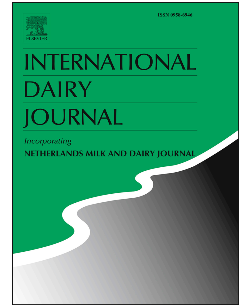
CORA Cork Open Research Archive
Cartlann Taighde Oscailte Chorcaí

Title	Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese
Authors	Bertuzzi, Andrea S.;Kilcawley, Kieran N.;Sheehan, J. J.;O'Sullivan, Maurice G.;Kennedy, D.;McSweeney, Paul L. H.;Rea, Mary C.
Publication date	2017-04-17
Original Citation	Bertuzzi, A. S., Kilcawley, K. N., Sheehan, J. J., O'Sullivan, M. G., Kennedy, D., McSweeney, P. L. H. and Rea, M. C. (2017) 'Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese', International Dairy Journal, 72, pp. 44-54. doi:10.1016/j.idairyj.2017.04.001
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1016/j.idairyj.2017.04.001
Rights	© 2017 Elsevier Ltd. This manuscript version is made available under the CC-BY-NC-ND 4.0 license. - http://creativecommons.org/licenses/by-nc-nd/4.0/
Download date	2024-05-30 03:59:12
Item downloaded from	https://hdl.handle.net/10468/3960

UCC
University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

Accepted Manuscript



Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese

A.S. Bertuzzi, K.N. Kilcawley, J.J. Sheehan, M.G. O'Sullivan, D. Kennedy, P.L.H. McSweeney, M.C. Rea

PII: S0958-6946(17)30078-X

DOI: 10.1016/j.idairyj.2017.04.001

Reference: INDA 4168

To appear in: *International Dairy Journal*

Received Date: 12 December 2016

Revised Date: 3 April 2017

Accepted Date: 4 April 2017

Please cite this article as: Bertuzzi, A.S., Kilcawley, K.N., Sheehan, J.J., O'Sullivan, M.G., Kennedy, D., McSweeney, P.L.H., Rea, M.C., Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese, *International Dairy Journal* (2017), doi: 10.1016/j.idairyj.2017.04.001.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

1 **Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese**

2

3

4

5

6 A. S. Bertuzzi[a,b] K. N. Kilcawley[a], J. J. Sheehan[a], M. G. O'Sullivan[b], D. Kennedy[a], P. L. H.
7 McSweeney[b], M. C. Rea[a,c]*

8

9

10

11

12

13 [a]*Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland,*
14 [b]*School of Food and Nutritional Science, University College Cork, Ireland,*
15 [c]*APC Microbiome Institute, University College Cork, Ireland*

16

17

18

19

20

21 *Corresponding author. Tel.:
22 *E-mail address*: (M. C. Rea)

23

ACCEPTED MANUSCRIPT

24 ___

25 ABSTRACT

26

27 The strains *Staphylococcus saprophyticus* DPC5671 and *Corynebacterium casei* DPC5298
28 were applied in combination with *Debaryomyces hansenii* DPC6258 to the surface of young
29 Cheddar cheese curd to obtain two different smear-ripened cheeses. A surface microbiota
30 developed over the incubation period, comprising of both yeast and bacteria; pulsed field gel
31 electrophoresis confirmed that the inoculated strains of *S. saprophyticus* DPC5671 or *C. casei*
32 DPC5298 were the dominant bacterial strains on the surface of the cheese at the end of the
33 ripening period. The smear cultures changed the appearance and aroma, which were
34 significantly different from the control cheese. The approach presented in this study
35 represents a method for the development of new cheese varieties with novel aromas within a
36 short ripening time.

37 ___

38

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

39 **1. Introduction**

40

41 Smear cheese is a traditional dairy product, which plays an important role in both
42 small and industrial scale dairy production. Smear cheese is characterised by a short ripening
43 time and strong aroma produced by the growth of smear microbiota on the cheese surface.
44 Smear-ripened cheeses are manufactured by inoculating the surface of the cheese curd,
45 dipping, spraying or brushing with a mixture of bacteria and yeasts. The traditional method of
46 production is called "old-young smearing" and consists of washing young curds with the
47 brine from old cheese, to encourage the transfer of the microbiota from the old to the young
48 cheeses (Desmasures, Bora, & Ward, 2015; Fox, Guinee, Cogan, & McSweeney, 2017a).
49 The microbiota on the surface of the smear cheese is composed of a variety of
50 microorganisms that coexist in symbiotic relationships. Yeasts are normally the first resident
51 microorganisms to establish themselves on the surface of the cheese due to their tolerance to
52 low pH and salt. Yeasts metabolise lactate, producing H_2O and CO_2 and increase the pH
53 (Cholet, Hénaut, Casaregola, & Bonnarme, 2007; Corsetti, Rossi, & Gobbetti, 2001).
54 Moreover, they produce metabolites and growth factors (vitamins and amino acids) which
55 encourage the growth of Gram-positive bacteria, such as *Corynebacterium*, *Staphylococcus*
56 and *Brevibacterium* species (Cogan et al., 2014; Larpin et al., 2011).
57 The growth of smear microorganisms on the surface of cheese curd modifies the
58 appearance, aroma, proteolysis and lipolysis of the cheese within a relatively short ripening
59 time (McSweeney, 2004). The combined growth of the bacteria and yeasts on the surface of
60 the cheese results in the production of proteolytic and lipolytic enzymes, increasing the
61 amount of free amino acids (FAAs) and free fatty acids (FFAs) (McSweeney & Sousa, 2000;
62 Sousa, Ardö, & McSweeney, 2001). Yeasts and Gram-positive bacteria isolated from smear
63 cheeses have a wide range of proteolytic enzymes that display various peptidase activities,

64 with FAAs increasing within the cheese as a consequence. Additionally, yeasts and Gram-
65 positive bacteria possess esterolytic/lipolytic enzymes capable of catabolising triacylglycerols
66 in cheese, producing FFAs (Curtin, Gobbetti, & McSweeney, 2002; Fox, Guinee, Cogan, &
67 McSweeney, 2017b).

68 The further metabolism of FAAs and FFAs during the ripening produces flavour
69 compounds important for cheese aroma. The catabolism of FAAs, especially of branched
70 chain amino acids, aromatic amino acids and sulphur amino acids, produces mainly
71 aldehydes, alcohols, carboxylic acids and sulphur compounds. Moreover, FFAs are involved
72 in reactions leading to the production of flavour compounds such as secondary alcohols,
73 carboxylic acids, esters, lactones and ketones (McSweeney & Sousa, 2000; Singh, Drake, &
74 Cadwallader, 2003; Smit, Smit, & Engels, 2005; Yvon & Rijnen, 2001).

75 The characteristics of smear-ripened cheese are not strictly controlled inside artisanal
76 smear cheese plants. The resulting product is also affected by the final microbial consortia of
77 the cheese, which is influenced by the individual in-house microbiota of the cheese-making
78 facilities. Microorganisms detected in the environment of artisanal cheese-making plants
79 have also been found on the surface of smear cheeses, indicating a strong relationship
80 between product and the environment in which the cheese is manufactured and ripened
81 (Bokulich & Mills, 2013; Goerges et al., 2008; Mounier et al., 2006a).

82 In previous studies, smear strains were added to the cheese surface or as adjunct
83 cultures to the milk during manufacture of smear-ripened cheese; however, some of the added
84 strains were not detected at the end of ripening (Feurer, Vallaeys, Corrieu, & Irlinger, 2004;
85 Goerges et al., 2008). These commercial smear strains have to compete with the in-house
86 microbiota and do not always successfully establish themselves on the cheese surface
87 (Bokulich & Mills, 2013; Feurer et al., 2004; Goerges et al., 2008). It is likely that the

ACCEPTED MANUSCRIPT

88 relationship within the smear microbiota promotes the survival of a particular group of
89 microorganisms to the detriment of others.

90 With the abolition of the milk quotas within the EU in 2015 there is a renewed interest
91 in developing novel cheeses with a range of flavours. There is a progressive increase in
92 global cheese consumption, with an annual production in Ireland of 207,100 tonnes in 2015
93 (data from Eurostat). Therefore the aim of this work was to develop a novel cheese with
94 diverse aromas and short ripening time using cheese curd made in a traditional Cheddar
95 cheese plant. Ripening time for Cheddar cheese can be from a little as 3 months for mild
96 cheese up to > 9–12 months for mature/extra mature varieties. In this study, the ability of
97 smear bacteria and yeast to grow on the surface of young Cheddar cheese curd was
98 investigated to produce a cheese variety with different flavour and appearance compared with
99 Cheddar cheese within a short time frame of 35 days.

100

101 **2. Materials and methods**

102

103 *2.1. Preparation of smear suspensions*

104

105 For the preparation of *Debaryomyces hansenii* DPC6258 suspension, the strain was
106 streaked onto yeast extract glucose chloramphenicol agar (YGC agar; Becton, Dickinson and
107 Company, City West, Dublin, Ireland) and incubated aerobically at 25 °C for 96 h. Using a 5
108 μL loop, the strain was inoculated into 10 mL of trypticase soy broth (TSB; Becton,
109 Dickinson and Company) and incubated, shaking at 100 rpm, at 25 °C. When the OD_{600}
110 reached ~1, the cells were centrifuged at 6000 × *g* at 4 °C for 15 min, washed twice with
111 sterile 0.75% NaCl and the pellet was resuspended in sterile 0.75% NaCl to obtain a
112 suspension of ~10^6 cfu mL^{-1}.

ACCEPTED MANUSCRIPT

113 For the preparation of the *Corynebacterium casei* DPC5298 and *Staphylococcus*
114 *saprophyticus* DPC5671 suspensions, the strains were streaked onto trypticase soy agar
115 (TSA; Becton, Dickinson and Company) and incubated aerobically at 30 °C for 48 h. Using a
116 5 µL loop, the strains were inoculated into 10 mL of trypticase soy broth (TSB; Becton,
117 Dickinson and Company) and incubated, shaking at 100 rpm, at 30 °C. When the OD_{600}
118 reached ~1, the cells were centrifuged at 6000 × *g* at 4 °C for 15 min, washed twice with
119 sterile 0.75% NaCl and the pellets resuspended in sterile 0.75% NaCl to obtain a suspension
120 of ~10^5 cfu mL^{-1}.
121
122 *2.2. Smearing of cheese blocks*
123
124 Cheddar cheese was supplied by a commercial cheese company as 20 kg blocks, <24
125 h post manufacture. The large cheese block was aseptically cut into smaller blocks (~8 × 6.5
126 × 30 cm). These blocks were then inoculated by placing them in a saline suspension
127 containing *D. hansenii* DPC6258 (10^6 cfu mL^{-1}), ensuring an even coating of yeast.
128 Subsequently the blocks of cheese were placed on sterile, plastic coated racks and allowed to
129 drain. Once the excess liquid had completely drained, the cheese pieces were placed inside a
130 sterile plastic bag (Südpack Verpackungen, Ochsenhausen, Germany) on the rack ensuring
131 that the sides of the cheese did not make contact with the plastic bag. Relative humidity %
132 (RH%) was maintained by pouring 100 mL of sterile 0.75% NaCl into the base of the bag and
133 the bag was sealed. The cheese was ripened at 15 °C with a RH% of ~97%. After 5 days of
134 ripening, the blocks of cheese were removed from the bag and dipped in saline suspension
135 containing *C. casei* DPC5298, or *S. saprophyticus* DPC5671 (10^5 cfu mL^{-1}). The blocks of
136 cheese were placed on the sterile rack and incubated for a further 30 days (for a total ripening
137 period of 35 days), as described above, to produce a smear cheese with *D. hansenii* DPC6258

138 in combination with *S. saprophyticus* DPC5671 (cheese A) or *C. casei* DPC5298 (cheese B).
139 During the ripening period the surface of the cheese blocks was washed with a sterile sponge
140 soaked in a sterile brine solution (5% NaCl) at day 7, 10, 15 of ripening to ensure an even
141 growth of the smear microbiota. As a control, blocks of Cheddar cheese were vacuum packed
142 in sterile bags and incubated at 15 °C. These blocks were not smeared with either bacteria or
143 yeasts and were not washed with NaCl solution during the ripening period. However, the
144 control cheese differs from normal Cheddar cheese in that the temperature of ripening was
145 higher (15 °C) than the ripening temperature normally associated with Cheddar cheese (~8
146 °C). Three replicate cheese trials were performed.
147
148 *2.3. Sampling cheese*
149
150 The surface of the cheese A and B was aseptically sampled for enumeration and
151 isolation of bacteria and yeast at 3, 5, 7, 10, 15, 21, 25, 30 and 35 days of ripening.
152 When analysing the control cheese samples, a composite sample of core and surface was
153 analysed, while for the test cheeses both the surface and core were analysed separately. At
154 day 0 and day 35, samples were taken from the control cheese, cheese A and B for
155 composition, urea-polyacrylamide gel electrophoresis (urea-PAGE), free fatty acid and free
156 amino acid analysis. At day 35, samples were taken from the control cheese, cheese A and B
157 for sensory evaluation and volatile analysis. At days 0, 10, 15, 21, 25, 30, 35 samples were
158 taken from the control cheese, cheese A and B for proteolysis analysis and colorimetric
159 analysis.
160
161 *2.4. Enumeration of bacteria and yeast from cheese surface*
162

ACCEPTED MANUSCRIPT

163 During ripening, ~ 5 cm^2 of the surface of the test cheeses were aseptically removed
164 and resuspended in 2% trisodium citrate, serially diluted and plated on TSA 5% NaCl with 50
165 U mL^{-1} of nystatin (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) and YGC agar, for the
166 enumeration of smear bacteria and yeasts, respectively. Nystatin was added to TSA to
167 prevent the growth of yeast and moulds. The TSA plates were incubated at 30 °C for 48 h,
168 while YGC plates were incubated at 25 °C for 96 h. Colonies were counted and the results
169 expressed as log cfu g^{-1} of cheese. Five colonies from the highest countable dilution were re-
170 streaked onto TSA and incubated at 30 °C for 48 h. Isolates were stocked at –80 °C in
171 glycerol for further analysis.

172

173 2.5. *Pulsed field gel electrophoresis*

174

175 The cultures isolated from cheese A and B were grown on TSA, incubated aerobically
176 at 30 °C for 24 h and then inoculated in 8 mL of TSB, with shaking at 100 rpm at 30 °C for
177 24 h. Pulsed field gel electrophoresis (PFGE) was carried out as described by Bannerman,
178 Hancock, Tenover, and Miller (1995) for *S. saprophyticus*, while the method outlined by
179 Brennan et al. (2001) was used for *C. casei*. Before digestion the agarose plugs were cut into
180 small slices (1 by 2 mm), transferred into 100 µL restriction buffer containing 20 U of *Sma*I
181 for *S. saprophyticus*, and 20 U of *Spe*I (all from New England Biolabs, Hitchin, UK) for *C.*
182 *casei* and incubated over night at 25 °C or 37 °C, respectively. The gel was run in a CHEF-
183 DR III PFGE apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK) at 1 V (6
184 V cm^{-1}) at 14 °C for 20 h, with the pulse ramped from 5 to 40 s for *S. saprophyticus,* while 1
185 V (6 V cm^{-1}) at 14 °C for 16 h, from 1 to 20 s for *C. casei*. Gels were stained with ethidium
186 bromide (0.5 µg mL^{-1}) in water, destained in water and then photographed using a gel
187 imaging system (AlphaImager 2000, Alpha Immotech, San Leonardo, CA, USA).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

188

189 *2.6. Compositional analysis*

190

191 Cheese samples were analysed for moisture content by oven-drying 3 g of cheese
192 sample at 102 °C for 5 h and for salt content by a potentiometric method on a mixture of 2 g
193 of grated cheese and 60 g of water (IDF, 1988). Total protein was determined on 0.2 g of
194 cheese sample and total fat was determined on 3 g of cheese sample by standard methods
195 (IDF, 1993, 1996). The pH was measured with a standard pH meter (Mettler-Toledo MP220,
196 Schwerzenbach, Switzerland) on a mixture of 20 g of grated cheese and 12 g of water, as
197 described by British Standards Institution standard (BSI, 1976).

198

199 *2.7. Determination of colour*

200

201 The development of the colour during the ripening was measured in triplicate on the
202 surface of the cheese at room temperature, using a Minolta Colorimeter CR-300 (Minolta
203 Camera, Osaka, Japan). A white colour tile standard was used to calibrate the instrument and
204 the colour was analysed using L*, a* and b*-values, which describe the colour space. L*-
205 value measures the visual lightness (as values increase from 0 to 100), a*-value measures
206 from the redness to greenness (positive to negative values, respectively) and b*-value from
207 the yellowness to blueness (positive to negative values, respectively).

208

209 *2.8. Proteolysis*

210

211 Proteolysis was determined by measuring the levels of the non-casein nitrogen
212 content, soluble at pH 4.6 (pH4.6-SN) (Fenelon & Guinee, 2000) and total nitrogen (TN) on a

ACCEPTED MANUSCRIPT

213 water soluble extract of a mixture of 60 g of grated cheese and 120 g of water, using the
214 macro-Kjeldahl method (IDF, 1993). The levels of proteolysis were expressed as a
215 percentage of the ratio between non-casein nitrogen content and total nitrogen (% pH4.6-
216 SN/TN).

217

218 *2.9. Urea-polyacrylamide gel electrophoresis*

219

220 Urea-polyacrylamide gel electrophoresis (urea–PAGE) was performed according to
221 the method described by Rynne, Beresford, Kelly, and Guinee (2004). The gel system was
222 composed of a separating and stacking gel, using a PROTEANs II xi cell vertical slab gel unit
223 (Bio-Rad Laboratories Ltd). The samples were prepared, maintaining the same concentration
224 of protein (4.25 g of protein content L^{-1} sample buffer) to have a final volume of 1 mL. Ten
225 microlitres of sample solution were loaded into individual wells. The sample buffer (pH 8.7),
226 the sample preparation and the running conditions were as described by Henneberry,
227 Wilkinson, Kilcawley, Kelly, and Guinee (2015). After the run, the gel was removed from the
228 plates and stained overnight in an aqueous solution of Coomassie Blue G250 (0.25%, w/v),
229 destained in a destaining solution (acetic acid 10%, methanol 25%), and washed in distilled
230 water. The images were acquired by a gel imaging system (AlphaImager 2000; Alpha
231 Immotech).

232

233 *2.10. Free amino acid analysis*

234

235 Individual FAAs were determined on the soluble N extracts as described by
236 McDermott et al. (2016) using a Jeol JLC-500V AA analyser fitted with a Jeol Na+ high
237 performance cation exchange column (Jeol Ltd., Garden City, Herts, UK). The

238 chromatographic analyses were conducted at pH 2.2. Results are expressed as μg mg^{-1} of
239 cheese.
240
241 *2.11. Free fatty acid analysis*
242
243 FFA extraction was performed on 10 g of grated cheese, according to the method
244 described by De Jong and Badings (1990). The FFA extracts were aliquoted into amber glass
245 vials and capped with PTFE/white silicone septa (Agilent Technologies, Little Island, Cork,
246 Ireland). The FFA extracts were derivatised as methyl esters as outlined by Mannion, Furey,
247 and Kilcawley (2016) using a Sample Prep Workbench (Agilent Technologies). Fatty acid
248 methyl esters extracts were analysed using Varian CP3800 gas chromatograph (Aquilant,
249 Dublin, Ireland) with a CP84000 auto-sampler and flame ionisation detector (GC-FID) and a
250 Varian 1079 injector (Aquilant). For the GC-FID analysis, 0.7 μL were injected into a CP
251 FFAP CB capillary column (30 m × 0.25 mm × 0.32 μm) (Agilent Technologies). Results are
252 expressed as μg mg^{-1} of cheese.
253
254 *2.12. Volatiles analysis*
255
256 After sampling the cheese samples were wrapped in foil, vacuum packed and stored at
257 –20 °C. Before analysis, the samples were defrosted at room temperature and blended with a
258 cheese grater. For the analysis, 4 g of cheese sample were placed in a screw capped SPME
259 vial with a silicone/PTFE septum (Apex Scientific, Maynooth, Ireland). The SPME vials
260 were equilibrated to 40 °C for 10 min with pulsed agitation (5 s on, 2 s off) at 500 rpm.
261 Sample introduction was performed using AOC-5000 injection system (Shimadzu, Albert-
262 Hahn-Str., Duisburg, Germany) and a single 50/30 μm Carboxen TM137 / divinylbenzene /

ACCEPTED MANUSCRIPT

263 polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre was used for the volatile extraction
264 (Agilent Technologies). The SPME fibre was exposed to the headspace above the samples for
265 20 min at depth of 54 mm, then was retracted and injected into the GC inlet and desorbed for
266 2 min at 250 °C. Injections were made on a Shimadzu 2010plus with an Agilent DB-5ms
267 column (60 m × 0.25 mm × 0.25 μm) (Agilent Technologies), using a multipurpose injector
268 with a Merlin microseal. The temperature of the column oven was initially held for 30 s at 35
269 °C, increased to 230 °C at a rate of 6.5 °C min^{-1}, and to 320 °C at a rate of 15 °C min^{-1},
270 yielding at total GC run time of 41.5 min. The carrier gas, helium, was at a constant pressure
271 of 1.58 bar, performing a splitless injection. The detector, a Shimadzu TQ8030 MSD triple
272 quadrupole mass spectrometer, was used in single quadrupole mode. The ion source
273 temperature and interface temperature were set at 220 °C and 280 °C, respectively, and the
274 MS mode was electronic ionisation (70 v) with the mass range between 35 and 250 amu. The
275 chromatograms were deconvoluted and the peaks quantified with TargetView (Markes
276 International Ltd, Llantrisant, UK). The compounds were identified using mass spectra
277 comparisons to the NIST 2014 mass spectral library, and using an internal database with
278 known target and qualifier ions for each compound. Ten microlitres of the standard solution
279 [1-butanol, dimethyldisulphide, butyl acetate, cyclohexane, benzaldehyde at 10 ppm, and 2-
280 phenylethanol at 50 ppm, in methanol/:water (1:99)] in a SPME vial were run before and
281 after every series of samples to ensure that both the SPME extraction and MS detection were
282 performing within specification. An autotune of the GCMS was carried out prior to the
283 analysis to ensure optimal GCMS performance. All analyses were performed in triplicate.
284

285 *2.13. Sensory affective evaluation and ranking descriptive analysis*

286

287 The blocks of cheese sample (~500 g), vacuum packed and stored at -20 °C, were
288 defrosted at 4 °C the day before sensory evaluation. The surface of cheese A and B was
289 removed and the blocks were cut into portions for the sensory test. Twenty naive assessors
290 were recruited in University College Cork, Ireland. Sensory acceptance testing was
291 conducted using these untrained assessors, who were both Cheddar and smear cheese
292 consumers, (age 21–48 years). Assessors used the sensory hedonic descriptors for the control,
293 cheese A and B. Samples underwent monadic presentation to the panel at ambient
294 temperatures (~21 °C) and coded with a randomly selected 3 digit code. Each assessor was
295 asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely
296 dislike) to 10 (extremely like). Ranking descriptive analysis (RDA) was then undertaken
297 using the consensus list of sensory descriptors, which was also measured on a 10 cm line
298 scale. All samples were presented in duplicate.
299
300 *2.14. Statistical analysis*
301
302 The statistical analysis for cheese composition, sensorial analysis, FAAs and FFAs
303 were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab Inc.,
304 Coventry, UK). A split plot designed with SAS 9.3 (SAS, Dublin, Ireland) was used to
305 determine the singular effect of smear treatments, ripening time and their interaction on the
306 microbiology, pH, % pH 4.6-SN/TN, L*, a* and b* -values, measured at several time points
307 during the ripening. The statistical analysis of the volatile compounds was tested with
308 ANOVA-Partial Least Squares Regression (APLSR) using Unscrambler (The Unscrambler X
309 10.3, Camo Software, Oslo, Norway). From the results of the APLSR, the individual volatile
310 compounds positively correlated with the samples were tested with one-way analysis of
311 variance (ANOVA) using Minitab 17 (Minitab Inc.), to evaluate the significant differences

ACCEPTED MANUSCRIPT

312 among the samples for each volatile compound. The level of significance for all analyses was
313 determined at $P < 0.05$.

314

315 3. Results

316

317 3.1. *Growth of the strains and pH development*

318

319 PFGE analysis established that the inoculated cultures of *S. saprophyticus* DPC5671
320 and *C. casei* DPC5298 were the dominant bacterial strains isolated at the end of ripening (day
321 35) (supplementary data). The total count of yeasts and smear bacteria during ripening is
322 shown in the Fig. 1. A significant interactive effect ($P < 0.05$) between ripening time and
323 smear treatments was observed for the growth of the surface microbiota. No significant
324 differences were observed on the growth of yeast and bacteria between cheese A and cheese
325 B.

326 The variation in pH of the control, and the core and the surface of cheese A and B is
327 shown in Fig. 2. A significant interactive effect ($P < 0.05$) between smear treatments and
328 ripening time was observed for pH. From day 21 to 35, the pH was significantly higher (P
329 < 0.05) at the surface of cheese A compared with its respective core and the control. From day
330 15 to 35, the pH was significantly higher ($P < 0.05$) at the surface of cheese B compared with
331 the respective core and the control.

332

333 3.2. *Cheese composition*

334

335 The mean composition of the Cheddar cheese before the smearing process was typical
336 of a commercial Cheddar cheese after manufacture, but the smearing treatments influenced

ACCEPTED MANUSCRIPT

337 the compositional parameters at the end of the ripening (35 days). Compositional data and
338 significant differences (P <0.05) are presented in Table 1.

339

340 *3.3. Proteolysis*

341

342 The proteolysis was particularly high at the surface of cheese A and B, where the
343 smear treatments significantly (P <0.05) influenced the level of % pH 4.6-SN/TN
344 (supplementary data). Urea-PAGE (Fig. 3) confirmed the high level of proteolysis on the
345 surface of cheese A and B. α_{S1}-Casein was partially degraded to α_{S1}-CN (f102–199) and α_{S1}-
346 CN (f24–199) in the control cheese and in the core of cheese A and B. However there was
347 evidence of further breakdown products on the surface samples of the test cheeses. Similar β-
348 casein degradation patterns were observed for the control and core of test cheeses, while
349 samples from the surface of the test cheeses showed that the β-casein was almost totally
350 degraded producing β-CN (106–209) and β-CN (108–209) with higher intensity (Fig. 3).

351

352 *3.4. Free amino acids and free fatty acids*

353

354 Significant differences (P <0.05) on the total amount of FAAs were observed between
355 the control and the surface of cheese A and B at the end of ripening on day 35 (Fig. 4). In the
356 cheese A and B, some individual FAAs were significantly higher (P <0.05) on the surface
357 compared with their respective cores or the control. No significant difference was determined
358 between the surface samples of cheese A and B with respect to the total FAA content.
359 Significant differences (P <0.05) in the total amount of FFAs were observed between
360 the control and the surface of cheese A and B at day 35 (Fig. 5). In the cheese A and B, all
361 individual FFAs detected on the surface were significantly higher (P <0.05) compared with

ACCEPTED MANUSCRIPT

362 the levels of the respective cores or the control. Significantly higher levels (*P* <0.05) of total
363 FFAs were detected on the surface of cheese A (20169±2120 μg mg^{-1}) compared with the
364 surface of cheese B (12338±3382 μg mg^{-1}).
365

366 *3.5. Cheese colour*
367

368 The measurements of the colour on the surface of cheese A and B during ripening are
369 shown in Fig. 6. A significant interactive effect (*P* <0.05) between time and smear treatments
370 was observed on a* and b* values. From day 15, a* value was significantly higher (*P* <0.05)
371 compared with the control for the cheese A and B. At the end of the ripening (day 35), a*
372 value was significantly higher (*P* <0.05) for cheese B compared with cheese A, resulting a
373 redder colour on the surface of cheese B.
374

375 *3.6. Volatile compounds and sensory analysis*
376

377 The analysis of variance enabled the selection of 40 volatile compounds that were
378 significantly different (*P* <0.05) and positively correlated with the samples (Table 2). In total,
379 22 volatile compounds (7 acids, 4 alcohols, 5 esters, 4 sulphur compounds, 1 ketone and 1
380 aromatic hydrocarbon) were significantly associated (*P* <0.05) with the surface of cheese A.
381 Eight compounds (2 acids, 2 aldehydes, 1 alcohol 1 ester, 1 pyrazine and 1 sulphur
382 compound) were significantly associated with the surface of cheese B. Two compounds (1
383 ketone and 1 hydrocarbon) were significantly associated (*P* <0.05) with the core of cheese A.
384 Two compounds (1 alcohol and 1 ketone) were significantly associated (*P* <0.05) with the
385 core of cheese B. Nine compounds (4 alcohols, 4 ketones and 1 ester) were significantly
386 associated (*P* <0.05) with the control cheese.

387 Using hedonic sensory analysis (Fig. 7) the control cheese scored significantly higher
388 (*P* <0.05) for "Liking of Aroma" compared with the cheese A and B. Cheese A scored
389 significantly higher (*P* <0.05) for "Liking of Aroma" compared with cheese B. The control
390 and cheese A scored significantly higher (*P* <0.05) for "Liking of Flavour" compared with
391 cheese B. As seen in Fig. 8, the control cheese, as expected, scored significantly higher (*P*
392 <0.05) for "Cheddar flavour" compared with cheese A, which scored significantly higher (*P*
393 <0.05) for "Cheddar flavour" compared with cheese B. Cheese A and B scored significantly
394 higher (*P* <0.05) for "Pungent flavour" and "Mould cheese flavour" compared with the
395 control cheese. Cheese B scored significantly higher (*P* <0.05) for descriptors "Sweaty/Sour
396 Aroma", "Pungent Aroma" and "Off-Aroma" compared with the control cheese. The control
397 scored significantly higher (*P* <0.05) for "Crumbly texture" compared with cheese A and B.
398

399 **4. Discussion**

400

401 *C. casei* and *S. saprophyticus*, bacteria commonly isolated from smear-ripened
402 cheeses (e.g., Limburger, Reblochon, Livarot, Tilsit, Gubbeen) (Cogan et al., 2014; Larpin et
403 al., 2011), do not belong to the traditional microbiota of Cheddar cheese, although in this
404 study both strains established themselves on the surface of young Cheddar cheese curd and
405 they were the dominant population on the cheese surface throughout the ripening.
406 *C. casei* DPC5298 or *S. saprophyticus* DPC5671 in combination with *D. hansenii*
407 DPC6258 developed a coloured layer on the cheese surface after 15 days of ripening. Unlike
408 studies with *Brevibacterium linens* that showed that colour development was influenced by
409 the yeast strain used (Leclercq-Perlat, Corrieu, & Spinnler, 2004a), in this study the type of
410 colour developed was dependent on the bacteria used. The combination *D. hansenii*
411 DPC6258 with *C. casei* DPC5298 on cheese B developed a redder colour compared with the

412 combination of *D. hansenii* DPC6258 with *S. saprophyticus* DPC5671 on cheese A. Similar
413 results were shown by Mounier et al. (2006b) who reported in a cheese model an increase of
414 the colour after the 15th day of ripening at 14 °C and higher a* value for the combination *D.*
415 *hansenii* with *C. casei*, compared with *D. hansenii* combined with *S. saprophyticus*, resulting
416 in higher development of red colour.

417 The development of the typical aroma and flavour in cheese A and B is associated
418 with the lipolytic and proteolytic processes of the yeast and bacterial component of the smear
419 consortium. These processes were slower in the control cheese, which did not develop the
420 same levels of proteolysis, FAAs and FFAs in the short ripening time (35 days).

421 During cheese ripening, a gradual decomposition of caseins into small peptides and
422 FAAs occurs. FAAs are considered precursors of flavour compounds during the development
423 of cheese flavour. It is likely that the smear treatments increased the proteolysis of β-casein
424 by plasmin (more active at alkaline pH) on the surface of the test cheeses (Fig. 3), increasing
425 the pH levels. At the end of ripening the dominant FAAs detected in high amount on the
426 surface of the test cheeses were valine, leucine, proline, glutamate and lysine (Fig. 4), due to
427 their relative concentration in casein and the peptidase activity of the smear consortium,
428 especially *D. hansenii* DPC6258. Analysis of cell free supernatants of the cultures used in
429 this study confirmed that *D. hansenii* DPC6258 had high peptidase activities (PepX and
430 PepN), while activities were low for *S. saprophyticus* DPC5671 and *C. casei* DPC5298
431 (supplementary data). Similar results have been previously reported for other *D. hansenii*, *S.*
432 *saprophyticus* and *C. casei* strains (Bintsis, Vafopoulou-Mastrojiannaki, Litopoulou-
433 Tzanetaki, & Robinson, 2013; Casaburi, Villani, Toldrá, & Sanz, 2006; Curtin et al., 2002).

434 The hydrolysis of triglycerides is the main biochemical transformation of fat during
435 cheese ripening, which leads to the production of FFAs. Individual FFAs contribute to the
436 cheese aroma with their specific flavours and especially with their metabolites. At the end of

ACCEPTED MANUSCRIPT

437 ripening high amounts of FFAs were detected on the surface of cheese A and B. The levels of
438 all individual FFAs detected were higher on the surface of cheese A compared with cheese B,
439 especially for C10:0 (Fig. 5). It has been previously reported that the lipolysis by *D. hansenii*
440 is weak, while studies on Gram-positive bacteria showed good activity on substrates with
441 different glyceride chains length (Bintsis et al., 2003; Cardoso et al., 2015; van den Tempel &
442 Jakobsen, 2000). Experimental work showed that *S. saprophyticus* DPC5671 had greater
443 lipolytic activity on tributyrin than either *C. casei* DPC5298 or *D. hansenii* DPC6258
444 (supplementary data). These results are in agreement with what was previously reported by
445 Talon and Montel (1997), who detected lipolytic activity on tributyrin in a range of
446 staphylococcus strains, including *S. saprophyticus*. The higher lipolytic activity of *S.*
447 *saprophyticus* DPC5671 compared with *C. casei* DPC5298 may explain the higher amount of
448 FFAs in cheese A.

449 Numerous volatile compounds were significantly (P <0.05) associated to cheese A
450 and B, especially those particularly characterised by strong aroma notes, such as some
451 specific carboxylic acids, alcohols, esters, ketones and sulphur compounds, suggesting that
452 the smear treatments have modified the aroma profile of Cheddar cheese curd in only 35 days
453 of ripening (Table 2).

454 The metabolism of FAAs in cheese A and B in this study was responsible for the
455 development of specific branched alcohols and branched chain acids detected (3-methyl-1-
456 butanol, phenylethyl-alcohol, 3-methyl-butanoic acid). *D. hansenii* has been identified as a
457 possible producer of alcohols in previous studies (Arfi, Spinnler, Tache, & Bonnarme, 2002;
458 Gori, Sørensen, Petersen, Jespersen, & Arneborg, 2012; Leclercq-Perlat, Corrieu, & Spinnler,
459 2004b), while the production of carboxylic acids has been previously attributed to both yeasts
460 and smear bacteria (*Geotrichum candidum* and *B. linens*) (Jollivet, Bézenger, Vayssier, &
461 Belin, 1992; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994).

462 The high amount of esters detected in cheese A (ethyl acetate, ethyl octanoate, methyl
463 hexanoate, 3-methylbutyl acetate and isopentyl hexanoate) is likely related to the high FFA
464 content and the presence of alcohols in cheese A, considering they originate from the
465 esterification or alcoholysis of alcohols with carboxylic acids. While information on the
466 biosynthesis of esters by corynebacteria is sparse, numerous studies reported ester production
467 by staphylococci isolated from fermented foods, including *S. saprophyticus* strains (Talon,
468 Chastagnac, Vergnais, Montel, & Berdagué, 1998). The formation of esters in cheese A, not
469 detected in high amount in cheese B, is likely due to the metabolic activity of *S.*
470 *saprophyticus* DPC5671 rather than by *D. hansenii* DPC6258.

471 Other products of FFA metabolism such as ketones and alcohols were detected in all
472 cheeses. However 2-pentanone, 2-hexanone, 2-nonanone and 2-decanone were particularly
473 associated with the control cheese and not with cheese A and B, suggesting an involvement
474 of LAB rather than the smear cultures. It is known that methylketones result from the β-
475 oxidation of FFA, by lipolytic enzymes due to autolysis of the LAB during ripening (Collins,
476 McSweeney, & Wilkinson, 2003), although it is also postulated that methylketones can be
477 produced by the heating of milk or directly from esterification of β-keto acids (Alewijn,
478 2006; Forss, 1979).

479 Sensory analysis showed different results between cheese A, cheese B and the control
480 cheese. The sensory panel was not influenced by the colour of the cheese as the surface was
481 removed before sensory analysis. “Mould Cheese Flavour” and “Pungent Flavour” are
482 descriptors associated with smear-ripened cheeses and they can be correlated with a range of
483 volatiles with strong aroma notes (Table 2) detected on the surface and core of both cheese A
484 and B, namely butanoic, octanoic acid (originated from lipolysis of lipids), 2-heptanol (from
485 reduction of ketones), 2-methyl-propanoic, 3-methyl-butanoic, 2-methyl-butanoic, pentanoic
486 acid, 3-methyl-butanol, 2-methyl-butanol, phenylethyl-alcohol, 3-methyl-butanal (from

487 metabolism of branched chain amino acids or possibly phenylalanine for phenylethyl-
488 alcohol), methanethiol, dimethyldisulphide and dimethyldisulphide (from metabolism of
489 sulphur amino acids). As expected these descriptors were significantly associated (P <0.05)
490 with cheese A and/or B, suggesting that the activities of yeast and Gram-positive bacteria on
491 the cheese conferred a typical smear cheese flavour, not perceived in the control. The
492 descriptors significantly associated (P <0.05) with cheese B, such as "Sweaty/Sour Aroma",
493 "Pungent Aroma" and "Off-Aroma", are considered as "unclean" and off-odorants and were
494 associated with some compounds detected in abundance on the surface and core of cheese B,
495 such as 3-methyl-butanoic acid, octanoic acid, methanethiol and particularly 8-nonen-2-one
496 (originated from β-oxidation of fatty acids) which was identified only in cheese B.
497 Sensory analysis showed the smear cultures on the cheese surface affected the cheese
498 ripening giving strong and intense aroma and flavour to cheese A and B, while the control
499 cheese was characterised by mild aroma and flavour. In a short ripening time of 35 days, the
500 smear treatments induced the development of different aroma profiles.
501 Overall, the bacterial strains in conjunction with *D. hansenii* may have the potential to
502 modify cheese colour and produce novel cheeses with diverse aromas using a Cheddar cheese
503 curd.
504
505 **5. Conclusion**
506
507 The cheese-making method described in this paper gives a new approach for the
508 production of novel smear cheeses starting from a Cheddar cheese curd. Both the yeast and
509 bacterial cultures were able to establish themselves on the surface of the cheese and become
510 the dominant microbiota on the cheese surface, producing a cheese variety with acceptable
511 appearance and novel flavour aroma profiles. The method proposed could be used as model

512 to produce novel cheese types with a range of flavours and aromas through the growth of
513 combinations of yeast and bacterial cultures on the surface using cheese curd produced on a
514 traditional Cheddar cheese plant.
515

516 **Acknowledgements**

517

518 The authors wish to thank to Sarah Henneberry, David Mannion and Hope Faulkner
519 for their assistance in the analysis. Andrea Bertuzzi is currently in a receipt of Teagasc Walsh
520 Fellowship.

521

522 **References**

523

524 Alewijn, M. (2006). *Formation of fat-derived flavour compounds during the ripening of*
525 *Gouda-type cheese*. PhD thesis. Wageningen University, The Netherlands.
526 Arfi, K., Spinnler, H., Tache, R., & Bonnarme, P. (2002). Production of volatile compounds
527 by cheese-ripening yeasts: requirement for a methanethiol donor for S-methyl
528 thioacetate synthesis by *Kluyveromyces lactis*. *Applied Microbiology and*
529 *Biotechnology, 58*, 503–510.
530 Bannerman, T. L., Hancock, G. A., Tenover, F. C., & Miller, J. M. (1995). Pulsed-field gel
531 electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*.
532 *Journal of Clinical Microbiology, 33*, 551–555.
533 Barron, L., Redondo, Y., Aramburu, M., Pérez-Elortondo, F. J., Albisu, M., Nájera, A. I., et
534 al. (2005). Variations in volatile compounds and flavour in Idiazabal cheese
535 manufactured from ewe's milk in farmhouse and factory. *Journal of the Science of*
536 *Food and Agriculture, 85*, 1660–1671.

ACCEPTED MANUSCRIPT

537 Bintsis, T., Vafopoulou-Mastrojiannaki, A., Litopoulou-Tzanetaki, E., & Robinson, R. K.
538 (2003). Protease, peptidase and esterase activities by lactobacilli and yeast isolates
539 from Feta cheese brine. *Journal of Applied Microbiology, 95*, 68–77.
540 Bokulich, N. A., & Mills, D. A. (2013). Facility-specific "house" microbiome drives
541 microbial landscapes of artisan cheesemaking plants. *Applied and Environmental*
542 *Microbiology, 79*, 5214–5223.
543 Brennan, N. M., Brown, R., Goodfellow, M., Ward, A. C., Beresford, T. P., Simpson, P. J., et
544 al. (2001). *Corynebacterium mooreparkense* sp. nov. and *Corynebacterium casei* sp.
545 nov., isolated from the surface of a smear-ripened cheese. *International Journal of*
546 *Systematic and Evolutionary Microbiology, 51*, 843–852.
547 BSI. (1976). *Chemical analysis of cheese. Determination of pH value. BS 770: Part 5.*
548 London, UK: British Standards Institution.
549 Cardoso, V. M., Borelli, B. M., Lara, C. A., Soares, M. A., Pataro, C., Bodevan, E. C., et al.
550 (2015). The influence of seasons and ripening time on yeast communities of a
551 traditional Brazilian cheese. *Food Research International, 69*, 331–340.
552 Carpino, S., Mallia, S., La Terra, S., Melilli, C., Licitra, G., Acree, T. E., et al. (2004).
553 Composition and aroma compounds of Ragusano cheese: native pasture and total
554 mixed rations. *Journal of Dairy Science, 87*, 816–830.
555 Casaburi, A., Villani, F., Toldrá, F., & Sanz, Y. (2006). Protease and esterase activity of
556 staphylococci. *International Journal of Food Microbiology, 112*, 223–229.
557 Cholet, O., Hénaut, A., Casaregola, S., & Bonnarme, P. (2007). Gene expression and
558 biochemical analysis of cheese-ripening yeasts: focus on catabolism of L-methionine,
559 lactate, and lactose. *Applied and Environmental Microbiology, 73*, 2561–2570.
560 Cogan, T. M., Goerges, S., Gelsomino, R., Larpin, S., Hohenegger, M., Bora, N., et al.
561 (2014). Biodiversity of the surface microbial consortia from Limburger, Reblochon,

ACCEPTED MANUSCRIPT

562 Livarot, Tilsit, and Gubbeen cheeses. In C. W. Donnelly (Ed.), *Cheese and microbes*
563 (pp. 219–250). Washington, DC, USA :American Society of Microbiology.
564 Collins, Y. F., McSweeney, P. L. H., & Wilkinson, M. G. (2003). Evidence of a relationship
565 between autolysis of starter bacteria and lipolysis in Cheddar cheese during ripening.
566 *Journal of Dairy Research, 70*, 105–113.
567 Corsetti, A., Rossi, J., & Gobbetti, M. (2001). Interactions between yeasts and bacteria in the
568 smear surface-ripened cheeses. *International Journal of Food Microbiology, 69*, 1–
569 10.
570 Curioni, P. M. G., & Bosset, J. O. (2002). Key odorants in various cheese types as
571 determined by gas chromatography-olfactometry. *International Dairy Journal, 12*,
572 959–984.
573 Curtin, Á. C., Gobbetti, M., & McSweeney, P. L. H. (2002). Peptidolytic, esterolytic and
574 amino acid catabolic activities of selected bacterial strains from the surface of smear
575 cheese. *International Journal of Food Microbiology, 76*, 231–240.
576 De Jong, C., & Badings, H. T. (1990). Determination of free fatty acids in milk and cheese
577 procedures for extraction, clean up, and capillary gas chromatographic analysis.
578 *Journal of High Resolution Chromatography, 13*, 94–98.
579 Desmasures, N., Bora, N., & Ward, A. (2015). Smear ripened cheeses. In N. Bora, C. Dodd
580 & N. Desmasures (Eds.), *Diversity, dynamics and functional role of Actinomycetes on*
581 *European smear ripened cheeses* (pp. 1–18). Cham, Switzerland: Springer
582 International Publishing AG.
583 Fenelon, M. A., & Guinee, T. P. (2000). Primary proteolysis and textural changes during
584 ripening in Cheddar cheeses manufactured to different fat contents. *International*
585 *Dairy Journal, 10*, 151–158.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

586 Feurer, C., Vallaeys, T., Corrieu, G., & Irlinger, F. (2004). Does smearing inoculum reflect
587 the bacterial composition of the smear at the end of the ripening of a French soft, red-
588 smear cheese? *Journal of Dairy Science, 87*, 3189–3197.
589 Forss, D. A. (1979). Mechanisms of formation of aroma compounds in milk and milk
590 products. *Journal of Dairy Research, 46*, 691–706.
591 Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017a). Principal families
592 of cheese. In P. F. Fox, T. P. Guinee, T. M. Cogan & P. L. H. McSweeney (Eds.),
593 *Fundamentals of cheese science* (pp. 27–69). Cham, Switzerland: Springer
594 International Publishing AG.
595 Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. H. (2017b). Biochemistry of
596 cheese ripening. In P. F. Fox, T. P. Guinee, T. M. Cogan & P. L. H. McSweeney
597 (Eds.), *Fundamentals of cheese science* (pp. 391–442). Cham, Switzerland: Springer
598 International Publishing AG.
599 Goerges, S., Mounier, J., Rea, M. C., Gelsomino, R., Heise, V., Beduhn, R., et al. (2008).
600 Commercial ripening starter microorganisms inoculated into cheese milk do not
601 successfully establish themselves in the resident microbial ripening consortia of a
602 South German red smear cheese. *Applied and Environmental Microbiology, 74*,
603 2210–2217.
604 Gori, K., Sørensen, L. M., Petersen, M. A., Jespersen, L., & Arneborg, N. (2012).
605 *Debaryomyces hansenii* strains differ in their production of flavor compounds in a
606 cheese-surface model. *MicrobiologyOpen, 1*, 161–168.
607 Gürbüz, O., Rouseff, J. M., & Rouseff, R. L. (2006). Comparison of aroma volatiles in
608 commercial Merlot and Cabernet Sauvignon wines using gas chromatography-
609 olfactometry and gas chromatography-mass spectrometry. *Journal of Agricultural and*
610 *Food Chemistry, 54*, 3990–3996.

ACCEPTED MANUSCRIPT

611 Henneberry, S., Wilkinson, M. G., Kilcawley, K. N., Kelly, P. M., & Guinee, T. P. (2015).
612 Interactive effects of salt and fat reduction on composition, rheology and functional
613 properties of mozzarella-style cheese. *Dairy Science and Technology*, 1–26.

614 IDF. (1988). *Cheese and processed cheese: Determination of chloride content*
615 *(potentiometric titration method). Standard 88A*. Brussels, Belgium: International
616 Dairy Federation.

617 IDF. (1993). *Milk. Determination of nitrogen content. Standard 20B.* Brussels, Belgium:
618 International Dairy Federation.

619 IDF. (1996). *Milk. Determination of the fat content (Rose Gottlieb gravimetric method).*
620 *Standard 1D.* Brussels, Belgium: International Dairy Federation.

621 Jollivet, N., Bézenger, M. -C., Vayssier, Y., & Belin, J. -M. (1992). Production of volatile
622 compounds in liquid cultures by six strains of coryneform bacteria. *Applied*
623 *Microbiology and Biotechnology, 36*, 790–794.

624 Jollivet, N., Chataud, J., Vayssier, Y., Bensoussan, M., & Belin, J. -M. (1994). Production of
625 volatile compounds in model milk and cheese media by eight strains of *Geotrichum*
626 *candidum*. *Journal of Dairy Research, 61*, 241–248.

627 Kilcawley, K. N. (2017). Cheese flavour. In P. F. Fox, T. P. Guinee, T. M. Cogan & P. L. H.
628 McSweeney (Eds.), *Fundamentals of cheese science* (pp. 443–474): Springer.

629 Kubíčková, J., & Grosch, W. (1997). Evaluation of potent odorants of Camembert cheese by
630 dilution and concentration techniques. *International Dairy Journal, 7*, 65–70.

631 Larpin, S., Imran, M., Bonaïti, C., Bora, N., Gelsomino, R., Goerges, S., et al. (2011).
632 Surface microbial consortia from Livarot, a French smear-ripened cheese. *Canadian*
633 *Journal of Microbiology, 57*, 651–660.

ACCEPTED MANUSCRIPT

634 Leclercq-Perlat, M. N., Corrieu, G., & Spinnler, H. E. (2004a). The color of *Brevibacterium*
635 *linens* depends on the yeast used for cheese deacidification. *Journal of Dairy Science,*
636 *87*, 1536–1544.
637 Leclercq-Perlat, M. N., Corrieu, G., & Spinnler, H. E. (2004b). Comparison of volatile
638 compounds produced in model cheese medium deacidified by *Debaryomyces hansenii*
639 or *Kluyveromyces marxianus*. *Journal of Dairy Science, 87*, 1545–1550.
640 Mannion, D. T., Furey, A., & Kilcawley, K. N. (2016). Comparison and validation of 2
641 analytical methods for the determination of free fatty acids in dairy products by gas
642 chromatography with flame ionization detection. *Journal of Dairy Science, 99*, 5047–
643 5063.
644 McDermott, A., Visentin, G., De Marchi, M., Berry, D., Fenelon, M., O'Connor, P., et al.
645 (2016). Prediction of individual milk proteins including free amino acids in bovine
646 milk using mid-infrared spectroscopy and their correlations with milk processing
647 characteristics. *Journal of Dairy Science, 99*, 3171–3182.
648 McSweeney, P. L. H., & Sousa, M. J. (2000). Biochemical pathways for the production of
649 flavour compounds in cheeses during ripening: a review. *Lait, 80*, 293–324.
650 McSweeney, P. L. H. (2004). Biochemistry of cheese ripening. *International Journal of*
651 *Dairy Technology, 57*, 127–144.
652 Mounier, J., Goerges, S., Gelsomino, R., Vancanneyt, M., Vandemeulebroecke, K., Hoste,
653 B., et al. (2006a). Sources of the adventitious microflora of a smear-ripened cheese.
654 *Journal of Applied Microbiology, 101*, 668–681.
655 Mounier, J., Irlinger, F., Leclercq-Perlat, M. N., Sarthou, A. S., Spinnler, H. E., Fitzgerald, G.
656 F., et al. (2006b). Growth and colour development of some surface ripening bacteria
657 with *Debaryomyces hansenii* on aseptic cheese curd. *Journal of Dairy Research, 73*,
658 441–448.

659 Poveda, J. M., Sánchez-Palomo, E., Pérez-Coello, M. S., & Cabezas, L. (2008). Volatile
660 composition, olfactometry profile and sensory evaluation of semi-hard Spanish goat
661 cheeses. *Dairy Science and Technology, 88*, 355–367.

662 Qian, M. C., & Reineccius, G. (2002). Identification of aroma compounds in Parmigiano-
663 Reggiano cheese by gas chromatography/olfactometry. *Journal of Dairy Science, 85*,
664 1362–1369.

665 Qian, M. C., Burbank, H. M., & Wang, Y. (2006). Preseparation techniques in aroma
666 analysis. In R. T. Marsili (Ed.), *Sensory-directed flavor analysis* (pp. 111–154). Boca
667 Raton, FL, USA: CRC Press.

668 Rynne, N. M., Beresford, T. P., Kelly, A. L., & Guinee, T. P. (2004). Effect of milk
669 pasteurisation temperature and in situ whey protein denaturation on the composition,
670 texture and heat-induced functionality of half-fat Cheddar cheese. *International Dairy*
671 *Journal, 14*, 989–1001.

672 Singh, T. K., Drake, M. A., & Cadwallader, K. R. (2003). Flavor of Cheddar cheese: a
673 chemical and sensory perspective. *Comprehensive Reviews in Food Science and Food*
674 *Safety, 2*, 166–189.

675 Smit, G., Smit, B. A., & Engels, W. J. M. (2005). Flavour formation by lactic acid bacteria
676 and biochemical flavour profiling of cheese products. *FEMS Microbiology Reviews,*
677 *29*, 591–610.

678 Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis
679 during cheese ripening. *International Dairy Journal, 11*, 327–345.

680 Talon, R., & Montel, M. C. (1997). Hydrolysis of esters by staphylococci. *International*
681 *Journal of Food Microbiology, 36*, 207–214.

682 Talon, R., Chastagnac, C., Vergnais, L., Montel, M. C., & Berdagué, J. L. (1998). Production
683 of esters by Staphylococci. *International Journal of Food Microbiology, 45*, 143–150.

684 Thomsen, M., Martin, C., Mercier, F., Tournayre, P., Berdagué, J. -L., Thomas-Danguin, T.,
685 et al. (2012). Investigating semi-hard cheese aroma: Relationship between sensory
686 profiles and gas chromatography-olfactometry data. *International Dairy Journal, 26*,
687 41–49.
688 Urbach, G. (1993). Relations between cheese flavour and chemical composition.
689 *International Dairy Journal, 3*, 389–422.
690 van den Tempel, T., & Jakobsen, M. (2000). The technological characteristics of
691 *Debaryomyces hansenii* and *Yarrowia lipolytica* and their potential as starter cultures
692 for production of Danablu. *International Dairy Journal, 10*, 263–270.
693 Varming, C., Andersen, L. T., Petersen, M. A., & Ardö, Y. (2013). Flavour compounds and
694 sensory characteristics of cheese powders made from matured cheeses. *International*
695 *Dairy Journal, 30*, 19–28.
696 Yvon, M., & Rijnen, L. (2001). Cheese flavour formation by amino acid catabolism.
697 *International Dairy Journal, 11*, 185–201.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure legends

Fig. 1. Enumeration of cfu g^{-1} of the cheese surface of cheese A and cheese B during the ripening time; yeasts total count in cheese A (●) and in cheese B (⊗), smear bacteria total count in cheese A (△) and in cheese B (☐). Values presented are the means and standard deviations from three replicate trials.

Fig. 2. Change in pH overtime; control cheese (●), surface of cheese A (△), core of cheese A (▲), surface of cheese B (☐) and core of cheese B (■). Values presented are the means and standard deviations from three replicate trials.

Fig. 3. Urea-PAGE electrophoretogram of sodium caseinate (lane C), control cheese at day 0 (lane 1), control cheese at day 35 (lane 2), core of cheese B at day 35 (lane 3), surface of cheese B at day 35 (lane 4), core of cheese A at day 35 (lane 5) and surface of cheese A at day 35 (lane 6). The urea-PAGE was performed on the basis of fixed weight protein for all three replicate trials.

Fig. 4. Individual free amino acids content of (A) the control (■), core cheese A (⚁), surface cheese A (▤), and (B) the control (■), core cheese B (▩) and surface cheese B (▤) at day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different (*P* <0.05).

Fig. 5. Individual free fatty acids content of (A) the control (■), core cheese A (⚁) and surface cheese A (▤), and (B) the control (■), core cheese B (▩) and surface cheese B (▤) at

ACCEPTED MANUSCRIPT

day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different (P <0.05).

Fig. 6. Colour development on the surface of the cheeses during the ripening. The colour values (L^*, a^*, b^*) of the control cheese (●), of cheese A (▲) and of cheese B (□). Values presented are the means and standard deviations from three replicate trials.

Fig. 7. Sensory affective (hedonic) analysis performed in duplicate on cheese A (▲), cheese B (■) and control (●) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher (P <0.05) than cheese A and B; (e) cheese A and control significantly higher (P <0.05) than cheese B.

Fig. 8. Ranking descriptive analysis performed in duplicate on cheese A (▲), cheese B (■) and control (●) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher (P <0.05) than cheese A and B; (b) cheese A and B significantly higher (P <0.05) than control; (c) cheese B significantly higher (P <0.05) than cheese A and control; (d) cheese A significantly higher (P <0.05) than cheese B and control.

ACCEPTED MANUSCRIPT

Table 1

ACCEPTED MANUSCRIPT

Composition of the control at day 0 and 35 and composition of the surface and core of cheese A and cheese B at day 35. [a]

Composition	Control d0	Control d35	Surf A d35	Core A d35	Surf B d35	Core B d35
Moisture (%, w/w)	38.80±0.84	38.32±0.62[c]	39.71±1.20	40.49±0.86	39.61±1.43	41.27±0.39[a]
Fat (%, w/w)	30.45±0.33	30.41±0.27	28.30±0.14[b]	30.09±0.69	28.01±0.25[b]	29.80±0.30
Protein (%, w/w)	24.87±0.21	25.17±0.11	24.28±0.57	24.5±0.83	24.94±0.32	24.72±0.44
MNFS (%, w/w)	55.79±1.44	55.07±1.02[c]	55.39±1.77	57.89±1.33	55.02±2.13[b]	58.79±0.39[a]
FDM (%, w/w)	49.77±1.16	49.32±0.80	46.97±1.13[b]	50.51±1.33	46.41±1.44[b]	50.74±0.24
S/M (%, w/w)	4.02±0.10	4.17±0.26[a]	3.74±0.20	3.81±0.33	3.58±0.10[b]	3.88±0.51
Salt (%, w/w)	1.56±0.02	1.60±0.09	1.48±0.11	1.55±0.12	1.42±0.08	1.60±0.22

[a] The compositional values are for moisture, fat, protein, MNFS (moisture in non-fat substances), FDM (fat in dry matter), S/M (salt in moisture) and salt of control at day 0 (Control d0), for control at day 35 (Control d35), for the surface of cheese A at day 35 (Surf A d35), for the core of cheese A at day 35 (Core A d35), for the surface of cheese B at day 35 (Surf B d35) and for the core of cheese B at day 35 (Core B d35). Values presented are the means±standard deviations of three replicate trials; values with different superscript letters differ significantly ($P < 0.05$).

ACCEPTED MANUSCRIPT

Table 2

ACCEPTED MANUSCRIPT

Volatile compounds detected with SPME-GCMS in cheese A, cheese B and control, and relative aroma notes. [a]

Volatile compound	CAS number	Aroma note
Aldehydes		
3-Methyl-butanal[b]	590-86-3	Malty, powerful, cheese, green, dark chocolate (Kilcawley, 2017)
2-Methyl butanal[b]	96-17-3	Malty, dark chocolate, almond, cocoa (Qian et al., 2006; Singh et al., 2003; Urbach, 1993)
Alcohols		
Ethanol[e]	64-17-5	Dry, dust, alcohol (Kilcawley, 2017)
2-Butanol[e]	78-92-2	Sweet, fruity, fusel oil, wine-like (Kilcawley, 2017)
3-Methyl-1-butanol[a]	123-51-3	Fresh cheese, breath-taking, alcoholic, fruity, grainy (Kilcawley, 2017)
2-Methyl-1-butanol[a]	137-32-6	Malty, wine, onion (Kilcawley, 2017)
2,3-Butanediol[e]	513-85-9	Fruity (Singh et al., 2003)
2-Heptanol[a,b]	543-49-7	Fruity, earthy, green, sweetish, dry (Kilcawley, 2017)
2-Ethyl-1-hexanol[d,e]	104-76-7	Animal, cardboard (Thomsen et al., 2012)
Phenylethyl-alcohol[a]	60-12-8	Unclean, rose, violet-like, honey, floral (Kilcawley, 2017)
Ketones		
2,3-Butanedione[c]	431-03-8	Buttery, strong (Kubíčková & Grosch, 1997; Singh et al., 2003)
2-Pentanone[e]	107-87-9	Orange peel, sweet, fruity (Kilcawley, 2017)
3-Methyl-2-pentanone[a]	565-61-7	Minty-camphoraceous, sharp (Barron et al., 2005)
2-Hexanone[e]	591-78-6	Floral, fruity (Qian et al., 2006)
8-Nonen-2-one[d]	5009-32-5	Animal, stinky (Poveda et al., 2008; Varming et al., 2013)
2-Nonanone[e]	821-55-6	Malty, rotten fruit, hot milk, green, earthy (Kilcawley, 2017)
2-Decanone[e]	693-54-9	Fruity, musty (Qian et al., 2006; Varming, et al., 2013)
Acids		
Acetic acid[a]	64-19-7	Vinegar, peppers, green, fruity, floral (Kilcawley, 2017)
2-Methyl-propanoic acid[a]	79-31-2	Rancid butter, sweaty, sweet, apple-like (Curioni & Bosset, 2002)
Butanoic acid[a]	107-92-6	Sweaty, butter, cheese, strong, acid (Kilcawley, 2017)
3-Methyl-butanoic acid[b]	503-74-2	Cheesy, sweaty, socks, rancid, rotten fruit (Kilcawley, 2017)
2-Methyl-butanoic acid[a]	116-53-0	Fruity, waxy, sweaty (Singh et al., 2003)
Pentanoic acid[a]	109-52-4	Rain, wood, vegetable, spicy, nutty, grain, Swiss cheese, stable, sweaty, sheep (Curioni & Bosset, 2002)
Heptanoic acid[a]	111-14-8	Soapy, fatty, goaty, rancid (Curioni & Bosset, 2002)
Octanoic acid[b]	124-07-2	Cheesy, rancid, pungent, sweat (Kilcawley, 2017)
n-Decanoic acid[a]	334-48-5	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
Esters		
Ethyl acetate[a]	141-78-6	Solvent, pineapple, fruity (Kilcawley, 2017)
Ethyl propionate[e]	105-37-3	Pineapple, solvent, fruity (Barron et al., 2005; Qian et al., 2006)
3-methylbutyl acetate[a]	123-92-2	Fruity, banana, candy, sweet (Barron et al., 2005; Curioni & Bosset, 2002; Qian et al., 2006)
Methyl hexanoate[a]	106-70-7	Pineapple, fruity (Qian et al., 2006; Varming et al., 2013)
Ethyl hexanoate[b]	123-66-0	Pineapple, sweet, fruity, banana (Kilcawley, 2017)
Ethyl octanoate[a]	106-32-1	Pear, apricot, sweet, fruity, banana, pineapple (Kilcawley, 2017)
Isopentyl hexanoate[a]	2198-61-0	Sweet, fruity (Gürbüz et al., 2006)
Sulphur compounds		
Methionol[a]	505-10-2	Orange (Carpino et al., 2004)
Methanethiol[a,b]	74-93-1	Rotten cabbage, cheese, vegetative, sulphur (Kilcawley, 2017)
Dimethyldisulphide[a]	624-92-0	Green, sour, onion (Kilcawley, 2017)
Dimethyltrisulphide[a]	3658-80-8	Vegetable-like, sulphurous, garlic, putrid, cabbage-like (Kilcawley, 2017)
Aromatic hydrocarbons		
Benzaldehyde[c]	100-52-7	Bitter almond, sweet cherry (Singh et al., 2003; Smit et al., 2005)
Benzeneacetaldehyde[a]	122-78-1	Honey-like, rose, violet-like, hyacinth, green (Kubíčková & Grosch, 1997; Qian et al., 2006; Singh et al., 2003; Smi, et al., 2005; Varming et al., 2013)
Pyrazines		
3-Ethyl-2,5-dimethyl-pyrazine[b]	13360-65-1	Roasted, baked (Qian & Reineccius, 2002)

[a] Superscript letters denote that volatile compounds are significantly different (*P*<0.05) and positively correlated to: ([a]) the surface of cheese A; ([b]) the surface of cheese B; ([c]) the core of cheese A; ([d]) the core of cheese B; ([e]) the control cheese.

ACCEPTED MANUSCRIPT

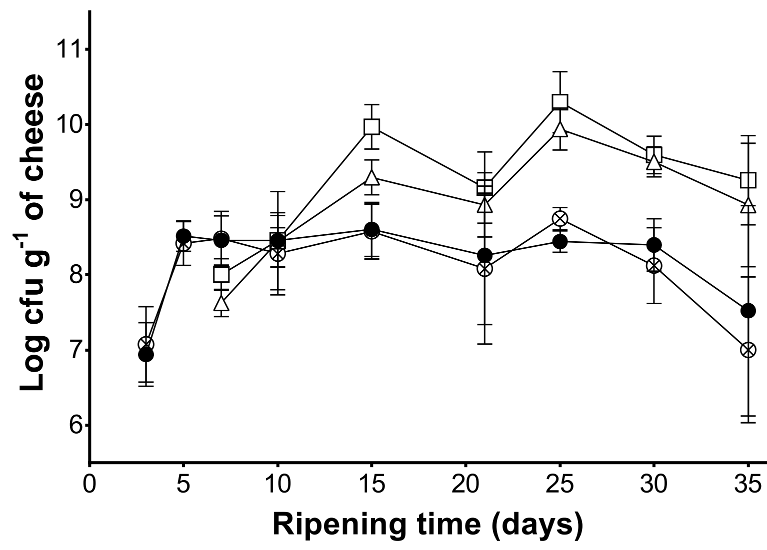


Fig. 1.

ACCEPTED MANUSCRIPT

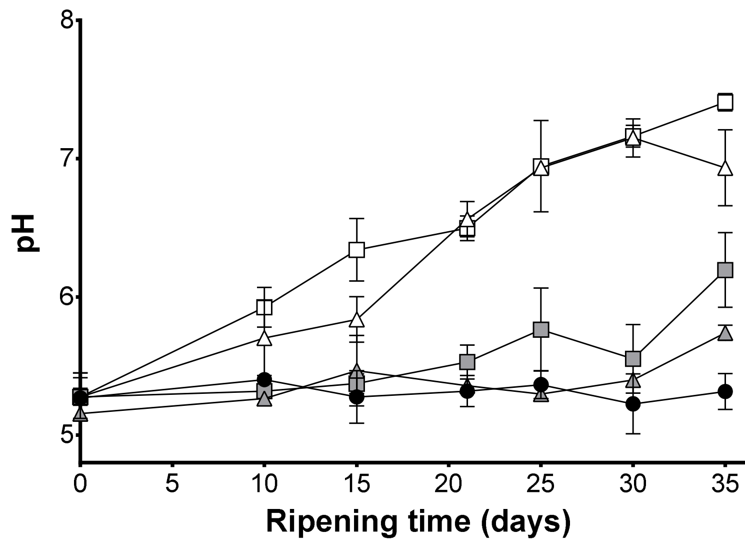


Fig. 2.

ACCEPTED MANUSCRIPT

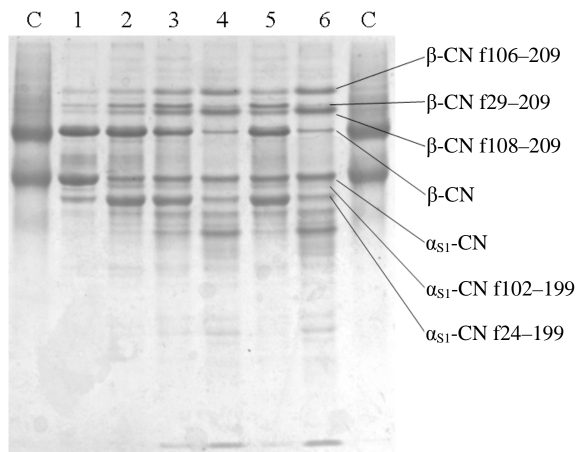


Fig. 3.

ACCEPTED MANUSCRIPT

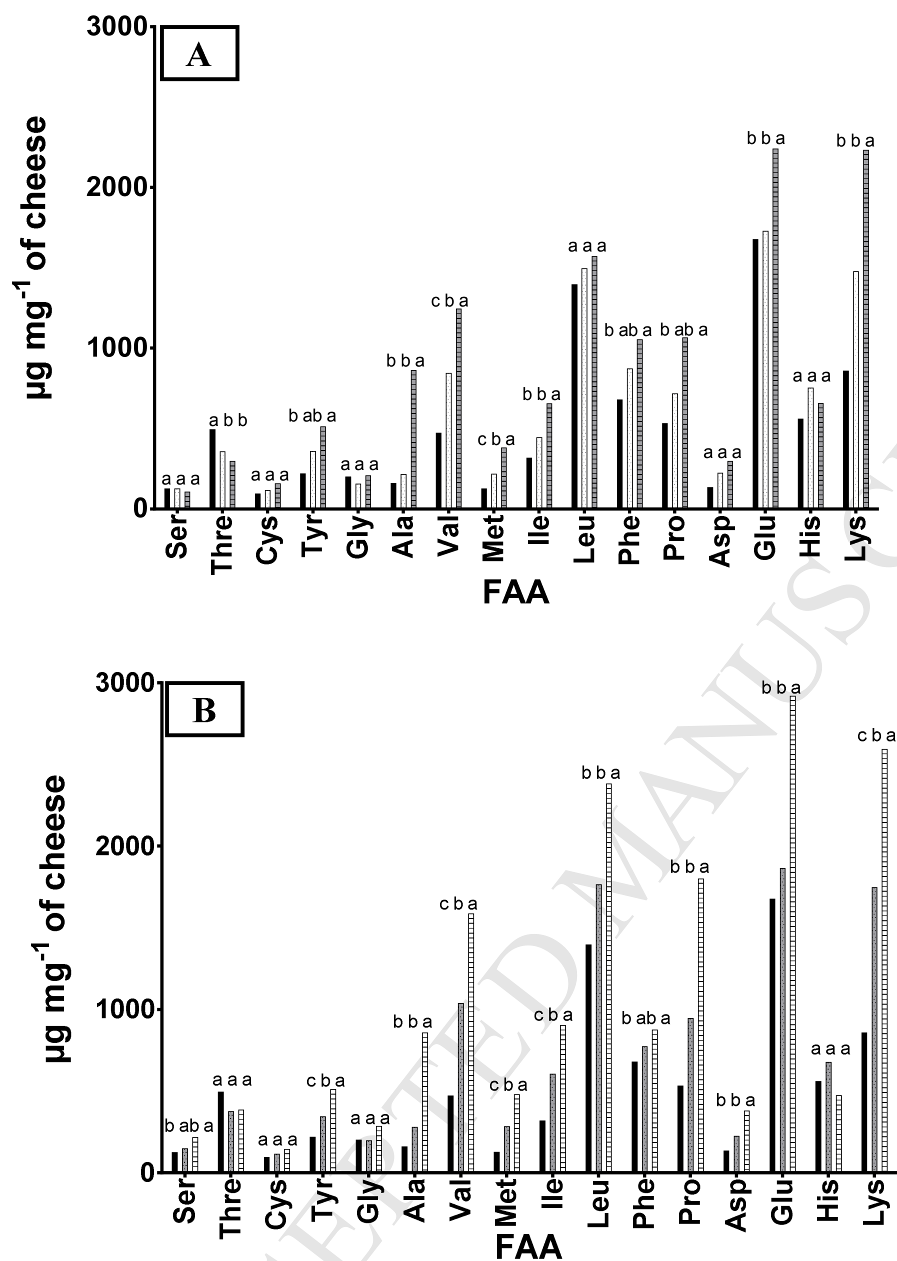


Fig. 4.

ACCEPTED MANUSCRIPT

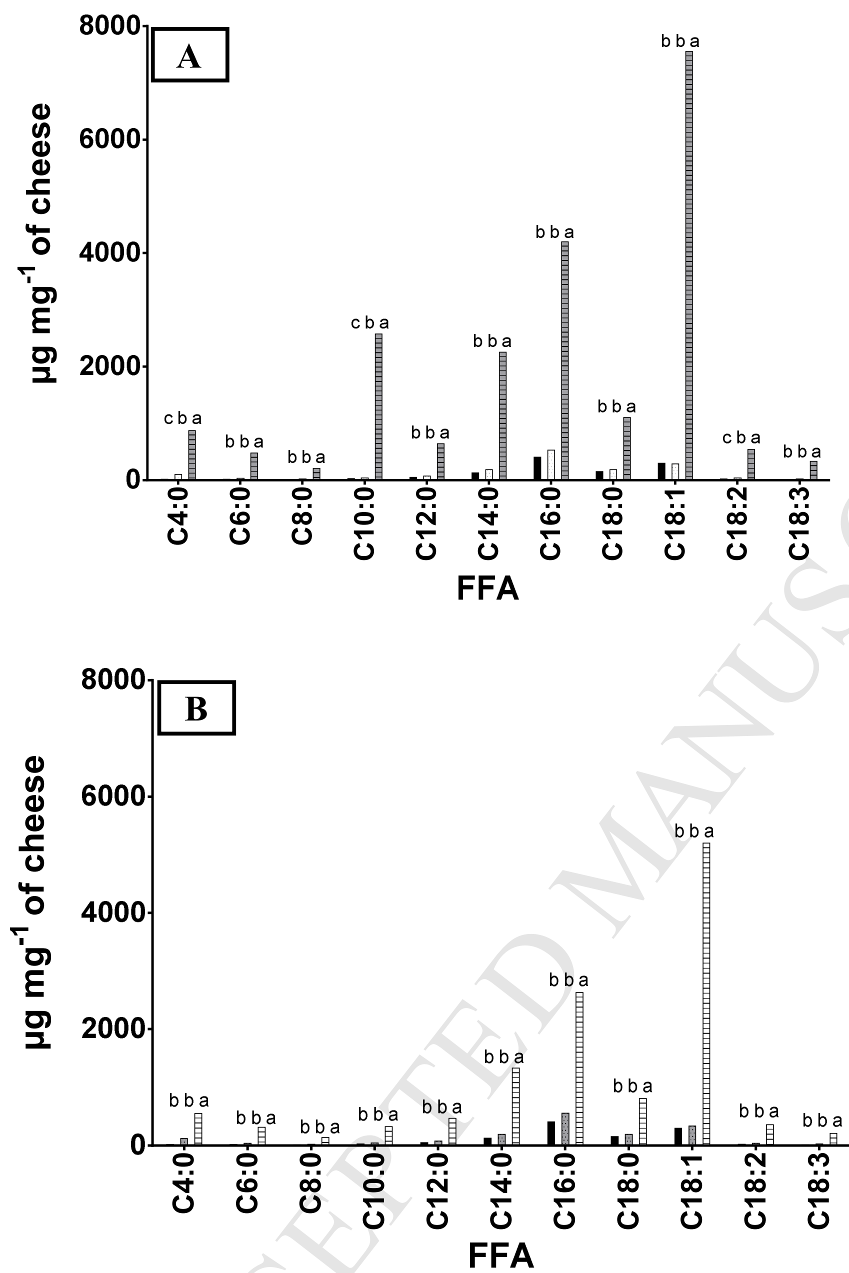


Fig. 5.

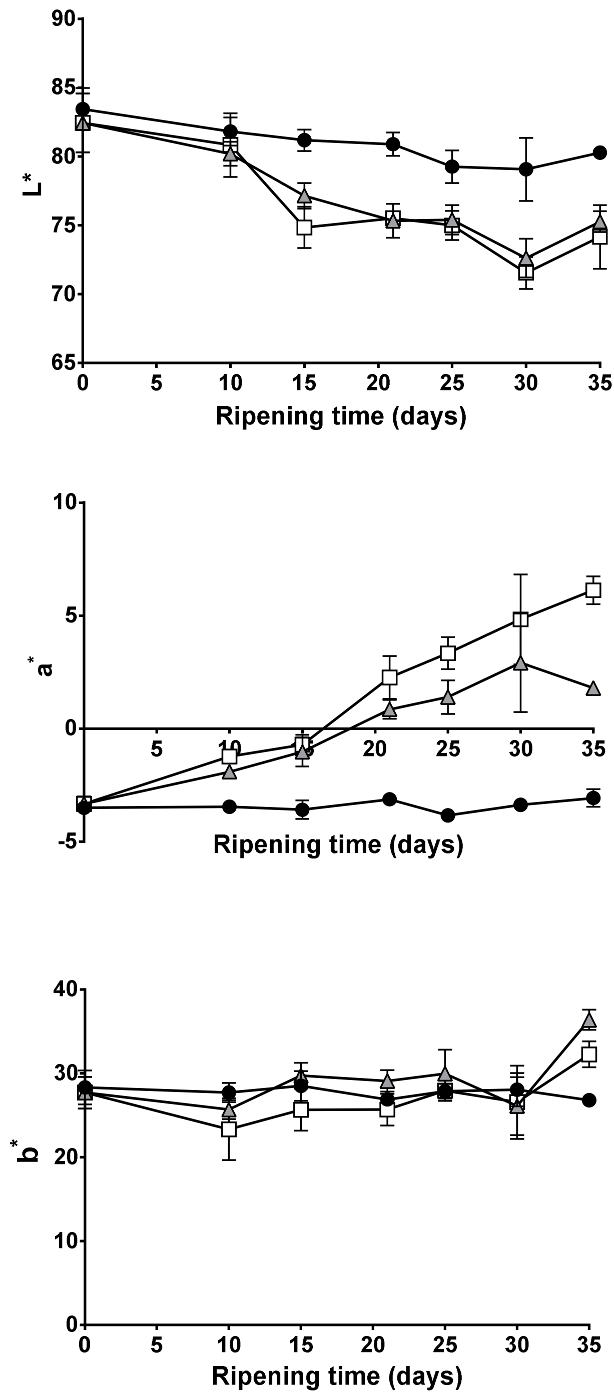


Fig. 6.

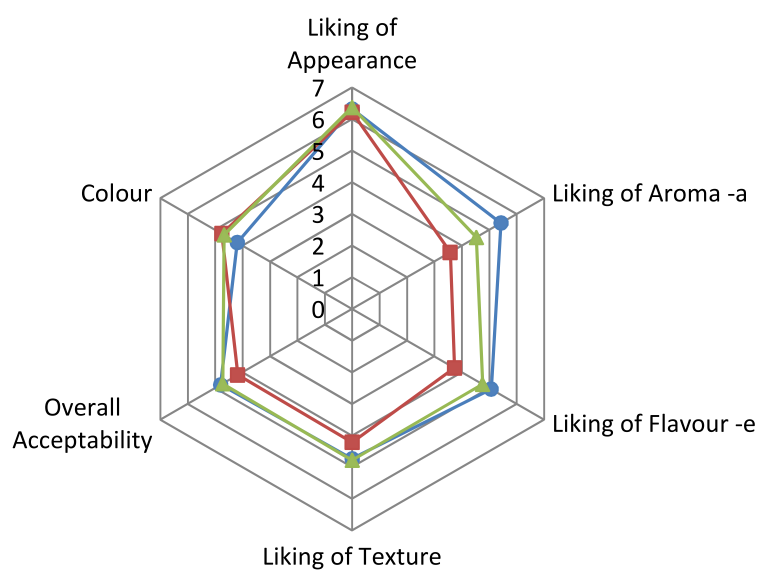


Fig. 7.

ACCEPTED MANUSCRIPT

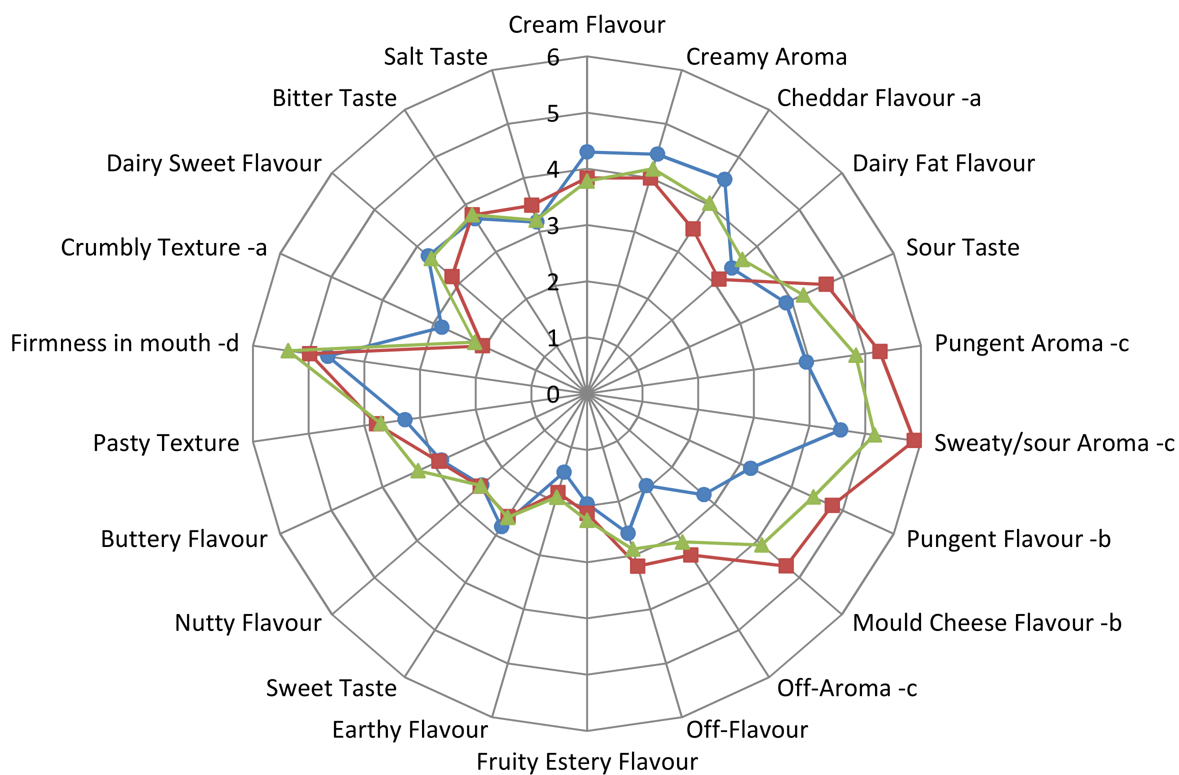


Fig. 8.

ACCEPTED