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Key Words:	Pasta filata cheese, Scamorza, adjunct, proteolysis

ADJUNCTS IN SCAMORZA CHEESE

Effect of adjuncts on microbiological and chemical properties of Scamorza cheese

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INTERPRETIVE SUMMARY

Effect of adjuncts on microbiological and chemical properties of Scamorza cheese - Parente

Scamorza is similar to low-moisture Mozzarella cheese, it is used as a table cheese or as pizza topping and it is characterized by a short ripening time. The use of a peptidolytic adjunct (*Lact. lactis*, *L. helveticus* and *L. paracasei*) in addition to the primary starter (*S. thermophilus*) significantly affected pH, microbial composition, proteolysis and volatile profile in Scamorza cheese produced with Italian Friesian milk, while use of 10% Jersey cattle milk had only a minor effect. Therefore, adjuncts can be used to accelerate ripening and manipulate the properties of the cheese, thus providing a tool for product diversification.

ADJUNCTS IN SCAMORZA CHEESE

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ABSTRACT

Scamorza is a semi-hard pasta filata cheese resembling low moisture Mozzarella cheese, with a short ripening time (<30 d). Scamorza has a bland flavor and, in order to provide diversification from similar cheeses, it was manufactured using two types of milk (100% Italian Friesian milk, F, or 90% F and 10% Jersey cow milk, M) and two types of starter (*S. thermophilus* or *S. thermophilus* with peptidolytic *Lact. lactis*, *L. helveticus* and *L. paracasei* strains as adjuncts). The cheese was ripened for 30 days. The adjunct did not significantly affect acid production or growth of the primary

starter; two of the species used in the adjunct (*L. paracasei* and *L. helveticus*) rapidly colonized the cheese and persisted until the end of ripening, while the number of non-starter lactic acid bacteria in the control cheese was low until the end of ripening. The adjunct affected pH, microbial composition (as assessed by both culture dependent and culture-independent methods), total free amino acid content and volatile profile (measured using an electronic nose) while milk type had only a minor effect. Although differences in primary proteolysis were found, they were probably indirect and related to the effects on pH and moisture.

We conclude that, even with a short ripening time (30 d), use of a peptidolytic adjunct may significantly affect important features of Scamorza and may be used for product differentiation.

Key words: Pasta filata cheese, Scamorza, adjunct, proteolysis, aroma

INTRODUCTION

A large variety of pasta filata cheeses (De Angelis and Gobbetti, 2011) are produced in Italy from cow and buffalo milk. While some cheeses have a Protected Designation of Origin (e.g. fresh cheeses like Mozzarella di Bufala Campana, and semi-hard or hard cheeses like Provolone Valpadana, Caciocavallo Silano, Provolone del Monaco, Ragusano; http://ec.europa.eu/agriculture/quality/door/list.html) according to standards of identity which require the use of raw milk and natural starter cultures or no starter at all (Randazzo et al., 2002; Piraino et al., 2005; Aponte et al., 2008; Ercolini et al., 2008; De Angelis and Gobbetti, 2011; De Filippis et al., 2014), several others, including a number of fresh, semi-hard and hard varieties, are produced using either direct acid addition or defined starter cultures composed of *Streptococcus*

thermophilus alone or in combination with thermophilic lactobacilli (De Angelis and Gobbetti, 2011). Scamorza and Provola are two short ripened varieties, which have evolved from traditional cheeses produced throughout southern Italy (Baruzzi et al., 2002; Cronin et al., 2007); they are similar in texture to low moisture Mozzarella cheese (Kinstedt 2004, Kinstedt et al., 2004) and are used as table cheese or pizza topping. Due to short ripening (<30 d), these cheeses cannot undergo significant proteolysis compared with cheeses with longer ripening times (such as Caciocavallo and Provolone; Gobbetti et al., 2002; Piraino et al., 2005). Use of adjunct cultures is a common method to accelerate ripening of cheese (Chamba and Irlinger, 2004) and proteolysis, petidolysis and amino acid metabolism are three important functions of adjuncts which can be used as viable or attenuated cultures, the latter to minimize their interference with starter activity (El Soda et al., 2000). Mesophilic non-starter lactic acid bacteria (NSLAB) such as *Lactobacillus casei*, *L. paracasei*, *L. rhamnosus* and *L. plantarum* are most frequently used as adjuncts in several cheese types (El Soda et al., 2000; Chamba and Irlinger, 2004); however *L. helveticus* (Kenny et al., 2006; Lee et al., 2007) and wild *Lactococcus lactis* strains (Ayad et al., 2000) have also been used. Cheese aroma is complex and derives from several biochemical and chemical processes (Marilley et al., 2004a). Metabolism of amino acids from starter and non starter bacteria provide a significant contribution (Yvon and Rjinen, 2001; Marilley et al. 2004a; Chamba and Irlinger, 2004). Different types of adjuncts have been shown to significantly affect the volatile compounds of several cheeses (Ayad et al., 2000; Lee et al., 2007; Van Hoorde et al., 2010).

Curd stretching in hot water, which is a characteristic step of pasta filata cheese making, may significantly reduce the viability and activity of starter and non-starter bacteria (Coppola et al., 2006) at the beginning of ripening, although the effect may

be influenced by different process parameters, such as screw speed and stretching temperature (Yun et al., 1995; Petersen et al., 2000). Adjunct cultures have been used with some success to accelerate the ripening of a hard pasta filata cheese (Caciocavallo Pugliese, Morea et al., 2007; Di Cagno et al., 2012) with a significant increase in secondary proteolysis. Stretching curd in hot water causes significant starter lethality, but autochthonous *Lactobacillus paracasei* adjuncts were found to grow well in cheese and contributed to proteolysis. With the exception of the use of probiotic adjuncts (Albenzio et al., 2013a and 2013b), no study concerning Scamorza cheese has been published on this subject.

The most common dairy cattle breed in Italy is the Italian Friesian. Despite the high production levels the milk is characterized by a moderate dry matter content, which may limit cheese yield. As a consequence, many farmers include into the herd 5-20% animals producing less milk but with higher dry matter contents. Due to the low maintenance requirements and high adaptability to different environmental conditions, in the hilly areas of southern Italy, Italian Jersey cows are often used to this aim.

Perna et al. (2014) showed that cow's genetic type (Italian Friesian vs. Italian Brown) significantly affected gross composition of Caciocavallo cheese produced using raw milk and a natural starter culture. Caciocavallo cheese produced with milk from Italian Brown cows showed higher moisture and a consequent higher proteolysis, although the role of autochthonous NSLAB was not evaluated. De Marchi et al. (2008) evaluated the effect of milk type (Holstein Friesian and Swiss Brown, used alone or in 50:50 mixtures) on the quality of three Italian cheeses (Casoletti, Vezzena and Grana Trentino) and found that milk type significantly affected yield, color and

fatty acid composition with intermediate values between the two milk types for 50:50 mixtures.

The objective of this study was to evaluate the effect of milk type and addition of a peptidolytic adjunct culture on microbiological and chemical properties of Scamorza cheese. In addition, the effect on volatile compounds production was evaluated. The adjunct included three strains (*Lactococcus lactis* C4F11, *Lactobacillus helveticus* L206 and *L. paracasei* C3D7), which had been selected in a previous study on pasta filata cheeses because of their proteolytic, peptidolytic and autolytic properties (Piraino et al., 2008). The impact on sensory properties and consumer liking is reported in Braghieri et al. (unpublished data).

MATERIALS AND METHODS

Starter cultures

A direct-to-vat freeze-dried culture of *Streptococcus thermophilus* (Lyofast ST051, Sacco Srl Cadorago, CO, Italy) was used for all cheeses as directed by the manufacturer. The use of ST051 alone (ST) was compared with the use of ST051 plus an adjunct culture (ST+A). The latter was prepared by cultivating *Lactococcus lactis* subsp. *lactis* C4F11 and *Lactobacillus helveticus* L206 in reconstituted (11% w/v) skim milk (RSM, Oxoid, Basingstoke, UK) for 16 h at 30 and 37°C, respectively, and *L. paracasei* subsp. *paracasei* C3D7 in RSM containing 0.5% glucose and 0.25% yeast extract (Oxoid) for 16 h at 30°C. All strains had been previously isolated from traditional pasta filata cheeses and were selected for their technological properties (Piraino et al., 2008). The cultures were frozen at -24°C until needed, and viable cells after thawing were measured by plate counts in LM17 (M17 + 0.5% lactose, 30°C 48

150 h) for C4F11 and in MRS agar (48 h, 37°C) for L206 and C3D7. Prior to use the
151 cultures were mixed to obtain an adjunct with 10^8 cfu/mL with equal proportions of
152 the three strains and inoculated in cheese milk at 0.2%.
153
154 ***Cheese-making trials and experimental design***
155 Cheese-making trials were carried out according to a randomized block design using
156 four 40 L vats on each cheese-making day, with three replicates. Two types of milk
157 were used: F (100% from Italian Friesian cattle) and M (90% from Italian Friesian
158 cattle and 10% from Jersey), both obtained from local dairy farms. Each was
159 inoculated with two different starters (ST or ST+A). The four treatments were
160 therefore FST, FST+A, MST, MST+A. Milk composition was determined using
161 Milkoscan FT1 (Foss Italia, Padova).
162 The cheese-making recipe for "Scamorza", a semihard pasta filata cheese, was used.
163 The milk was heat treated at 65°C for 10 min and cooled at 38°C. Liquid veal rennet
164 (1:18,000, Caglificio Clerici SpA, Cadorago, CO, Italy) was added (30 mL/100 L
165 milk). Coagulation occurred within 16 min and the curd was manually cut to 2 cm.
166 Cooking was immediately started under agitation until the temperature of 42°C was
167 reached. The curd was then ripened under whey until the pH for stretching (5.2) was
168 reached (2.8 h from the addition of rennet). Stretching and molding were performed
169 manually to obtain pear shaped cheeses with a small head (500 g fresh weight). After
170 cooling in tap water (1 h), the cheese was salted in brine (20 Bé, 2 h) and ripened at
171 10-12°C at 75-80% RH for 30 days. Samples were taken at 0 (curd), 0.2 (immediately
172 after salting), 7, 15 and 30 days for chemical and/or microbiological analyses. At the
173 end of ripening the cheeses were vacuum packaged and stored at 4°C.
174

175 ***Gross composition of cheeses and proteolysis***

176 pH was measured using a spear-tip electrode (Hamilton Bonaduz AG, Bonaduz,
177 Switzerland) and a pH-meter (Orion 420A plus, Thermo Fisher Scientific, Rodano,
178 Italy). Moisture (% w/w) was measured by oven drying (IDF, 1982) and chlorides by
179 a potentiometric method (Fox, 1963). Fat in cheese was measured using a standard
180 method (IDF, 1996).
181 pH 4.6 soluble and insoluble fractions of cheese at 7 and 15 d were obtained using the
182 method of Kuchroo and Fox (1982). Free amino acids were measure using the TNBS
183 method (Adler-Nissen, 1976) on the pH 4.6-soluble fraction. The pH 4.6-insoluble
184 fraction was freeze-dried and used to assess primary proteolysis by urea-PAGE as
185 previously described (Piraino et al., 2005). After staining with Coomassie Brilliant
186 Blue (Blakesley and Boezi, 1977) the gels were digitized to .tif images using a
187 scanner.
188
189 ***Microbial counts***
190 Microbial counts were performed on milk, milk after the addition of starter, curd
191 before stretching and on cheese at 7, 15 and 30 days. Cheese samples were
192 homogenized in 2% (w/v) trisodium citrate solution and further decimal dilutions
193 were prepared in sterile quarter-strength Ringer's solution, while all dilutions were
194 carried out in Ringer for milk samples. Total mesophilic counts were carried out by
195 pour plating in Plate Count Agar standard (PCA, Oxoid) after 48 h at 30°C.
196 Thermophilic streptococci were enumerated in LM17 agar (M17 broth, Oxoid, with
197 1% lactose and 1.2% Agar bacteriological) after incubation for 2 days at 42°C. Non-
198 starter lactic acid bacteria were differentially enumerated in mMRS-BPB (Lee and
199 Lee, 2008) after incubation in anaerobiosis (GenBox Jars, bioMérieux Italia, Firenze,

with AnaeroGen bags, Oxoid) at 25 or 37°C for 48 h. Coliforms were enumerated in milk and cheese by pour plating in VRBA (Oxoid), after incubation for 24 h at 37°C.

DNA extraction and PCR-DGGE

DNA was extracted from a 1:4 suspension of cheese in 2% (w/v) trisodium citrate using Power food Bacterial DNA Extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) (Quigley et al., 2012) as described in the manufacturer's instructions. PCR-DGGE of the V3 region of 16S ribosomal DNA was carried out as described by (Ercolini et al. 2004) but Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) was used and staining was performed using 25 µL of SYBR Gold 10,000X (Invitrogen, Thermo Fisher Scientific, Rodano, Italy) in 250 mL of TAE 1X. Gel images were digitized using a GelDocXR apparatus with and XcitaBlueTM conversion screen, ChemiDocTM XRS filter and Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, Ca, USA) and converted to .tif images. For the identification of bands two procedures were used: two ladders including respectively amplified DNA from pure cultures of *L. plantarum* DSM20174, *Enterococcus faecium* DSM20477, *L. helveticus* ATCC15807, *E. faecalis* DSM20478, *Lact. lactis* subsp. *lactis* DSM20481, and *L. paracasei* DSM5622 were included in all gels, and bands which did not match with those of the ladder were eluted, re-amplified using the same primers, checked for purity and sequenced if a single amplification product was obtained.

222 ***Electronic nose analysis***

223 Cheese samples were analyzed using a ten-MOS electronic device (PEN-3,
224 AIRSENSE, Analytics GmbH, Schwerin, Germany). The air flux method was used in
225 this trial. The fluxed aroma was obtained using an output needle inserted into a Teflon
226 50 mL vial containing 1 g of cheese at 20°C with an air flow of 400 mL/min The
227 sample run lasted 60 sec and was followed by 300 sec flush time. Each measurement,
228 carried out in triplicate, was controlled and recorded in a text file by Win Munster
229 v.1.6.2.2 software.
230
231 ***Statistical analysis***
232 Statistical analyses were performed using Systat 13 (Systat Software Inc., San Jose,
233 CA) unless otherwise stated. Analysis of Variance (ANOVA), Analysis of covariance
234 (ANCOVA) and multiple mean comparisons with Tukey's HSD were used to test the
235 significance of differences caused by the treatments and ripening time on gross
236 composition and microbial counts. Multivariate statistical methods (Principal
237 Component Analysis, Partial Least Square regression) were used for the analysis of
238 the response of the electronic nose. Image analysis of electrophoretic patterns (PCR-
239 DGGE, urea-PAGE) and band matching were performed using GelCompar II
240 (Applied Maths BV, St-Martens-Latem, Belgium). Cluster analysis (using the Dice
241 coefficient and Unweighted Pair Group Method with Averages, UPGMA) was used to
242 group PCR-DGGE patterns. Principal Component Analysis was carried out on
243 relative band intensities in urea-PAGE gels and principal component scores were used
244 in ANOVA.
245
246 **RESULTS AND DISCUSSION**

247 ***Milk quality and cheese composition***

248 Gross composition of milk was (mean and standard error for the three replicate trials)
249 3.51±0.28% fat, 3.18±0.10% protein, 4.72±0.09 lactose for F, and 5.10±0.78% fat,
250 3.62±0.18% protein, 4.75±0.10 lactose for Jersey. Microbiological quality of milk
251 was within legal limits for both milks with no significant differences among cheese-
252 making trial replicates.
253 The gross composition of cheese at the end of ripening is shown in Table 1.
254 Significant differences due to milk and milk x starter interaction were found only for
255 salt in moisture. Significant differences due to starter were found for pH (lower for
256 cheeses produced with the adjunct), total free amino acids (higher for samples
257 produced with the adjunct). Although significant differences in cheese composition as
258 a function of milk type have been found by De Marchi et al. (2008) for Casolet,
259 Vezzena, Grana Trentino, and by Perna et al. (2014) for Caciocavallo cheese
260 produced with pure milk of different breeds (De Marchi et al. 2008; Perna et al. 2014)
261 or with 50:50 mixtures (De Marchi et al., 2008), in our study the lack of significant
262 differences due to milk type may be caused by the relatively low amount of milk for
263 Jersey cows used in treatments MST and MST+A. Differences in pH are most likely
264 due to differences in adjunct activity on residuals sugars in the curd, and differences
265 in free amino acid content are due to the higher proteolytic and peptidolytic activity of
266 the adjuncts. The small, but systematic, differences in salt in moisture content of the
267 cheeses produced with mixed milk are difficult to explain. All cheeses had identical
268 pH (5.3) at stretching and when they were salted in brine, and the salt content was not
269 significantly different at 7 and 15 d (data not shown). Although not significant, the
270 slightly higher fat content of cheese made with mixed milk may have contributed to
271 reducing salt diffusion during brining (Guinee and Fox 2004) and this difference

appeared as significant only at the end of ripening when the loss of moisture was highest.

pH, microbial counts and PCR-DGGE

Evolution of pH and microbial counts on LM17 and mMRS-BPB are shown in Figure 1. pH was significantly ($p<0.05$) lower for cheeses produced with adjuncts from day 7 onward. Counts on LM17 (which reflect the presence of the primary starter, *S. thermophilus* at least in the first 15 days of ripening) were close to 10^9 cfu/g throughout ripening. The slight initial decrease may be due to the effect of stretching in hot water. All species of the adjunct grew on mMRS-BPB at 37°C, as shown by the presence of colonies with the typical morphology of the three species used, whereas the primary starter was unable to form colonies on this medium. As expected, counts on mMRS-BPB were significantly higher for the cheeses made with adjuncts until the end of ripening, when high counts were found in all cheeses. Figure 2 shows the proportion of the different species recovered on mMRS-BPB at 30 d, based on colony morphology. Most colonies for all cheeses had the typical morphology of *L. paracasei*, whereas colonies with the morphology of *L. helveticus* were detectable for all cheeses made with adjuncts and for one replicate of cheese made with mixed milk without adjuncts. *Lact. lactis* colonies were never found for cheeses made without adjuncts and their proportion was very low (usually <1%) even for cheeses made with adjuncts. To confirm the results from enumerations, total DNA was extracted from cheese at 7 and 30 days of ripening, and used for PCR-DGGE analysis. An example of a typical gel is shown in Supplementary Figure 1. Except for the bands that matched those of the ladder (all of which were confirmed by extraction, re-amplification and sequencing), other bands were aspecific amplification products or

297 matched with the pattern for the *L. paracasei* group (bands below the *L. paracasei*
298 band). Cluster analysis of the PCR-DGGE patterns resulted in three main clusters and
299 two smaller ones (Figure 3). Two clusters included most of the cheeses produced
300 without the adjunct, and one cluster included only 7 d cheeses in which the only
301 identifiable band was that of the primary starter (*S. thermophilus*) while the other
302 included 30 d cheeses with the primary starter band and that of the *L. casei/paracasei*
303 group. Most of the cheeses produced with the adjunct showed the bands for all 3
304 adjunct species at both 7 and 30 days. One sample (MST replicate 3) produced
305 without adjunct showed *L. paracasei* and *L. helveticus* at 7 days, but only the *L*
306 *paracasei* band was present at 30 days. Although a contamination of the milk with the
307 adjunct is possible, it is unlikely.
308 To confirm that the differences in pH were due to activity of the adjunct, lactose,
309 lactic acid and acetic acid concentration were assayed at 15 d. Residual lactose
310 concentrations were (mean ±standard error for the three replicate cheese-making
311 trials) 13.8±0.07, 1.22±0.07, 8.12±0.32, 2.15±0.26 g/kg DM for treatments FST,
312 FST+A, MST, MST+A respectively. L-lactic acid concentrations were 13.1±0.8,
313 14.2±0.3, 15.6±0.6, 14.6±1.2 g/kg DM for treatments FST, FST+A, MST, MST+A,
314 respectively and D-lactic acid concentrations were <0.2, 4.7±0.3, 1.2±0.1, 3.2±0.2
315 g/kg DM for treatments FST, FST+A, MST, MST+A, respectively. Acetic acid
316 concentration was below the detection limit of the method used. This pattern confirms
317 that the lower pH may be due to increased lactose consumption by the DL-lactic acid
318 producing species (*L. helveticus* and *L. paracasei*) early during ripening.
319 Of the three adjuncts used, only two (*L. helveticus* and *L. paracasei*) were found by
320 both culture dependent and independent methods. Although we did not use strain
321 typing to confirm that the strains found in cheese were indeed those added with the

322 adjunct, the low number of NSLAB found in cheese produced without adjuncts until
323 day 15 suggests that the differences are due to the addition of the adjunct rather than
324 to adventitious NSLAB, which, on the other hand, were able to develop to high
325 numbers in all cheeses at 30 d. (Table 1, Figure 1). *L. helveticus* persisted in all
326 cheeses produced with adjuncts until the end of ripening and was also found in one
327 single replicate of cheese produced with mixed milk together with a higher content of
328 *L. paracasei*. This might have been due to contamination with the adjunct during
329 cheese making, although this is unlikely, but also to a higher NSLAB content in the
330 milk (microbial counts of the Jersey milk for this single replicate were significantly
331 higher). In the cheeses produced without adjunct only bands identified as *S.*
332 *thermophilus* were found in 7 d cheeses, while in 30 d cheese a band corresponding to
333 the *L. casei* group was always found. Our results are similar to those obtained for
334 Caciocavallo Pugliese produced with adjuncts (Di Cagno et al., 2012) in which the
335 addition of viable adjuncts resulted in high numbers (>10^8 cfu/g) of mesophilic
336 lactobacilli early during ripening (15 d) while the number of mesophilic NSLAB was
337 significantly lower in control cheese and increased to values close to 10^7 only after 30
338 d of ripening. NSLAB and *L. helveticus* are frequently found in ripened pasta filata
339 cheeses (Gobbetti et al., 2002, Piraino et al., 2005). Although the latter is not
340 dominant in the mature cheese it as been shown to survive and contribute to
341 proteolysis in other semi-hard cheeses (Kenny et al., 2006). *Lact. lactis* has also been
342 found as a subdominant member of the microbiota of ripened pasta filata cheeses
343 (Piraino et al. 2005) but it is apparently outnumbered during cheese ripening (Aponte
344 et al., 2008). Its lack of ability to colonize the cheese may be either due to a lower
345 survival to the stretching step or to a low competitiveness on the cheese matrix.

Lower pH is frequently observed in cheese produced with NSLAB adjuncts (Ong et al., 2006; Ong et al., 2007; Ciocia et al., 2014) and this is usually due to the production of acetic acid by NSLAB (Ong et al., 2006; Ong et al., 2007). However, in our study this is clearly not the case, because acetic acid was not detected. *S. thermophilus* is usually unable to ferment galactose (Iyer et al., 2010) and use of galactose negative strains in the production of low moisture Mozzarella cheese results in residual galactose in the curd (Kinstedt et al., 2004). On the other hand, all strains used as adjuncts in this study were galactose positive and salt tolerant (Piraino et al., 2008). As a result more lactic acid and lower pH were found in cheese produced with adjuncts as well as a higher concentration of the D-lactic acid isomer, produced by *L. helveticus* and *L. paracasei*. Di Cagno et al. (2012) obtained similar results for Caciocavallo Pugliese produced with live adjuncts.

Proteolysis

pH 4.6-soluble and -insoluble nitrogen fractions were obtained for cheeses at 15 and 30 days of ripening. pH 4.6-insoluble nitrogen fraction was separated by urea-PAGE. Gel images are shown in Supplementary figures 2a and 2b. Attempts at using a PLS-DA model to assess the effect of time, milk and starter were unsuccessful. However, univariate ANOVA showed that the intensity of several bands was affected by the type of starter (relative front, RF, 0.211, 0.262, 0.292, 0.55 - corresponding to α-casein -, 0.635 - likely corresponding to α_{s1}-CN f24-199 -, 0.651, 0.714, 0.801, 0.888), type of milk (RF 0.211, 0.391, 0.635), or time (0.147, 0.211, 0.454, 0.635). A Principal Component Analysis was carried out on the correlation matrix of relative band intensities and the first three factors explained 75.7% of the variance. Score and loading plots are shown in Figure 4. High variability among replicate cheese-making

371 trials is evident. ANOVA was carried out on the factor scores and showed that factor
372 1 was significantly affected by time (p=0.001, with lower values at t=30 d) and starter
373 (p=0.03, with lower values with the adjunct), factor 2 was significantly affected by
374 time (p=0.0006, with lower average values at 30 d) and factor 3 by milk type
375 (p=0.003, with lower values for the mix).
376 The TNBS method was used to estimate total amino acid content of cheese. No
377 significant differences were found at 15 d, whereas the effect of the adjunct culture
378 (with more secondary proteolysis in cheeses made with the adjunct) and block (with
379 more proteolysis in cheeses made in the third cheese-making day) were significant.
380 Proteolysis is one of the most important phenomena during cheese ripening
381 (Upadhyay et al., 2004) and adjunct cultures are often used to increase it because of
382 their proteolytic and peptidolytic activity (Chamba and Irlinger, 2004; El Soda et al.,
383 2000). Although significant proteolysis is only found in ripened pasta filata cheeses,
384 such as Provolone and Caciocavallo (De Angelis and Gobbetti, 2011), proteolysis is
385 also important in short-ripened varieties, including low moisture Mozzarella cheese,
386 in which it affects the functional properties of the cheese (Kinstedt et al., 2004)
387 because of its effect on functional properties. Although adjuncts rarely contribute to
388 primary proteolysis in cheese (Ciocia et al., 2013; Di Cagno et al., 2006), differences
389 were found in the urea-PAGE profiles of Caciocavallo Pugliese cheese produced with
390 or without adjunct cultures at 30 d (Di Cagno et al., 2012). However, it was not clear
391 if the differences were due to direct or indirect effects. Perna et al. (2014) found that
392 milk type (Holstein vs. Italian Brown) significantly affected primary proteolysis in
393 Caciocavallo cheese and attributed the differences to differences in pH and moisture.
394 In our study pH and, to a lesser extent, moisture differences due to adjunct addition
395 possibly affected the residual activity of plasmin. Plasmin is likely to be the main

proteolytic enzyme in pasta filata cheeses (Gobbetti et al., 2002) since chymosin is usually inactivated at high temperatures (Hayes et al., 2002). However, residual chymosin activity may be present in some pasta filata cheeses stretched at low temperatures (De Angelis and Gobbetti, 2011) and this may have caused the limited degradation of a_{S1}-casein observed in our study.

The amount of total amino acids estimated by the TNBS method in Scamorza cheese after 30 days of ripening was significantly lower (from 13 to 21 mg/g) than that found in ripened pasta filata cheeses (De Angelis and Gobbetti, 2011; Di Cagno et al., 2012) and this is likely due to the short ripening time. However, adjuncts significantly increased the amount of free amino acids. Adjunct starters are known to contribute to the release of free amino acids in several cheeses (El Soda et al., 2000), and the addition of adjuncts or attenuated adjuncts has been shown to significantly increase the amount of total amino acids in Caciocavallo Pugliese cheese ripened for two months. Albenzio et al. (2013b) found that even the addition of probiotic cultures (*Bifidobacterium longum* and *B. lactis* or *L. acidophilus*) significantly affected both primary and secondary proteolysis in ewe's milk Scamorza cheese ripened for 15 d. Both species were used at a very high inoculum level (2%) and their addition resulted in a lower pH in cheese. Since it is unlikely that the probiotic strains grew in cheese, the effect may have been largely indirect.

Volatile compounds

Headspace analysis of cheese was performed using an electronic nose with ten sensors. Although within each replicate cheese-making trial and time the built in software of the electronic nose was able to separate quite clearly the treatments on the basis of milk and starter, separation was poor when all samples (for each of which 3

421 replicate measurements were performed) were included. A PCA was performed on the
422 correlation matrix of sensor responses and the results are shown in Figure 5. As
423 before, variability between cheese-making trials obscured the pattern. However,
424 Analysis of covariance (ANCOVA) showed that no sensor was significantly affected
425 by milk type while six sensors were significantly affected by starter and time ($p<0.01$,
426 W1C, W5C, W3C, W5S, W1W, W2W) and two by time only (W1S, W3S).
427 A PLS-DA was also used to evaluate the effect of time, starter and milk type on
428 response. Cross-validation R^2 values were generally low (0.55-0.60), but the same
429 sensors whose response was significantly affected by starter and time in ANCOVA
430 were significantly affected in PLS.
431 Different species of lactic acid bacteria used as adjuncts, including the species we
432 used in our study, have been shown to affect the volatile compounds of several
433 cheeses (Ayad et al., 2000; Lee et al., 2007; Van Hoorde et al., 2010). Sensor arrays
434 have been widely used for the evaluation of the authenticity and quality of foods
435 (Reid et al., 2006), including cheese (Ampuero and Bosset, 2003), screen lactic acid
436 bacteria for aroma production (Marilley et al., 2004b; Gutiérrez-Méndez et al., 2008)
437 and to evaluate the effect of adjunct in Swiss cheese (Kocaoglu-Vurma et al., 2008).
438 Although electronic noses lack selectivity, they are a fast and relatively less expensive
439 tool to assess cheese aroma, compared to GC/MS. Because of their multivariate
440 nature, electronic nose data are treated by multivariate statistical methods, including
441 Principal Component Analysis, Discriminant analysis and Artificial Neural Networks
442 and the purpose is often discrimination of groups rather than inference on the effect of
443 design variables. Although it is difficult to compare our data with those of other
444 studies because of differences in the set-up and type of sensors, we confirmed that
445 addition of adjuncts significantly affects the aroma of a pasta filata cheese even

during short ripening times. Since MOS sensors have low specificity it is impossible to correlate their response with the production of specific volatile aroma compounds by the adjuncts. However, the sensor which were affected most by use of adjuncts and/or milk type in our study were reported to have a broad range of response (W5S, W1S) or to respond to aromatic (W1C, W3C, W5C, W2W) or non-polar organic compounds (W5C, W3S), to sulphur containing organic compounds (W1W) or to terpenes (W1W) (Baietto et al., 2013). Organic compounds belonging to these classes are frequently found in the volatile organic fraction of cheese (Marilley et al., 2004a) and may derive from either milk or starter culture activity (including metabolism of amino acids). An attempt to identify the volatile organic compounds by dynamic headspace gas chromatography was carried out on cheese samples at 15 and 30 days of ripening using the techniques described by Fedele et al. (2005). Unfortunately, due to high variability between cheese-making trials, no significant difference was found as a function of milk type, use of adjunct or time and it is therefore impossible to clearly identify the contribution of adjuncts and milk type on individual or groups of aroma compounds.

CONCLUSIONS

The addition of a complex adjunct and, to a lesser extent, the addition of a small amount of Jersey cow milk to Italian Friesian milk significantly affected composition, microbiological properties, proteolysis and volatile compounds profile in a semi-hard pasta filata cheese, Scamorza, produced using a *S. thermophilus* primary starter, over a relatively short (30 d) ripening time. Only two of the components of the adjunct (*L. helveticus* and *L. paracasei*) were consistently found in cheese, and the most

noticeable effects were on pH and release of free amino acids, while the effect on primary proteolysis was probably indirect. Although the changes caused by the addition of the adjunct are not necessarily desirable for all consumers (Braghieri et al., unpublished data) this work confirms that adjuncts can be used to accelerate ripening, manipulate cheese chemical, microbiological and volatile properties and differentiate the product.

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Figure captions

Figure 1. Evolution of pH (right), primary starter counts (LM17, 42°C, middle) and adjunct counts (mMRS-BPB, 37°C, right) in "Scamorza" during ripening. Cheese was made with two types of milk (F, circles: 100% Italian Friesian cattle; M, triangles: 90% F + 10% Jersey,) and two types of starter culture (ST, empty symbols: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A, closed symbols: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei* and *L. helveticus*).The four treatments were FST, FST+A, MST, MST+A. Mean and standard error for three replicate cheese-making trials.

Figure 2. Stacked bar plot showing the presumptive counts of adjunct species recovered in "Scamorza" cheese after 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei*

665 and *L. helveticus*). The four treatments were FST, FST+A, MST, MST+A. LPAR = *L.*
666 *paracasei*, LHEL= *L. helveticus,* LLAC= *Lact.* lactis. The values for the three
667 replicate cheese-making trials are shown.
668 **Figure 3**. Dendrogram showing the similarity relationships (Dice coefficient was used
669 and clustering was performed using Unweighted pair Group Method with Averages,
670 UPGMA) among PCR-DGGE patterns of the V3 region of 16S rDNA extracted from
671 "Scamorza" cheese at 7 and 30 d of ripening. Cheese was made with two types of
672 milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of
673 starter culture (ST: a defined *S. thermophilus* starter culture, Lyofast ST051, used
674 alone; ST+A: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei*
675 and *L. helveticus*). The four treatments were FST, FST+A, MST, MST+A. The
676 identity of bands and the patterns for the three replicate cheese-making trials are
677 shown. Values at nodes are bootstrapped confidence values.
678 **Figure 4**. Score and loading plots for the Principal Component Analysis carried out
679 on the correlation matrix of relative surfaces of bands detected by Urea-PAGE of pH
680 4.6 insoluble nitrogen fraction extracted from "Scamorza" cheese at 15 and 30 d of
681 ripening. Cheese was made with two types of milk (F, circles: 100% Italian Friesian
682 cattle; M, triangles: 90% F + 10% Jersey,) and two types of starter culture (ST, empty
683 symbols: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A,
684 closed symbols: ST + an adjunct containing *Lactococcus lactis, Lactobacillus*
685 *paracasei* and *L. helveticus*). The four treatments were FST, FST+A, MST, MST+A.
686 **Figure 5**. PCA score and loading plot for the Principal Component Analysis carried
687 out on the correlation matrix of responses of sensors of an electronic nose to the
688 headspace VOC from "Scamorza" cheese made with two types of milk (100% Italian
689 Friesian cattle, F, circles; 90% F + 10% Jersey, M, triangles) and two types of starter

culture (a defined *S. thermophilus* starter culture, Lyofast ST051, used alone, ST, empty symbols, and adjunct containing *Lactococcus lactis, Lactobacillus paracasei e L. helveticus,* ST+A, closed symbols) at 7, 15 and 30 d of ripening. The size of symbols is made proportional to ripening time.

Supplementary Figure 1. A sample gel showing electrophoretic patterns for PCR-DGGE patterns of the V3 region of 16S rDNA extracted from "Scamorza" cheese at 7 and 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei* and *L. helveticus*). The four treatments were FST, FST+A, MST, MST+A. The identity of bands and the patterns for the three replicate cheese-making trials are shown. The identity of bands in the ladder are shown.

Supplementary figure 2. Urea -PAGE patterns of pH 4.6 insoluble nitrogen extracted from "Scamorza" cheese at 15 and 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei* and *L. helveticus*). The four treatments were FST, FST+A, MST, MST+A. Bovine sodium caseinate (Sigma) was loaded on the first and last lane of each gel.

Table 1. Gross composition, pH and microbial counts of "Scamorza" at 30 days of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined *S. thermophilus* starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing *Lactococcus lactis, Lactobacillus paracasei* and *L. helveticus,*). The four treatments were FST, FST+A, MST, MST+A. Mean and standard error for three replicate cheese-making trials. The results of ANOVA for milk type (MT), starter (S), block (B, cheese-making day) and interaction (MTxS) are shown

	DM (%)	Fat (% DM)	pH	NaCl (% DM)	S/M (%)	FAA (mg leu/g DM)	LM17 log(cfu/g)	MRS37 log(cfu/g)
FST	59±1.5	40.3±1.8	5.07±0.01	2.31±0.06	3.18±0.05	3.9±0.3	8.6±0.3	7.6±0.2
FST+A	62±0.8	41.6±1.5	4.77±0.12	2.54±0.07	3.53±0.04	6.3±1.0	9.0±0.2	8.5±0.1
MST	62±1.0	43.7±1.5	4.92±0.06	2.08±0.08	2.96±0.15	4.6±0.2	9.4±0.1	8.6±0.1
MST+A	60±0.8	44.9±1.5	4.71±0.03	2.20±0.25	2.55±0.05	6.2±0.5	9.2±0.1	8.8±0.1
				ANOVA				
MT	n.s.	n.s.	n.s	n.s.	p<0.001	n.s.	n.s.	p<0.001
S	n.s.	n.s.	p=0.006	n.s.	n.s.	p<0.001	n.s.	p=0.002
MTxS	n.s.	n.s.	n.s.	n.s.	p<0.001	n.s.	n.s.	p=0.023
B	n.s.	n.s.	n.s.	n.s.	n.s.	p<0.001	n.s.	n.s.

LM17 thermophilic streptococci, enumerated on LM17 at 42°C; MRS37 non starter lactic acid bacteria enumerated on mMRS-BPB at 37°C. n.s. not significant (p>0.05)

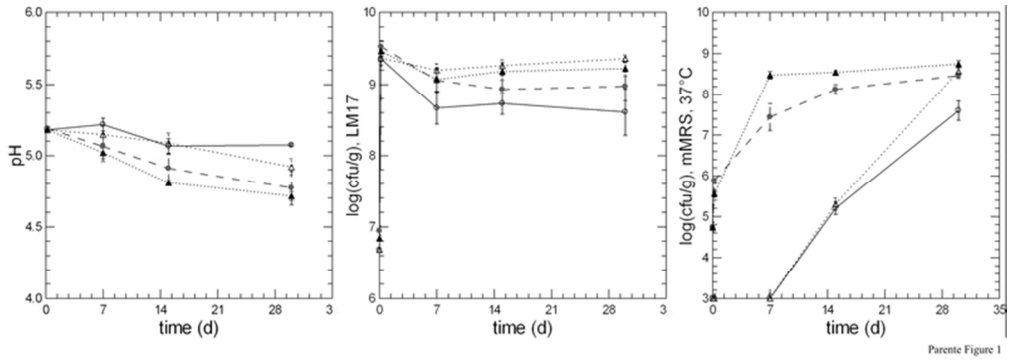
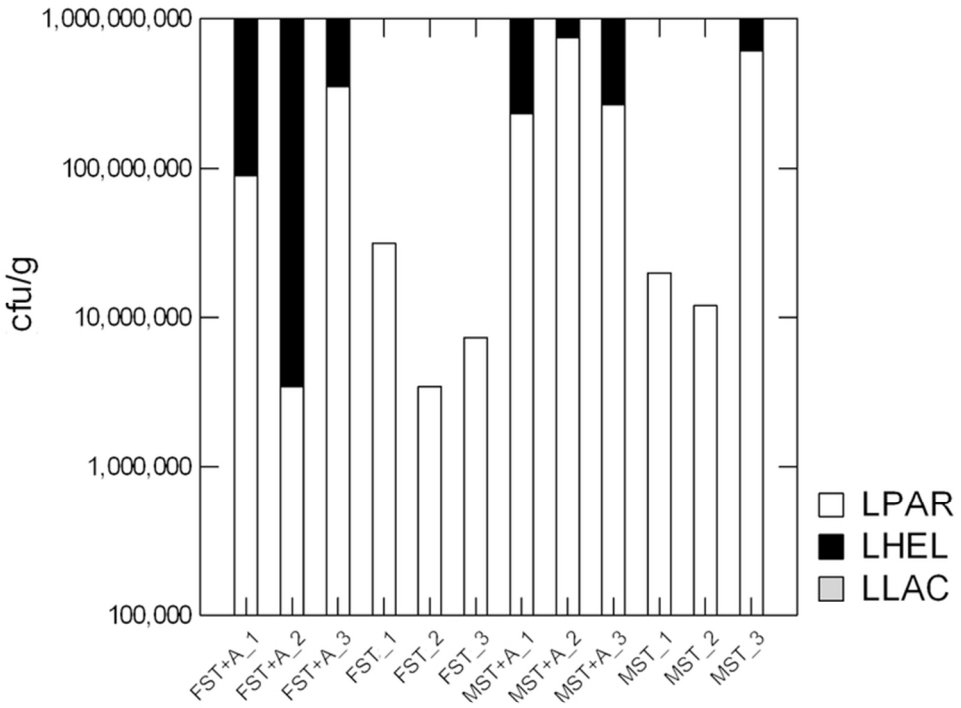
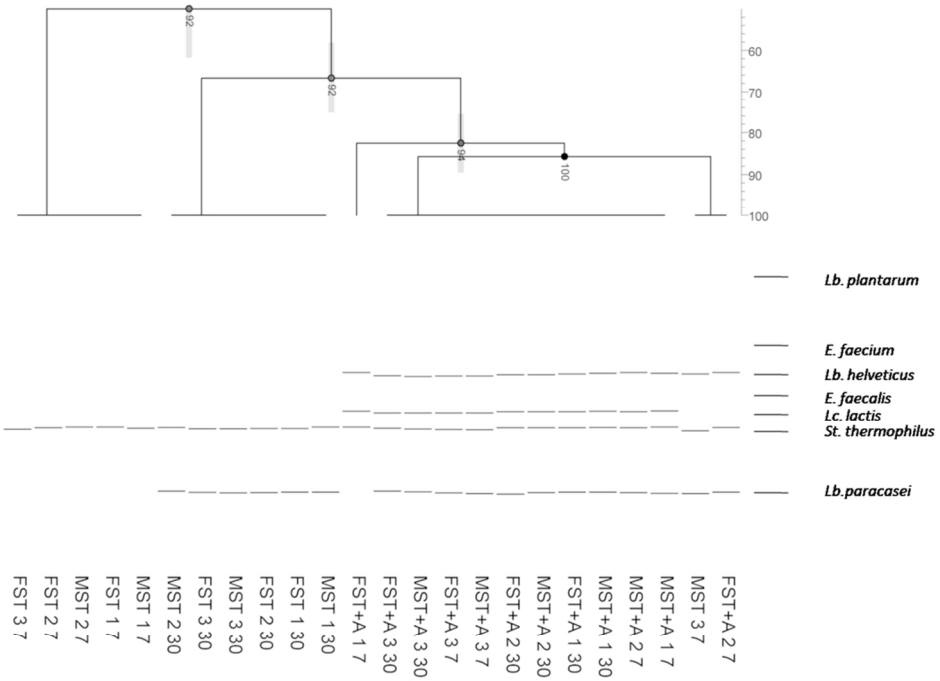


Figure 1. Evolution of pH (right), primary starter counts (LM17, 42°C, middle) and adjunct counts (mMRS-BPB, 37°C, right) in "Scamorza" during ripening. Cheese was made with two types of milk (F, circles: 100% Italian Friesian cattle; M, triangles: 90% F + 10% Jersey,) and two types of starter culture (ST, empty symbols: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A, closed symbols: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus).The four treatments were FST, FST+A, MST, MST+A. Mean and standard error for three replicate cheese-making trials.
67x23mm (300 x 300 DPI)



Parente Figure 2

Figure 2. Stacked bar plot showing the presumptive counts of adjunct species recovered in "Scamorza" cheese after 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A. LPAR = L. paracasei, LHEL= L. helveticus, LLAC= Lact. lactis. The values for the three replicate cheese-making trials are shown.
71x56mm (300 x 300 DPI)



Parente Figure 3

Figure 3. Dendrogram showing the similarity relationships (Dice coefficient was used and clustering was performed using Unweighted pair Group Method with Averages, UPGMA) among PCR-DGGE patterns of the V3 region of 16S rDNA extracted from "Scamorza" cheese at 7 and 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A. The identity of bands and the patterns for the three replicate cheese-making trials are shown. Values at nodes are bootstrapped confidence values.
105x78mm (300 x 300 DPI)

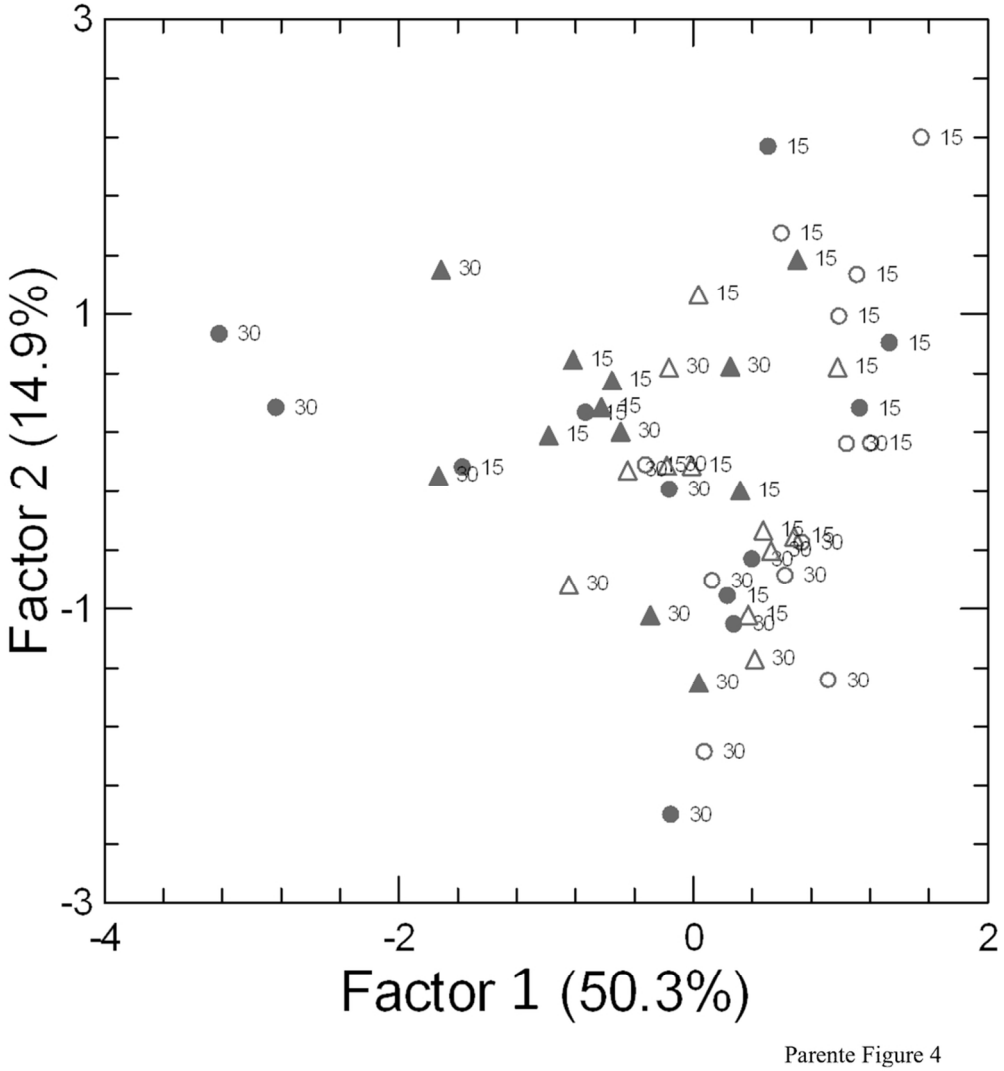
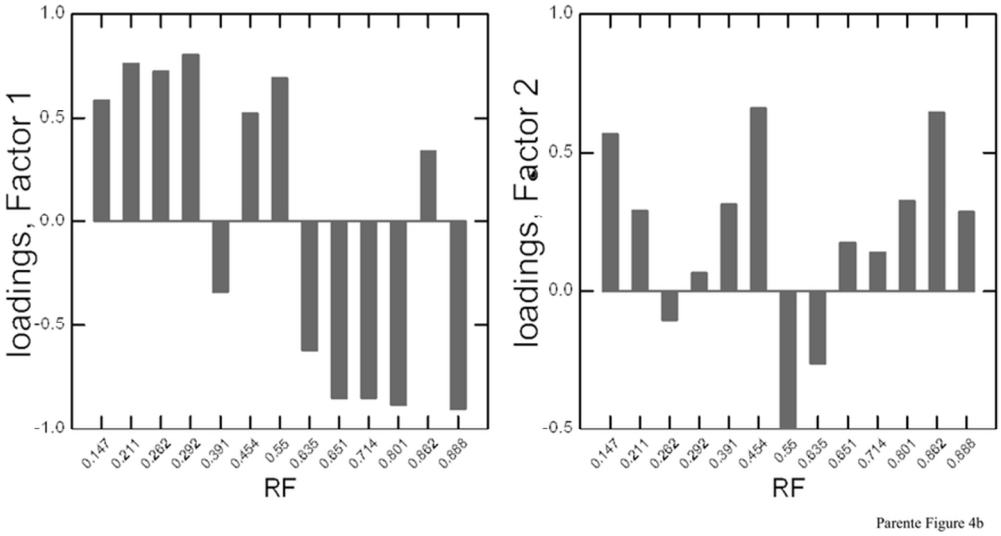


Figure 4. Score (a) and loading plots (b) for the Principal Component Analysis carried out on the correlation matrix of relative surfaces of bands detected by Urea-PAGE of pH 4.6 insoluble nitrogen fraction extracted from "Scamorza" cheese at 15 and 30 d of ripening. Cheese was made with two types of milk (F, circles: 100% Italian Friesian cattle; M, triangles: 90% F + 10% Jersey,) and two types of starter culture (ST, empty symbols: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A, closed symbols: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A.
95x102mm (300 x 300 DPI)



Parente Figure 4b

74x39mm (300 x 300 DPI)

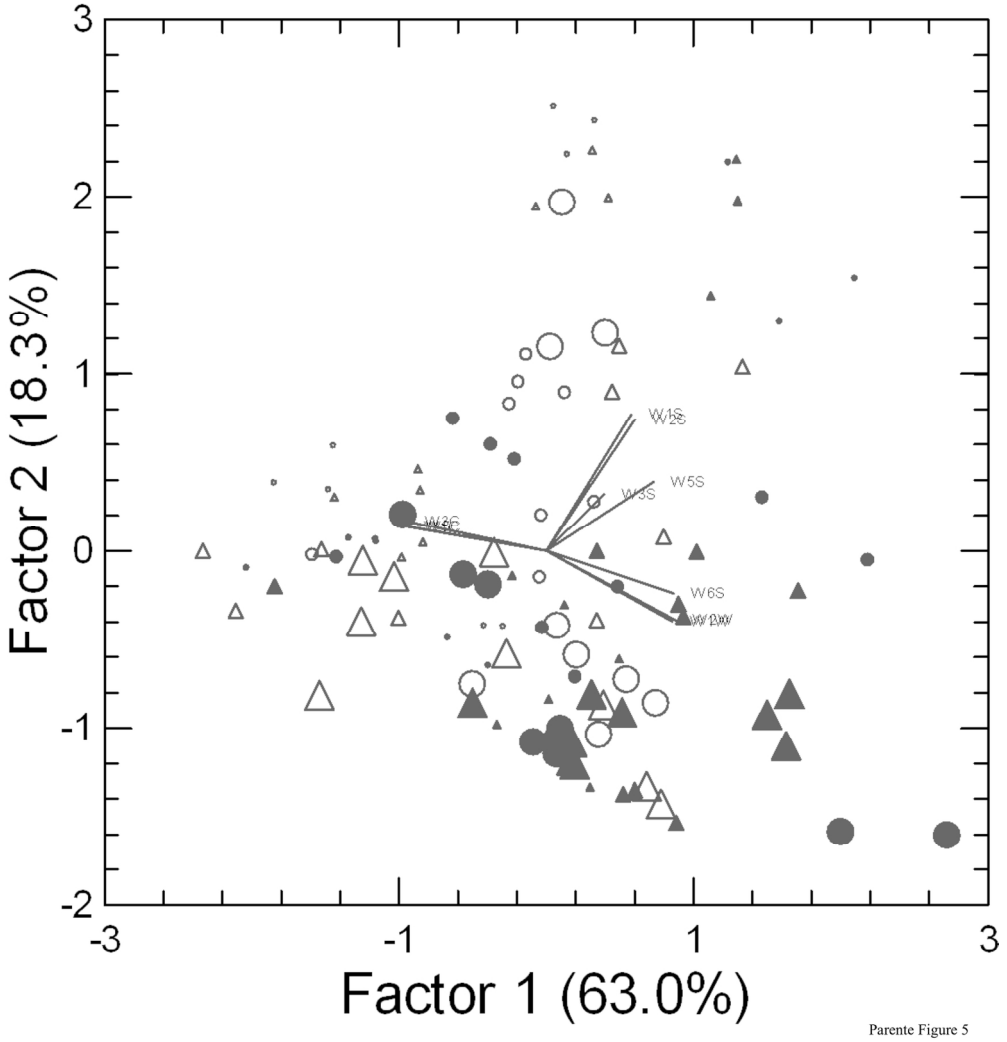
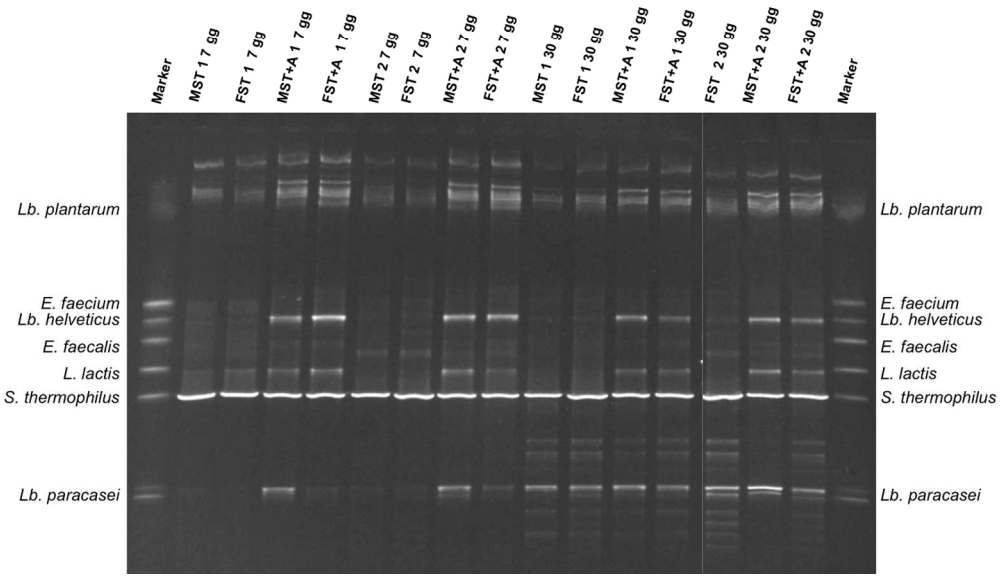
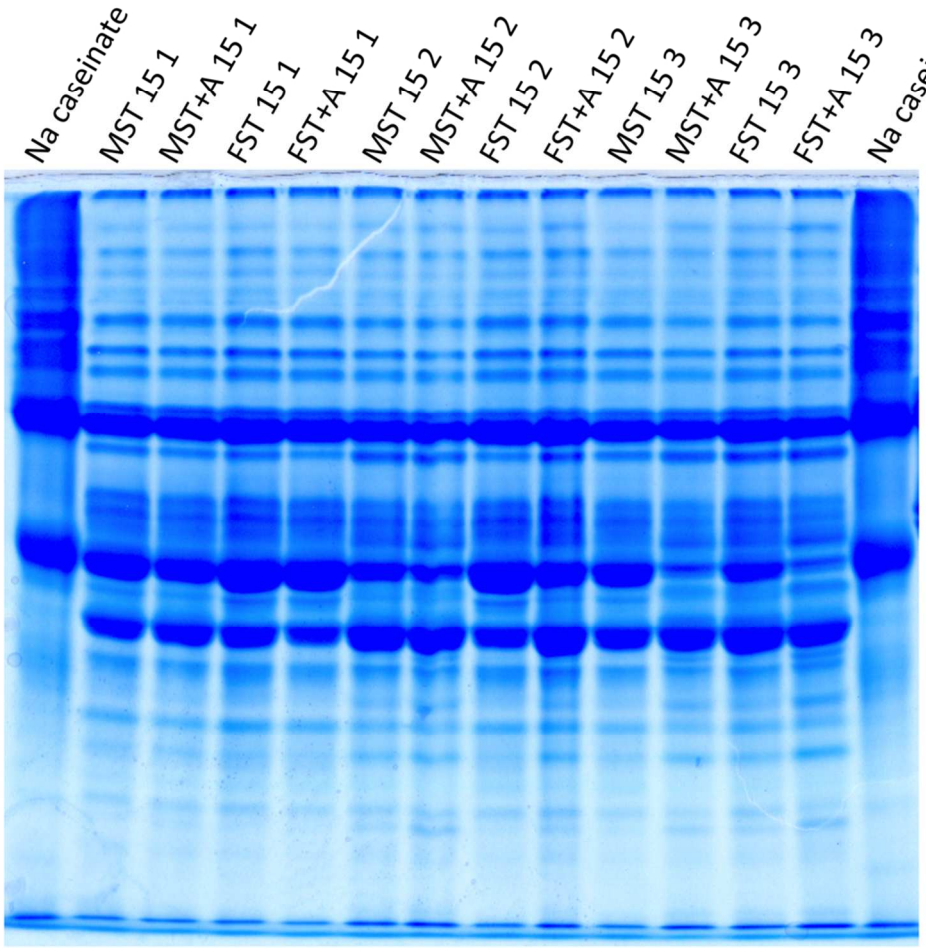


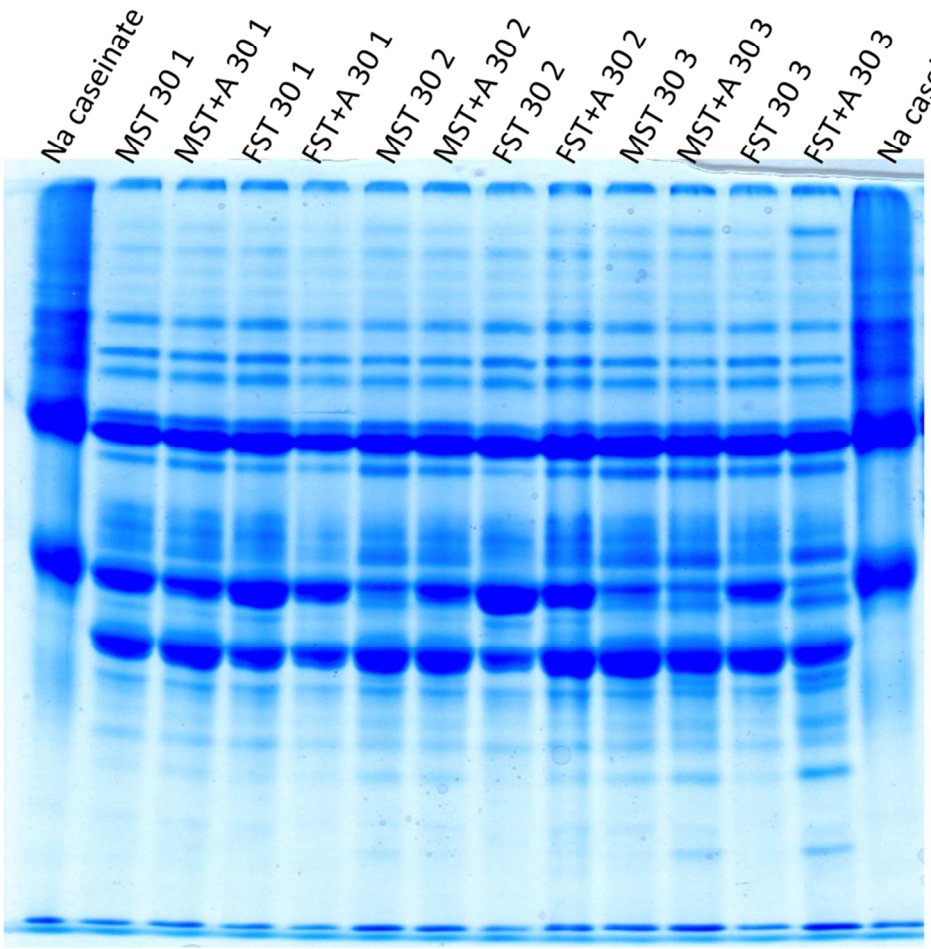
Figure 5. PCA score and loading plot for the Principal Component Analysis carried out on the correlation matrix of responses of sensors of an electronic nose to the headspace VOC from "Scamorza" cheese made with two types of milk (100% Italian Friesian cattle, F, circles; 90% F + 10% Jersey, M, triangles) and two types of starter culture (a defined S. thermophilus starter culture, Lyofast ST051, used alone, ST, empty symbols, and adjunct containing Lactococcus lactis, Lactobacillus paracasei e L. helveticus, ST+A, closed symbols) at 7, 15 and 30 d of ripening. The size of symbols is made proportional to ripening time.
147x156mm (300 x 300 DPI)



Supplementary Figure 1. A sample gel showing electrophoretic patterns for PCR-DGGE patterns of the V3 region of 16S rDNA extracted from "Scamorza" cheese at 7 and 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A. The identity of bands and the patterns for the three replicate cheese-making trials are shown. The identity of bands in the ladder are shown.
508x381mm (72 x 72 DPI)



Supplementary figure 2a. Urea -PAGE patterns of pH 4.6 insoluble nitrogen extracted from "Scamorza" cheese at 15 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A. Bovine sodium caseinate (Sigma) was loaded on the first and last lane of each gel.
391x376mm (72 x 72 DPI)



Supplementary figure 2. Urea -PAGE patterns of pH 4.6 insoluble nitrogen extracted from "Scamorza" cheese at 30 d of ripening. Cheese was made with two types of milk (F: 100% Italian Friesian cattle; M: 90% F + 10% Jersey,) and two types of starter culture (ST: a defined S. thermophilus starter culture, Lyofast ST051, used alone; ST+A: ST + an adjunct containing Lactococcus lactis, Lactobacillus paracasei and L. helveticus). The four treatments were FST, FST+A, MST, MST+A. Bovine sodium caseinate (Sigma) was loaded on the first and last lane of each gel.
394x394mm (72 x 72 DPI)