (S. thermophilus)* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) in lyophilized form; and ii) an experimental Caciotta (EC) was produced with Lyofast Y080 B and the adjunct culture AJ4341 (Fig. 1).

For both Caciotta production, a standardized raw milk (3,45% protein, 4,05 % fat, w/w) was kept at 5°C overnight in an insulated tank and then was pasteurized at 73°C for 28 s. 400L of pasteurized milk, divided in two batches, were heated at 39.5°C and inoculated with Lyofast Y080 B (according to Sacco

144 System indication) for CC and Lyofast Y080 B plus AJ4341 for EC (Fig. 1). After 1 h, calf rennet
145 (Caglio Bellucci, Modena, Italy) containing 80% of chymosin and 20% of pepsin, having a clotting
146 activity of 1:12,500 Soxhlet units, was added and kept at 39.5°C for 20 min, until a pH of 6.2 was
147 reached. Thus, the curd was cut and rested under whey for 40 min. The curd was transferred into
148 cylindrical molds with a diameter of 7.5 cm and a height of 13 cm (sample E1 or C1), and transferred
149 to a ventilated, refrigerated cell at 5°C with a relative humidity (RH)> 90% where it remained for 2
150 days to complete whey drainage and reach a pH of 5.18 (sample E2 or C2). Fourteen wheels for each
151 type of Caciotta were stored for ripening at 4°C up to four weeks. After a short ripening time: 2 and 4
152 weeks, two cheese samples (EC2W or CC2W and EC4W or CC4W, respectively) were stored for the
153 analyses (Fig.1).

154 **2.3 DNA extraction and quantification**

155 Microbial DNA extraction, from curds (C1, C2, E1 and E2) and from cheeses (CC2W, CC4W, EC2W
156 and EC4W) was performed, in duplicate, using DNeasy Blood and Tissue Kit (Qiagen, Hilden,
157 Germany) modified as follows. To remove fat and milk impurities: 10 g of curd or cheese sample was
158 mixed with 90 ml of 2 % (w/vol) sodium citrate and homogenized for 2 min by means of Stomacher®
159 400 Circulator (VWR International Srl, Milan). Subsequently, the sodium citrate was incubated at 50°C
160 for 30 min. After the incubation, the homogenate was centrifuged at 500 rpm for 4 min at 4°C, and the
161 supernatant was transferred to a new tube, to partly separate it from contaminating fat layers. The
162 supernatant was centrifuged at 10000 rpm for 10 min at 4°C, and the pellet was resuspended in 20 ml
163 of 2 % (w/vol) sodium citrate and incubated for further 10 min at 50°C. The solution was centrifuged at
164 10000 rpm for 10 min at 4°C. Subsequently, the manufacturer's protocol for DNA extraction from
165 Gram+ bacteria was followed, by doubling the reagents volumes. Briefly, the cells were lysed in 360
166 µL of lysis buffer containing 25 mg/mL of lysozyme for 30 min at 37 °C. The lysed cell suspension
167 was protease treated for 30' at 56 °C. At the end of the spin-column protocol, the DNA was eluted with

50 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).

2.4 RealTime qPCR assay

The absolute quantification of the species *L. bulgaricus* and *S. thermophilus* was performed on curds (C1, C2, E1 and E2) and from cheeses (CC2W, CC4W, EC2W and EC4W), using specific primers (Table 1) designed on pheS gene sequences (Bottari et al., 2013). For absolute quantification of the strain *L. paracasei* 4341 specific primers designed on spxB gene sequence were used (Table 1), as previously described (Bancalari et al., 2017). Reaction mix for each primer pairs contained: 1× PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Milan, Italy), forward and reverse primers at concentration of 250 nM and nuclease-free water to a total of 20 µL per well. All the reactions were performed on biological replicates of the samples in duplicate, and no template controls (NTC) were included in each experiment. The total DNA, previously extracted, was diluted 10-fold 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), the thermal cycle was 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 absolute quantification, standard curves have been constructed using purified genomic DNA of type strains of *L. delbrueckii* ssp. *bulgaricus* LMG 6901, *S. thermophilus* LMG 6896 and *L. paracasei* ATCC 334, and copy number was calculated 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 of magnitude, and plotting the resulting threshold cycles (Ct), against the

192 logarithm of the target gene copy number. The copy number of target gene of each species was
193 calculated for all the samples by comparing the Ct of the sample with that of the respective standard
194 curve.
195
196
197 **2.5 Organic acid measurement and volatile molecule profiles**
198 Organic acids were determined on curds (C1, C2, E1 and E2) and cheeses (CC2W, CC4W, EC2W and
199 EC4W) with an HPLC (PU-2089 Intelligent HPLC quaternary pump, UV-VIS multiwavelength
200 detector UV 2070 Plus, Jasco Corp., Tokyo, Japan) and a manual Rheodyne injector equipped with a
201 20 μL loop (Rheodyne, Rohnert Park, Calif., U.S.A.). The extraction was performed on 10 g of samples
202 according to Tabanelli et al. (2018) and the analytical conditions were those reported by the same
203 authors.
204 Volatile compounds were monitored, on milk (M), curds (C1, C2, E1 and E2) and cheeses (CC2W,
205 CC4W, EC2W and EC4W) through GC-MS coupled with a solid phase micro-extraction (GC-MS-
206 SPME) technique by using an Agilent Hewlett–Packard 7890 GC gas-chromatograph and a 5975 MSD
207 MS detector (Hewlett–Packard, Geneva, Switzerland). The analysis was performed on 3 g of samples
208 according to Montanari et al. (2018). The volatile compounds were identified by computer matching of
209 mass spectral data with those of compounds contained in NIST 2011 mass spectral library (Scientific
210 Instrument Services, Ringoes, NJ, United States). The compounds are reported as ratio between each
211 peak area and the area of internal standard (4-methyl-2-pentanol), added to a concentration of 3.3
212 mg/kg.
213 **2.6 pH, moisture and water activity measurements**
214 The pH values of the control and experimental cheeses after 4 weeks of ripening (CC4W and EC4W),
215 were measured with a Portamess pH-meter mod. 913 (Knick Elektronische, Berlin, Germany) equipped

with a Double Pore F electrode (Hamilton Company, Reno, Nevada, USA) in four different random spots of the inner part of the cheese. Moisture content of the same samples was determined in quadruplicate by oven-drying samples at 102°C (AOAC, 1990). Water activity (a_w) was measured in quintuplicate with an AquaLab 4TE water activity meter (Decagon, Pullman, WA, USA).

2.7 Color measurements

Color measurements of control and experimental cheeses after 4 weeks of ripening (CC4W and EC4W), were performed using a CR-2600d spectrophotometer (Minolta Co., Osaka, Japan) equipped with a standard illuminant D65. The instrument was calibrated prior of each analysis using a white color tile standard. International Commission on Illumination (CIE) $L^*a^*b^*$ color space was chosen to describe colorimetric characteristics of the cheeses.

Lightness of color (L^* that ranges between 100 of white to 0 of black), redness (a^*, that ranges between +120 of red to -120 of green), yellowness (b^*, that ranges between +120 of yellow to -120 of blue) were measured in specular component included (SCI) mode. Moreover, hue angle (h°), chroma (C) were calculated according to equation (1) and (2). Ten measurements were conducted on random points in the inner part of the cheeses.

$$h^\circ = arc\ tan \frac{b^*}{a^*} \quad (1)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (2)$$

2.8 Texture analysis and dynamic rheological analysis

The textural properties of control and experimental cheeses after 4 weeks of ripening (CC4W and EC4W), were measured using a TA.XT2plus Texture Analyzer equipped with a 30 kg load cell (Stable Micro Systems, Godalming, UK), a force resolution of 0.01 N and an accuracy of 0.025%. A texture profile analysis (TPA) double compression test was performed using a stainless-steel cylindrical probe with a diameter of 30 mm; a crosshead speed of 1.5 mm/s was applied to compress the cube samples (15 mm side) to 60% strain. The textural parameters considered were hardness (N), cohesiveness,

springiness and gumminess (N). Prior to be analyzed, samples were equilibrated in a temperature-controlled climate chamber set at 25°C (mod. ICH256, Memmert, Schwabach, Germany). Ten replicates were measured for each sample.

Frequency sweep tests were performed on control and experimental cheeses after 4 weeks of ripening (CC4W and EC4W), according to Alinovi et al. (2018) using an ARES rheometer (TA instruments, New Castle, Delaware, USA) equipped with a 25 mm parallel plate geometry. Measurements were performed applying a constant strain of 0.05%; this strain value was included into the linear viscoelastic region of the cheese as determined by strain sweep measurements prior to frequency sweep tests. Dynamic analyses were performed at 25°C in the range between 0.1 and 12.5 Hz and temperature of the sample was controlled using a Peltier device. Frequency dependence of rheological moduli G' and G'' was evaluated using laws equations (1) and (2) (Steffe, 1992):

$$G' = k'(f)^{n'} \quad (3)$$

$$G'' = k''(f)^{n''} \quad (4)$$

Measurements were performed in quintuplicate.

2.9 Sensory triangle test

Triangle discriminant analysis of control and experimental cheeses after 4 weeks of ripening (CC4W and EC4W), was performed as reported by Alinovi et al., (2018) with a panel group of 27 people. The panel was asked to identify the different cheese sample and to indicate one or more sensory attributes that were perceived different between the samples.

2.10 Statistical analysis

One-way analysis of variance (ANOVA) using SPSS Statistics v.25 (IBM, Armonk, USA) was carried out to estimate the effect of treatments among physical, chemical and microbiological observations ($\alpha = 0.05$). Concerning the discrimination triangle test a binomial test was carried out to assess if the correct

263 classification by the panel gave a higher probability level (P) than a random classification process (P >
264 1/3, $\alpha = 0.05$).
265
266 ## 3. Results and discussion
267 ### 3.1 Bacterial dynamics of starter and adjunct cultures in cheese
268 Analysis of qPCR data allowed to reconstruct the dynamics of acidifying starter culture and aromatic
269 adjunct *L. paracasei* 4341 during cheese manufacturing and ripening. The two acidifying starter
270 species were present in the curd after extraction, with a 50 fold prevalence of *S. thermophilus*, in both
271 the experimental (E1), (Fig. 2A) and control (C1), (Fig. 2B) cheeses, with comparable measured values
272 of 6.83 ± 0.03 (mean ± standard deviation) and 6.51 ± 0.35 Log copy number/g of cheese, respectively.
273 The other acidifying starter species *L. delbrueckii* had lower concentrations in the curd after extraction,
274 *i.e.* 5.09 ± 0.13 and 5.45 ± 0.07 Log copy number/g of cheese for control (C1) and experimental (E1)
275 cheese, respectively. The small differences in quantity of both starter culture species between the two
276 types of curds can be due to the reasonable differences that occur when working in two different vats in
277 parallel. In the experimental cheese, the selected adjunct culture was present at an intermediate value of
278 6.08 ± 0.05 Log copy number/g of cheese.
279 During curd acidification (two days at 5°C until reaching a pH value of 5.18), the two acidifying
280 species in the control curd C2, (Fig. 2B) developed in a similar way, reaching the maximum values of
281 5.76 ± 0.13 and 7.18 ± 0.17 Log copy number/g for *L. delbrueckii* and *S. thermophilus*, respectively. In
282 the experimental curd, (Fig. 2A), the presence of the adjunct culture did not influence the replication of
283 *S. thermophilus*, that reached values comparable to those of the control curd (7.29 ± 0.11 Log copy
284 number/g), but led to a slight restraint of *L. delbrueckii*, that reached values of 5.56 ± 0.01 Log copy
285 number/g (Fig. 2A).

In this cheese-making step, the presence of adjunct *L. paracasei* 4341 did not influence curd acidification, as expected from Bancalari et al. (2017), and confirmed by the produced amounts of lactic acid (Table 2). The adjunct culture underwent a small reduction in its concentration, to values of 5.82 ± 0.13 Log copy number/g. After two weeks of ripening (EC2W), (Fig. 2A), adjunct starter concentration continued to decrease, but, confirming their ability to survive in ripening condition (Bancalari et al. 2017), its value stabilized at a concentration of 5.49 ± 0.09 Log copy number/g, after two further weeks of ripening (EC4W). Even though, the main starter species *S. thermophilus* decreased during ripening of Caciotta cheese, it was always the majority lactic acid bacteria species, in both the conventional and experimental production lines.

L. delbrueckii, instead, was affected by the presence of the adjunct culture during ripening. While in the control cheese C2 this species reached its maximum concentration at the end of the acidifying step (Fig. 2B), it started to decrease until the end of ripening. Conversely, in the experimental cheese *L. delbrueckii* growth was partly slowed down, reaching the highest values only after two weeks of ripening (Fig. 2A), and stabilizing at the end of four weeks (Fig. 2A) to values comparable to that of the adjunct culture, and higher with respect to the control cheese. For these sampling points, EC2W and EC4W, (Table 2), the measured lactic acid concentration resulted to be significantly higher ($P<0.05$) than that of control cheese (Table 2), probably ascribed to the metabolic activity interaction of *L. paracasei* 4341 and *L. delbrueckii.*

Thus, while the two types of Caciotta cheese show similar trends in the evolution of the main LAB population, there is a small effect on the *Lactobacillus* moiety, not only for the presence of the adjunct culture, but also for the observed difference in the starter development. These differences and the presence of the adjunct *L. paracasei* 4341 lead to a clear differentiation of the experimental cheese compared to the control one, due to the activation of metabolic pathways, as confirmed by the measured lactic acid production (Table 2) and the identified volatile compounds (see next paragraph).

3.2 Aroma profile

The volatile profile of the samples as determined by GC-MS-SPME analysis allowed to identify 32 compounds belonging to different chemical classes, and namely aldehydes, alcohols, ketones, esters and acids (Table S1). Data are reported in the table as ratio between each peak area and the area of internal standard (4-methyl-2-pentanol), which was added in constant amount.

The aroma profile of milk showed, as expected, relatively few volatiles that were present in low amounts. The most important were ketones (mainly acetone and 2-butanone) and aldehydes (hexanal). The aroma profile of the curds immediately after their transfer into the molds (C1 and E1) presented small differences compared to milk with regard to some aldehydes (nonanal, decanal) and hexanol. The most relevant difference concerned 3-hydroxy-2-butanone (acetoin) which resulted the most important volatile compound,without significant differences in relation to the presence of the adjunct culture *L. paracasei* 4341. The importance of acetoin production during cheese production lies in its characteristic aromatic notes, related to a pleasant buttery/creamy odor (Bancalari et al.,2017).

At the end of curd acidification, after 48 hours at 5°C, the presence of volatiles increased in both cheeses (C2 and T2) and a relevant accumulation of ketones was observed. These two groups remained the most important also in cheeses during ripening, accompanied by an increase of alcohols, while aldehydes remained quite constant. The presence of esters (ethyl acetate) was detected mainly during ripening.

Among ketones, diacetyl (2,3-butanedione) and acetoin (3-hydroxy-2-butanone) were the most represented molecules. While the first mainly accumulated during the first step of cheese-making and then decreased during ripening, the latter increased mainly during ripening, due to the chemical reduction of diacetyl. These compounds are extremely important for cheese flavor formation and, noteworthy, their presence was higher in the cheeses obtained with the use of the adjunct aromatic strain *L. paracasei* 4341. Also 3-hydroxy-3-methyl,2-butanone showed a similar trend. Acetone and 2-

334 butanone, the most important components of milk, remained constant or slightly decreased during the
335 process.
336 Acetic acid increased during ripening and its concentration was significantly higher ($P<0.05$) in the
337 experimental samples after 4 weeks rather than in the control, consistently with data obtained by HPLC
338 (Table 2). Also, other acids (butanoic, hexanoic and octanoic) accumulated during ripening: for
339 instance, butanoic and hexanoic acids were significantly higher ($P<0.05$) during ripening in
340 experimental cheeses, while octanoic acid showed no differences between cheeses.
341 Ethanol was the principal alcohol detected and its amount was significantly higher in the 4 weeks
342 ripened cheeses obtained with the addition of *L. paracasei* 4341. The same trends were observed for 3-
343 methyl-3-buten-1-ol, 3-methyl-2-buten-1-ol and 2-butanol.
344 To better evidence the relationships between the formation of aroma profile in cheeses in relation to the
345 starter cultures, a principal component analysis (PCA) was carried out on the correlation matrix based
346 on the volatile compounds detected in the samples. In Fig. 3 the case coordinates on the sample score
347 plot of the Factors 1 and 2 (representing 47.72% and 16.82% of the total variability, Fig.3A) and the
348 PCA loadings plot of the volatiles on the same two factors (Fig. 3B) are reported.
349 The Factor 1 discriminated milk from the different steps of cheese-making moving from positively
350 correlated (mainly acetone) to negatively correlated compounds (ethanol, 3-methyl-3-buten-1-ol, 3-
351 methyl-2-buten-1-ol, 3-hydroxy-3-methyl-2-butanone, acetoin, 2-nonanone, 2-heptanone and organic
352 acids). Interestingly, while no differences between the control cheeses after 2 and 4 weeks of ripening
353 (CC2W and CC4W) were observed, relevant differences were present in the experimental cheeses,
354 obtained with the addition of the aromatic strain *L. paracasei* 4341 (ECW2 and ECW4). This fact
355 seems to indicate that the activity of the adjunct culture persisted throughout the ripening. The Factor 2
356 is correlated positively with 2-butanone and negatively with diacetyl, benzaldehyde and 2,3-
357 pentanedione.

358 The volatile molecules able to discriminate the cheeses are attributable to metabolic pathway
359 influenced by microbial activities. In particular, pyruvate (deriving from citrate and other metabolisms)
360 can lead to the production of diacetyl which can be successively reduced to acetoin. These compounds,
361 characterized by butter and nut notes, are key aroma component of several cheeses. The diketone 2,3-
362 pentanedione can be produced by two distinct routes, the first starting from pyruvate and acetate and
363 the second from pyruvate and threonine (Smid & Kleerebezem, 2014). In this case, the samples
364 obtained with *L. paracasei* 4341 are discriminated mainly by the presence of acetoin. 3-methyl-3-
365 buten-1-ol, 3-methyl-2-buten-1-ol (isoprenol and prenol, respectively) have already been detected in
366 cheese (Mariaca, Fernandez-Garcia, Mohedano & Nunez, 2001; Bergamaschi et al., 2015) and may be
367 the result of the dehydratation of 3-methyl-3-butanediol and 3-methyl-2-butanediol (Morino, Yamada
368 & Sato, 2014), which derive from isoleucine and leucine metabolism.
369 The increasing presence of acetate can be related to pathways starting from pyruvate which can be
370 provided by lactic acid, residual sugars or amino acid metabolisms (Smid & Kleerebezem, 2014; Zotta,
371 Parente & Ricciardi, 2017).

372 **3.3 Physico-chemical properties**

373 pH values of control and experimental Caciotta 4 weeks ripened cheeses (CC4W and EC4W) were
374 significantly different ($P<0.05$), respectively 4.90 and 4.51, respectively, (Table 3) were both lower
375 than those of acidified curds (5.18) (data not shown). In particular, EC4W had a lower pH than the
376 control CC4W accordingly to the measured higher concentration of organic acid after four weeks of
377 aging (Table 2 and 3). The fermentation products lactic and acetic acids were detected in both samples
378 and lactic acid was present at higher amounts already in the curds at the beginning of the process in the
379 experimental cheese line (Table 2). During fermentation and ripening lactic acid accumulated, and in
380 ECW4 reached a concentration of 16.34 that was significantly higher than CCW4 (9.58 g/kg) (Table 2).

381 Acetic acid was present at low concentrations (about 0.4-0.6 g/kg) and its amount slightly increased in
382 the experimental samples during 4 weeks of ripening (Table 2).
383 For ECW4, a significantly lower ($P<0.05$) moisture content and a significantly higher value of a_w
384 ($P<0.05$) were observed (Table 3). This can be related to a lower water-holding capacity (WHC) of the
385 ECW4, accordingly with a study of Marchesseau and colleagues (1997). Furthermore, it was already
386 observed that higher pH values in cream cheese formulations could have promoted protein-to-water
387 interactions and swelling of the protein network, while lower pH values could have favored
388 hydrophobic protein-to-protein interactions and contraction of the protein network into denser and
389 more rigid fibers (Monteiro, Tavares, Kindstedt & Gigante, 2009). This phenomenon has also been
390 observed in Cheddar cheese (Pastorino, Hansen & McMahon, 2003). Moreover, higher acidification
391 measured for the ECW4, caused by the additional activity of the adjunct culture, could also have
392 promoted a decrease of the residual activity of rennet enzymes and endogenous proteases (i.e. plasmin)
393 as their activity is lowered at lower pH values (Picon et al., 2010; Børsting, Qvist & Ardö, 2014). Even
394 if, in the present work the degree of proteolysis was not measured, a higher degree of proteolysis and a
395 higher release of peptides, could have occurred in CCW4, co-promoting a lower moisture loss and a_w
396 because of the formation of free ionic groups that can bond free water (Ak & Gunasekaran, 1996) and
397 the release of low molecular weight peptides. In addition, it is known that the addition of adjunct
398 culture may lead to an increment of free amino acids a well as peptides and also free fatty acids that
399 could influence the physico-chemical characteristics, the overall aroma profile but also accelerate
400 cheese ripening (Crow, Curry & Hayes, 2001).
401 Colorimetric coordinates showed significant differences between the two Caciotta type. In particular,
402 L* and b* values were significantly higher and lower ($P<0.001$), respectively, in the ECW4 cheese
403 than in the control CCW4. A higher lightness of the cheese body which is related to a higher amount of
404 free water droplets and a lower degree of light scattering phenomena (Sánchez-Macías et al., 2010;

405 Sheehan et al., 2005), could be possibly associated to the decreased protein hydration in ECW4 caused
406 by the lower pH of the cheese or by the higher proteolysis. Moreover, yellowness (b*), that can be
407 related to oxidative reactions and a higher extent of ripening phenomena (Buffa, Trujillo, Pavia &
408 Guamis, 2001), was higher in the control cheese. Consequently, to the measured differences in color
409 coordinates, also values of C and h° angle showed statistical differences; both cheeses were
410 characterized by a dominant yellowish color.

411 **3.4 Rheological and textural properties**

412 Rheological properties measured in CC4W and in EC4W showed the predominance of the elastic
413 behavior in both cheeses, as the storage modulus G' was higher than the loss modulus G'' in the
414 measured frequency range and tanδ of the cheeses was between 0.29 and 0.40 (Fig. 4 A,4 B, 4C). Both
415 G' and G'' increased linearly with the increasing deformation rate (log-log scale). Power law equations
416 fitted experimental dynamic data with a good level of accuracy, as determination's coefficients (R^2)
417 were higher than 0.98.

418 Power law coefficients of G' and G'' rheological moduli showed significant differences between the
419 samples ($P<0.001$). EC4W was characterized by a higher k' than the control, that can be related to a
420 stronger protein network and to its lower moisture content. In facts, as water acts as plasticizer in a gel
421 or viscoelastic system, a decrease of its content can promote an increase of the structural rigidity of the
422 cheese (Perreault et al., 2017).

423 Tanδ, that express the ratio between the amount of energy dissipated as viscous dissipation and the
424 amount of energy that is stored or recovered (G''/G'), is showed in Figure 4C. Tanδ was higher in the
425 CC4W than in EC4W in the low frequency range (0.1-1 Hz), while it was similar in the measured
426 cheeses at higher frequency values. Because high frequency values mean low relaxation times, the
427 different cheeses could not have enough time to reflect the structural differences in terms of
428 viscoelastic behavior.

A lower frequency dependence of storage modulus than loss modulus in the case of treatment cheese can be observed from n' and n'' parameters reported in Table 4; moreover, frequency dependence of G' was also lower in EC4W than in CC4W. As a higher frequency dependence (higher n' values) is typical for weak gels (Tunick, 2011; Banville, Morin, Pouliot & Britten, 2014; Perreault et al., 2017), a lower structured cheese matrix was observed in control cheese and can be due to the lower degree of organization of casein micelles as a consequence of proteolysis or to the higher pH, as previously discussed.

Textural analyses confirmed rheological measurements, as EC4W had a higher hardness than the control cheese (16.93 ± 2.76 N vs 6.16 ± 2.16 N) and also gumminess, that in cheese texture is often related to hardness development (Irudayaraj, Chen & McMahon, 1999) showed the same behavior.

On the contrary, cohesiveness, that is a measurement of the strength of the internal bonds in the matrix but that can be also sensorially related to the amount of deformation undergone by a material before rupture (Meullenet, Carpenter, Lyon & Lyon, 1997), was significantly lower for the control cheese than for the EC4W. In fact, the latter was characterized by a harder, more rigid structure but that was also brittle when subjected to large, destructive deformations. A higher brittleness of EC4W can be related to its lower water content and availability (Creamer & Olson, 1982) causing the development of a less plasticized matrix but also to modifications of protein structure and functionality and to a lower colloidal calcium content, as previously highlighted for other kind of cheeses (Luyten, Vliet & Walstra, 1991; Kindstedt, Zielinski, Almena-Aliste & Ge, 2001).

3.5 Sensory triangle test

The different physical, chemical and aroma properties of the two cheeses were also sensorially perceived, as the panel group correctly classified a number of 21 cheese comparisons, corresponding to the 77.8% of total number of tested comparisons and to a P-value lower than 0.001. EC4W, when

452 correctly classified, mainly discriminated from the control because of a higher firmness (63.0% of the
453 times), different acidity (29.6%) and saltiness (22.2%).
454
455 **1. Conclusion**
456 In the present study a short-ripened Caciotta-type cheese was produced with and without the addition
457 of a strain which was previously isolated and suggested for its potential technological features
458 (Bancalari et al., 2017). The aim was to use *Lactobacillus paracasei* 4321, as adjunct aromatic culture,
459 to enhance the flavour of a Caciotta-type produced with pasteurized cow milk and ripened for less time
460 than the usual. The complete results obtained with a polyphasic approach are very interesting because it
461 was demonstrated how the adjunct strain was able to develop in curd and cheese interacting with
462 acidifying starter and producing higher amount of volatile compounds that lead to a clear
463 differentiation of the experimental Caciotta respect to the control in terms of aromatic profile and
464 physico-chemical and rheological properties. While the main acidifying starter species, *S.*
465 *thermophilus*, has shown comparable behaviour in all cheese-making steps of control and experimental
466 Caciotta. *L. delbrueckii*, growth was slowed down by the presence of the adjunct culture. In this way
467 the acid production by *L. delbrueckii* was prolonged over time, resulting in higher lactic acid
468 concentration in the experimental cheeses, ascribable to the metabolic activity interaction of *L.*
469 *paracasei* 4341 and *L. delbrueckii.*
470 As previously observed by Gobbetti and colleagues (2018), also in our case, despite the advantages, the
471 addition of adjunct NSLAB to cheese milk caused a slight over acidification of the curd in addition to
472 primary starters. This phenomenon increased whey drainage, which affect microbial and biochemical
473 activities during ripening and consequently physico-chemical, rheological and sensory characteristics
474 of the cheese. These over acidification could probably be solved by using attenuated adjunct culture.

However, the results obtained with this study allow us to support the use of aromatic adjunct starters in cheeses made with pasteurized milk. This strategy could represent a very promising technique for obtaining pasteurized milk cheeses with improved aromatic profiles and improved organoleptic characteristics. For this reason, the choice of the strain to be used for this purpose continues to be a very important topic.

Figure captions

Figure 1 Sampling point scheme. Sampling point are shown as red rhombus for control line (C), green for experimental line (T). For Experimental cheese production: E1 (Curd transferred into molds), E2 (curd at the end of acidification), EC2W (Experimental Cheese after 2 weeks of ripening), EC4W (Experimental Cheese after 4 weeks of ripening). For the Control cheese production: C1 (Curd transferred into molds), C2 (curd at the end of acidification), CC2W (Control Cheese after 2 weeks of ripening), CC4W (Control Cheese after 4 weeks of ripening).

Figure 2 Bacterial dynamics and lactic acid production in cheese. The graph shows the dynamics of the species *S. thermophilus* (blue line and triangles), *L. delbrueckii* (blue dashed line and squares) and the adjunct starter *L. paracasei* 4341 (orange dashed line and triangles) at various production stages, in both the experimental (A) and conventional (B) manufacturing. For each production stage the measured lactic acid concentration is reported (white bars). Error bars represent standard deviation.

Figure 3 PCA Results of Principal Component Analysis: a) projection of case coordinates on the sample score plot of the Factors 1 and 2; b) PCA loading plot of the aroma compounds selected on the first two factors obtained from PCA.

Figure 4 Storage modulus (G'), loss modulus (G''), tangent of the phase angle (tanδ) and complex viscosity (η*) frequency-dependent curves measured at 25°C of experimental cheeses manufactured with (ECW4) and the control (CCW4), without the addition of the secondary adjunct culture.

499

Table 1 Primer pairs used in this study, the same as reported in Bottari et al., 2013 (1), or Bancalari et al., 2017 (2).

Primer	Primer sequence (5'→3')	Lenght (bp)	Size (bp)	Reference
LlpheSF	ACGTTGACGCTGACCACC	18	51	(1)
LlpheSR	GGCTTGAACTGGTGAAGTCTG	21		
StpheSF	GAAGAAATCTTGCTTCGCACTC	22	50	(1)
StpheSR	AGTGTACGAGCTTGGACAGGA	21		
poxcDNAFw	CAGACGCAATGATCAAGGTG	20	150	(2)
poxPromRV	AATGCGCCyACTTCTTCATG	20		

505 **Table 2** Organic acid content (g/kg) in the samples of Caciotta during manufacturing and ripening.
506 Data are the mean of two cheeses, each analysed twice.
507

Organic acids	Sample	Experimental cheese	Control cheese
Lactic acid	Curd before acidification	2.21 (±0.46)	2.10 (±0.22)
	Curd after acidification	7.65 (±0.46)	7.18 (±0.31)
	Cheese after 2 weeks of ripening *	12.35 (±0.75)	9.25 (±0.09)
	Cheese after 4 weeks of ripening *	16.48 (±0.10)	9.76 (±0.55)
Acetic acid	Curd before acidification *	0.39 (±0.02)	0.59 (±0.11)
	Curd after acidification *	0.38 (±0.01)	0.30 (±0.01)
	Cheese after 2 weeks of ripening *	0.61 (±0.02)	0.37 (±0.07)
	Cheese after 4 weeks of ripening *	0.64 (±0.02)	0.47(±0.07)

508
509 The presence of an asterisk indicates significant difference ($p < 0.05$) between the two samples
510 (experimental cheese *vs.* control cheese) for that organic acid.
511

512 **Table 3.** Mean values of pH, moisture content (**MC**), water activity ($\mathbf{a_w}$) and color attributes (L*, a*, b*, C, h°) of experimental cheeses

513 manufactured with (ECW4) and the control (CCW4), without the addition of the secondary adjunct culture.

Sample	pH	MC (% w/w)	a_w	L*	a*	b*	C	h°
CCW4	4.90 ± 0.05	48.89 ± 0.86	0.9697 ± 0.0015	88.54 ± 1.01	-0.20 ± 0.09	18.50 ± 0.74	18.50 ± 0.74	90.62 ± 0.27
ECW4	4.51 ± 0.01	47.62 ± 0.55	0.9719 ± 0.0008	90.62 ± 0.65	-0.06 ± 0.14	16.48 ± 0.21	16.48 ± 0.21	90.22 ± 0.49
Sign.	***	*	*	***	*	***	***	*

514 *P<0.05, **P<0.01, ***P<0.001

515

516

517

Table 4. Textural parameters derived from Texture Profile Analysis (TPA) curves and rheological parameters derived from power-law equations for storage (G'), loss (G'') moduli and complex viscosity (η*) of experimental cheeses manufactured with (EC4W) and the control (CC4W), without the addition of the secondary adjunct culture.

Sample	**k' (Pa·s$^{n'}$)**	**n' (-)**	**k'' (Pa·s$^{n''}$)**	**n'' (-)**	**Hardness (N)**	**Cohesiveness (-)**	**Gumminess (N)**	**Springiness (mm)**
CC4W	20226±2834	0.219±0.015	6986 ±950	0.201±0.004	6.16 ±2.16	0.45 ±0.06	2.84±1.16	5.16± 0.85
EC4W	54315±7266	0.177±0.004	16830±2368	0.209±0.005	16.93±2.76	0.21±0.03	3.60±0.88	4.37± 0.98
Sign.	***	***	***	*	***	**	***	

*P<0.05, **P<0.01, ***P<0.001

Table S1. Volatile profile of milk and cheeses during manufacturing and ripening determined by GC-MS-SPME. Data are expressed as ratio between each peak area and the area of internal standard (4-methyl-2-pentanol).

Acknowledgements

This work was supported by Regione Emilia-Romagna in the framework of the project "Collezioni microbiche regionali: la biodiversità al servizio dell'industria agroalimentare" (CUP J12F16000010009).

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