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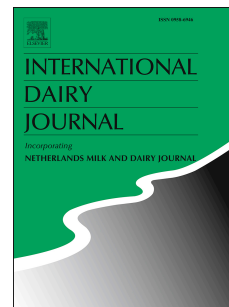
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1 **Integration of high and low field  $^1\text{H}$  NMR to analyse the effects of bovine dietary regime on**  
2 **milk metabolomics and protein-bound moisture characterisation of the resulting mozzarella**  
3 **cheeses during ripening**

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27

28 ABSTRACT

29

30 The influence of dairy cow feeding regime was investigated using  $^1\text{H}$  nuclear magnetic resonance  
31 (NMR). Two different NMR analytical systems were deployed: high field  $^1\text{H}$  NMR to investigate  
32 the influence on milk metabolomics and low field NMR to characterise proton relaxation linked to  
33 changes in the state of mozzarella cheese moisture during ripening. The metabolomics results  
34 showed that grass-based feeding increased the concentration of a biological marker that signifies  
35 near-organic milk production conditions. On the other hand, the investigation of cheese moisture  
36 distribution showed that grass-based diets reached final moisture partitioning in a shorter time,  
37 which implied the formation of a more compact protein structure in the cheese matrix. These results  
38 indicate that pasture-based dairying may be differentiated in terms of the provenance of milk  
39 produced along with the accrual of additional benefits during ripening of the resulting mozzarella  
40 cheeses.

41

42

## 43 1. Introduction

44

45 Milk production in temperate climates such as New Zealand (O'Brien et al., 1999) and  
46 Ireland relies substantially on grass growth as the principle bovine dietary source. Ideally matched  
47 to ruminant digestion needs, pasture grazing is valued at many levels such as animal welfare  
48 (Verkerk, 2003) and the promotion of natural animal foraging behaviour (Charlton, Rutter, East, &  
49 Sinclair, 2011; Legrand, von Keyserlingk, & Weary, 2009). However, this system is not practical in  
50 all other countries because of climatic extremes. Hence, an alternative milk production system  
51 widely practiced in mainland Europe utilises year-round indoor housing in which the dairy cows'  
52 dietary needs are addressed using a total mixed ration (TMR). It has been pointed out that in this  
53 system animal welfare presents some concern due to incidences of lameness and mastitis that  
54 diminish animal comfort and reduce milk output (Fregonesi, Veira, Von Keyserlingk, & Weary,  
55 2007; Haskell, Rennie, Bowell, Bell, & Lawrence, 2006). On the other hand, this system protects  
56 animals from extreme climatic conditions (heat, cold and wetness) and allows better management of  
57 animal nutrition formulations.

58 The influence of different feeding systems on the milk production has been extensively  
59 studied over the years (Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006; Lee, Theobald,  
60 Tweed, Winters, & Scollan, 2009; White et al., 2001). A more recent study (O'Callaghan et al.,  
61 2016b) is of particular interest as it compared the influence of three different feeding regimes: TMR  
62 with outdoor grazing of perennial ryegrass (*Lolium perenne* L.; GRO), and outdoor grazing of  
63 perennial ryegrass/white clover (*Trifolium repens* L.; GRC) on milk raw composition and quality  
64 during lactation. The results show that, in general, the GRO feeding system increased milk quality,  
65 particularly higher concentrations of protein and fat, and improved the nutritional profile of milk  
66 fat. In a related study (O'Callaghan et al., 2016a), butters produced from GRO and GRC milks had  
67 a higher nutritional value and improved sensory scores compared with TMR butter.

68            However, while the milk composition from Irish dairy farming and its influence on the dairy  
69 processing has been extensively investigated (Kelly, O'Keeffe, Keogh, & Phelan, 1982; Keogh,  
70 Kelly, O'Keeffe, & Phelan, 1982; Phelan, O'Keeffe, Keogh, & Kelly, 1982), the influence that  
71 feeding has on metabolomics and on industrial processes such as the cheese making remain open.  
72 Increasingly, more advanced analytical instrumentation is being applied to probe in greater detail  
73 compositional differences and other markers in milks produced as a result of different feeding  
74 regimes, e.g., HR  $^1\text{H}$  NMR spectroscopy has been already used successfully to investigate the  
75 influence of breeding on milk composition (Sundekilde, Frederiksen, Clausen, Larsen, & Bertram,  
76 2011), milk somatic cells, usually correlate with mastitis, with the changes in metabolomics profile  
77 (Sundekilde, Poulsen, Larsen, & Bertram, 2013b).

78            In this study, a 600 MHz NMR installed with a cryoprobe was used to obtain spectra of milk  
79 ultrafiltrates from the already cited three feeding regimes. At the same time, a LF NMR was used to  
80 investigate the relaxation time of the proton of water present within the microstructure of  
81 mozzarella cheese prepared from the aforementioned milks, since it is known that LF-NMR is able  
82 to distinguish between three different populations of water based on binding to the cheese protein  
83 matrix (Gianferri, Maioli, Delfini, & Brosio, 2007; Kuo, Gunasekaran, Johnson, & Chen, 2001).

84

## 85    **2.    Materials and methods**

86

### 87    *2.1.   Milk sampling*

88

89            Milk sampling was conducted in mid-May (15 May 2016) from the afternoon lactation, at  
90 the period of maximum production. Sixty spring-calving Friesian cows were allocated to three  
91 groups (n = 20) at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark,  
92 Fermoy, Co. Cork, Ireland. Groups were randomised based on milk yield, milk solids yield, calving  
93 date (mean calving date February 19, 2015), and lactation number. The feeding systems analysed

94 were the same as those used by O'Callaghan et al. (2016b) consisting of the first group housed  
95 indoors and fed a TMR diet, the second group was maintained outdoors on perennial ryegrass  
96 (*Lolium perenne* L.) pasture (GRO), whereas group 3 was also maintained outdoors on a perennial  
97 ryegrass/white clover (*Trifolium repens* L.) pasture (GRC). The daily feed dry matter allowance for  
98 the GRO, GRC and TMR herds was 18, 18 and 22.6 kg per cow, respectively. Dry matter  
99 consumption of TMR herd was obtained directly from the sum of dry matter of the grass silage,  
100 maize silage and concentrate used in the ration. Dry matter consumption for outdoor herds were  
101 measured by determining pre- and postgrazing sward heights daily using the rising plate meter  
102 (Jenquip, Feilding, New Zealand), while pregrazing herbage mass was measured with an Etesia  
103 mower (Etesia UK Ltd., Warwick, UK) (O'Callaghan et al. 2016b).

104 Sample of milk were collected from the individual cows, to which sodium azide was added  
105 ( $0.3 \text{ mg mL}^{-1}$ ) to prevent bacterial growing and stored at  $4 \text{ }^{\circ}\text{C}$  overnight. On the following day, the  
106 samples were mixed and filtered to remove the fat and the protein phase. Five millilitres of sample  
107 was ultrafiltered using a Vivaspin® 6 10 kDa (Sartorius Stedim Ireland LTD, Dublin, Ireland) and  
108 centrifuged at  $10000 \times g$  for 30 min to separate the serum phase. The data from four of the sixty  
109 cows introduced at the beginning of the experiment were discarded from the sampling due to animal  
110 health issues, thus, resulting in a reduction of the total number of samples to fifty-six.

111 Cheese was made separately from the bulked milks of each of the three different dairy herds,  
112 which were feed on perennial ryegrass (GRO), perennial ryegrass and white clover (GRC) or total  
113 mix ration (TMR) on four separate occasions/days (trials), between October 10 and November 5,  
114 based on each of the three dietary treatments. Between 800 and 1000 kg of milk from the three  
115 different herds (GRO, GRC and TMR) was collected in three different tanks from both morning and  
116 afternoon milkings. Milk was then standardised to a protein:fat ratio of 1.2. After overnight storage  
117 at  $4 \text{ }^{\circ}\text{C}$  the milk was pasteurised ( $72 \text{ }^{\circ}\text{C}$  for 15 s) to reduce bacterial load before cheesemaking.

118

119 2.2.  $^1\text{H}$  Nuclear magnetic resonance

120

121 Prior to  $^1\text{H}$  NMR analysis, sufficient  $\text{D}_2\text{O}$  was added to all filtered samples to produce a  
122 solution of composition 90:10  $\text{H}_2\text{O}/\text{D}_2\text{O}$  (v/v). TSP (sodium 3-trimethylsilyl-2,2,3,3-tetra- $^2\text{H}$ -  
123 propanoate) was employed as an internal chemical shift reference for each solution.  $^1\text{H}$  NMR  
124 spectra of the samples were obtained using a Bruker Avance III 600 MHz NMR spectrometer  
125 (Bruker UK Ltd., Coventry, UK) equipped with a helium gas cooled 5 mm Bruker Dual C-H  
126 cryoprobe (CRYOPLATFORM™ technology) and a Bruker SAMPLEJET™ sample changer at  
127 University College Cork. Spectra were measured at a sample temperature of 300/303 K using  
128 Bruker TopSpin 3.2 software for spectrometer control, sample handling, NMR data acquisition and  
129 processing. For all samples, 16 scans were collected into 65.5 K data points using an excitation  
130 sculpting pulse sequence with gradients for water signal suppression and a spectral width of 12.33  
131 KHz. Spectra were phased and baseline corrected in Topspin. Prior to Fourier transform, a line  
132 broadening function of 0.3 Hz was applied.

133

### 134 2.3. *Multivariate data*

135

136 The fifty-six  $^1\text{H}$  NMR spectra obtained from the filtered milk were analysed using the  
137 method already described (Sundekilde et al., 2011) apart from some exceptions. All spectra were  
138 aligned using the *icoshift* tool (Savorani, Tomasi, & Engelsen, 2010) according to the signal of TSP  
139 and adjusted to 0.0 ppm. Alignment was performed in MATLAB 7.13 (MathWorks Inc., Natick,  
140 MA, USA).

141 The chemical shift region between 0.5 and 10 ppm (excluding residual water signal at 4.7–5 ppm)  
142 was subdivided into 0.01-ppm integral regions and integrated, reducing each spectrum into 950  
143 separate variables in the region. Spectra were scaled using the Pareto scaling and meanscale.

144 Principal component analysis (PCA) was used to provide a transformation of the original variables  
145 (NMR resonances) into a substantially reduced set of uncorrelated variables, the principal

146 components (PC). Furthermore, the data were analysed by a partial least squares (PLS) regression  
147 model coupled with a discrimination analysis (PLS-DA) to address the variation in metabolites  
148 specifically associated with the feeding system. PLS-DA models were cross-validated using  
149 segmented ( $n = 10$ ) cross-validation. PLS-DA model robustness was valuated using correlation  
150 coefficient ( $Q^2$ ) and root mean square error of cross validation (RMSECV). PCA and PLS-DA  
151 were performed in PLSToolbox 7.9.3 (Eigenvector Research Inc., Manson, WA, USA).

152

#### 153 2.4. *Cheese manufacture*

154

155 After pasteurisation, 460 kg of milk was cooled to 36 °C and pumped into the cheese vats  
156 (500-L; APV Schweiz AG, CH-3076 Worb 1, Switzerland). Mozzarella cheese manufacture was as  
157 previously described by Guinee, Feeney, Auty, and Fox (2002). In the specific instance, a full  
158 description of the prepared cheeses description may be found in Gulati et al. (2018), where their  
159 compositions (GRO, GRC and TMR) were investigated and compared in detail.

160

#### 161 2.5. *Relaxometry study*

162

163 Water proton relaxometry was used to investigate the ripening of the mozzarella cheese.  
164 Samples were prepared for analysis by collecting a cylindrical plug of 1.5 cm of diameter and 5 cm  
165 of height from a cheese block. The samples were all collected using a cork borer from the bulk part  
166 of the cheese. Three samples were collected from any cheese block. The cheese samples were  
167 inserted in a NMR tube (diameter 1.5 cm) and closed with Parafilm-M to avoid dehydration during  
168 analysis.

169

170 Before commencing spectra collection, the temperature of the sample was equilibrated at 25  
171 °C in a thermostatically-controlled unit (Techne Dri Block DB3) for 1 h. The analysis was  
performed using a MQC23-benchtop NMR analyser instrument (Oxford instrument, Abington,

172 Oxfordshire, UK), with an operating frequency of 23.4 MHz for protons. Spectra were conducted at  
173 the operating temperature of the instrument (40 °C). The samples were maintained inside the  
174 machine for as short a period as possible to minimise any structural modification arising from  
175 exposure to high temperature.

176 Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to study the transverse  
177 relaxation times (T<sub>2</sub>). This pulse sequence reduces the influence of field inhomogeneity (Hills,  
178 Takacs, & Belton, 1990) for long relaxation times. A total of 1024 echos was collected, with a 90-  
179 180 Degree Pulse Gap ( $\tau$ ) value of 420 $\mu$ s. Eight scans were collected for each analysis. The  
180 resultant decays were analysed by tri-exponential fitting in the RI Win-DXP software (V. 1.2.3.  
181 Oxford Instruments, Abington, Oxfordshire, UK). Every sample was analysed in triplicate.

182 As in previous studies, four different populations of protons denoted as T<sub>4</sub>, T<sub>3</sub>, T<sub>2</sub> and T<sub>1</sub>  
183 according to decreasing order of relaxation time (Gianferri et al., 2007) were identified and  
184 monitored in the cheese samples over the course of ripening for 50 days. T<sub>4</sub> relating to expressible  
185 serum showed a relaxation time close to that of free water. T<sub>3</sub> was associated with the fat present in  
186 the cheese, while T<sub>2</sub> was correlated with entrapped water–water that is in close vicinity to the  
187 protein matrix and its relaxation time is defined by the diffusion between the water and the protein  
188 phase. T<sub>1</sub> was associated with junction water, i.e., the water directly bound to the protein matrix,  
189 with a relaxation time directly connected with the proton exchange within the protein proton  
190 (Gianferri et al., 2007).

191

## 192 2.6. *Urea and lactose content*

193

194 Analysis of lactose and urea contents was performed using a Milkoscan FT 6000 plus  
195 Fossomatic 300 (CombiFoss) (Foss Allé 1, DK-3400, Hilleroed, Denmark). Immediately after  
196 collection, samples were placed in a fridge at 4 °C during the course of transport to the laboratory.  
197 All samples were heated to 40 °C and mixed prior to testing on the same day of collection.

198

199 2.7. *Moisture and fat content*

200

201 Grated cheese samples were analysed in duplicate at one day (post production) for protein,  
202 moisture, fat, salt and pH. For the purpose of this paper, only selected results relating to moisture  
203 obtained by oven drying at 102 °C for 5 h (IDF, 1982) and fat determined by Röse-Gottlieb method  
204 (IDF, 1996) were shown. Additional details on production and composition of these cheeses are  
205 outlined in Gulati et al. (2018).

206

207 2.8. *Expressible serum*

208

209 Expressible serum of cheese was investigated by grating 120 g of cheese and centrifuging it  
210 at  $12,500 \times g$  for 75 min at 25 °C as described by Guo and Kindstedt (1995). The expressible serum  
211 was indicated as the weight of serum expressed for 100 g of cheese.

212

213 **3. Results**

214

215 3.1. *NMR-based milk metabolomics*

216

217 The influence of feeding system was investigated using spectra generated during  $^1\text{H}$  NMR  
218 spectroscopy of milk sera, an example of which is illustrated in Fig. 1. The spectrometer was fitted  
219 with a cryo-probe to improve the signal to noise ratio. Spectra obtained from this analysis were in  
220 accordance with those reported in a previous studies (Sundekilde, Larsen, & Bertram, 2013a) and  
221 metabolomics identification was based on previous literature (Chenomx, 2018; Sundekilde et al.,  
222 2013a,b; Wishart et al., 2009, 2012). The list of the detected metabolites (Table 1) correspond with  
223 those of a previous study (Klein et al., 2010).

224 The influence of feeding regime on milk metabolites was first analysed using PCA. Lactose  
225 dominated the  $^1\text{H}$  NMR spectrum of milk. Consequently, the PC1 explained lactose variation  
226 between samples. To identify milk metabolites other than lactose, resonances from lactose were  
227 removed before PCA modelling. Resonances between 0.5 and 10 ppm were analysed by excluding  
228 the main region of the lactose signals: 3.27–3.35, 3.51–4.01, 4.37–4.7 and 5.13–5.25 ppm. This  
229 exclusion of lactose was done to remove any PC due to a change in this signal particularly in light  
230 of the fact that no difference in lactose content was found between the three different feeding  
231 systems (Table 2). The scores and loadings of a PCA model on  $^1\text{H}$  NMR data (Fig. 2) showed a  
232 clear distinction between the GRO and the TMR feeding regime (Fig. 2a). The PCA loadings  
233 showed a tendency for GRO-based cow feeding to have a decreased milk urea content according to  
234 its NMR spectrum (Fig. 2c). This lower urea content was confirmed by further analysis made on the  
235 total sample (Table 2).

236 Further data investigation was conducted using PLS-DA statistical analysis. This analysis  
237 showed a clear separation of the TMR feeding system from the GRO and GRC population (Fig 3d)  
238 while separation between GRO and GRC results was compromised by the presence of some  
239 samples appearing to belong to the other population (Fig 3b,c). This was also confirmed by the  
240 value of RMSECV with TMR scoring a better value (0.232149) with respect to GRO (0.348863)  
241 and GRC (0.36038). However, sufficient separation between all three groups of samples was  
242 obtained to allow the interpretation of difference between them.

243 The results (Fig. 4a) confirm the lower content of urea in GRO samples, with the peak  
244 related to the urea showing a higher presence in the GRC samples. Results also showed an increase  
245 of orotate in the GRC samples that was not detected by PCA analysis. When contrasting TMR with  
246 the two outdoor feeding regimes (Fig. 4b) it is possible to observe that these latter samples possess  
247 higher concentrations of hippurate (7.8, 7.6 and 7.5 ppm), lactate (4.1 and 1.33 ppm), carnitine  
248 (3.23 ppm), creatinine (3.05 ppm) and orotate (6.19 ppm). At the same time, however, TMR is  
249 richer in citrate (2.72, 2.68, 2.54, 2.51 ppm) and choline (3.2 ppm).

250

251 3.2. *Cheese microstructure*

252

253 Mozzarella cheeses produced from the three different milk batches were ripened at 4 °C for  
254 50 days. Samples were collected after 1, 10, 20, 30 and 50 days following production and analysed  
255 for expressible serum and proton transversal relaxometry profile. Further study on the cheeses was  
256 done in parallel by Gulati et al. 2018.

257 The expressible serum and the total moisture contents are outlined in Table 3. No  
258 differences in the total moisture content and expressible serum were detected between the cheeses  
259 obtained from the three different feeding systems.

260 However, when the water transversal relaxometry signal of the cheeses was investigated it  
261 was possible to monitor changes occurring in the nature of the moisture distribution during  
262 ripening. Four different proton populations were identified from the spectra, namely T1, T2, T3 and  
263 T4 (see Materials and methods, section 2.5) in order of increasing relaxation time. The relative  
264 concentrations and relaxation times of these four moisture population changes during ripening may  
265 be observed in Fig. 5. Only T2 and T3 were found at all sampling times, while T4 and T1 occurred  
266 during the first and final stages of the ripening process, respectively.

267 With respect to the change of relaxation time and distribution of moisture populations (Fig.  
268 6) over ripening duration, the second population (T2) increased in all cheeses whereas populations  
269 three and four (T3 and T4) decrease. T4 was found only on the first day of the cheese obtained from  
270 GRO, but was more persistent in GRC and TMR cheeses where it was present until day 20. The  
271 first population (T1) was the more difficult to identify due to its low concentration, however it was  
272 found after 20 days cheese ripening for the GRO-fed cows and after 10 days of ripening in the case  
273 of the GRC-fed cows, and only on the Day 20 sample for the TMR cheese. GRO cheese showed a  
274 higher content of this population (T1) even if this increase was not statistically significant with  
275 respect to the values found in the other two cheeses ( $p < 0.005$ ). The relaxation time was found to

276 increase with ripening time for all the four populations, with GRO cheese showing a higher value  
277 compared with the other two cheeses for T1, T2 and T3 populations.

278

## 279 **4. Discussion**

280

281 The importance of dairy cow feeding system on milk composition has been recently studied  
282 (Gulati et al., 2018; O'Callaghan et al., 2016b). A unique Irish temperate climate condition favours  
283 a feeding regime based on outdoor grazing (O'Callaghan et al., 2016b). During a comparison of  
284 three different feeding regimes, sizeable changes to the fat content of the milks was observed  
285 (O'Callaghan et al., 2016b), as well as to the rumen metabolome of the individual lactating animals  
286 involved in the studies (O'Callaghan et al., 2018). In this paper, NMR-based analytical techniques  
287 are explored to examine milk metabolomics in the milks of the cows subjected to the feeding  
288 regimes already outlined.

289

### 290 *4.1. Metabolomics*

291

292 Using  $^1\text{H}$  NMR spectra it was possible to identify the difference in the metabolomics profile  
293 of the filtered milks obtained from the three different feeding regimes. This technique has already  
294 been of value in previous published work (Sundekilde et al., 2011, 2013b). However, the NMR  
295 instrumentation used in this paper was furnished with a cryoprobe to reduce the background noise  
296 on the final spectra. While the use of the cryoprobe has a beneficial influence on the signal to noise  
297 ratio of the spectra, no difference in the milk metabolomics (Table 1) was detected when compared  
298 with previous work (Sundekilde et al., 2013a), suggesting that this specific probe does not increase  
299 the sensitivity of this technique.

300

301 Further investigation of the metabolomics data was explored using PCA and PLS-DA

301

302 lower concentration of urea. This result was confirmed during PLS-DA analysis (Fig. 4a), where  
303 GRO showed a lower concentration compared with GRC samples. The difference in urea content  
304 was also confirmed by direct analysis on the sample (Table 2). This difference may be due to the  
305 presence of higher nitrogen in the GRC-feeding system compared with GRO (O'Callaghan et al.,  
306 2016a).

307 A PLS-DA comparison between the outdoor (GRO and GRC) and TMR showed an increase  
308 of hippurate, lactate, carnitine and orotate and a reduction of choline and citrate in the first group.  
309 On the other hand, TMR showed a higher content in choline and citrate (Fig.4b). These metabolites  
310 have different influences on milk's nutritional value and industrial application.

311 The hippurate metabolite was previously identified using gas chromatography in  
312 combination with mass spectrometry when comparing different brands of milk, where it was found  
313 to be in higher concentration in those brands associated with organic farming (Boudonck, Mitchell,  
314 Wulff, & Ryals, 2009). In addition, hippurate together with orotate, another metabolomic  
315 compound found at higher concentration in GRO and GRC samples, were associated with milk  
316 having a low concentration of somatic cells (Sundekilde et al., 2013b). Higher levels of lactate also  
317 occurred in GRO and GRC milks which raised a concern given that this metabolite was previously  
318 reported to be associated with elevated levels of somatic cells (Sundekilde et al., 2013b). However,  
319 it was not possible with the present set of results to associate positive or negative influences of  
320 feeding on somatic cell count of milk.

321 Both carnitine and choline are of importance nutritionally to milk (Campoy et al., 1998;  
322 Zeisel, Mar, Howe, & Holden, 2003). Choline is an important structural component in the formation  
323 of phospholipids, cell membrane signalling and lipid transporter. Its biosynthesis is insufficient to  
324 meet bodily needs, hence a reliance on supplementation from dietary sources. Carnitine plays an  
325 important role in the catabolism of long chain fatty acids, except that its biosynthesis during early  
326 life is less efficient. Hence carnitine deficiency may in extreme cases lead to infant mortality (Crill  
327 & Helms, 2007). Carnitine occurred at higher concentrations in GRO and GRC samples while

328 choline was more prominent in TMR-produced milk. Considering the high nutritional importance of  
329 both nutrients and their contrasting occurrences in both groups of samples, it is difficult to identify  
330 which feeding system has a higher positive influence on the nutritional value of milk.

331 Higher concentrations of citrate were found in the TMR milk. Those metabolomics were  
332 previously related to the coagulation properties of milk (Sundekilde et al., 2011) where a higher  
333 concentration of citrate reduces the storage module ( $G'$ ) during rennet coagulation. Impaired rennet  
334 gel properties as a result of a reduced storage module have a negative impact on cheese making and  
335 subsequent cheese quality.

336

#### 337 4.2. *Cheese microstructure*

338

339 Relaxometry studies were previously done on freshly-produced mozzarella cheese prepared  
340 from buffalo milk (Gianferri et al., 2007) and also with mozzarella cheese over the course of a  
341 relatively short ripening period (Kuo et al., 2001). In the current study, the authors conducted their  
342 investigations over a longer cheese ripening period (50 days). The four different proton populations  
343 found in our results are similar to those found in previous work (Gianferri et al., 2007) and can be  
344 related to the same moisture and fat populations.

345 During the ripening of mozzarella cheese, the water contained in the serum-fat channel is  
346 absorbed by the cheese protein matrix (McMahon, Fife, & Oberg, 1999). This is visible with the  
347 reduction of the T4 population and the increase of T2 and T1, which is evident for all three feeding  
348 systems. In comparing the three different feeding systems it is possible to notice that the GRO  
349 cheese indicated the T4 population (expressible serum-related protons) only during first sampling  
350 while the other two cheeses continued to indicate this moisture population until 20 days of ripening  
351 in agreement with the results obtained with the expressible serum (Table 3). GRO also shows a  
352 faster increase of T2 (entrapped water-related protons) during the first period of ripening (20 days)  
353 and a higher content of T1 (protein-bound water).

354 These results suggest that in the GRO cheese the ripening time to reach the final structure is  
355 reduced to 40 days compared with 50 days in the case of TMR- and GRC-produced cheeses. It was  
356 also shown that the protein microstructure contained more bound water with respect to the other  
357 two cheeses, thus suggesting a different protein microstructure. The higher relaxation time value of  
358 GRO probably indicates a slower diffusion between protein matrix and water, hence suggesting a  
359 protein matrix that is more compact with respect to the other two moisture populations.

360

## 361 5. Conclusion

362

363 Using <sup>1</sup>H NMR and Relaxometry instrumentation, it was possible to investigate the  
364 influence of the feeding regime on the metabolomics content of milk and on the microstructure of  
365 cheese, respectively. Both analytical techniques reveal useful scientific detail, which may be  
366 aligned with technological and commercial needs.

367 When identifying a feeding regime to promote the metabolomics value of milk from a  
368 nutritional perspective, GRO- and GRC-grass based diets stand out in terms of possessing higher  
369 concentrations of biological markers such as hippurate, which reflect near-organic milk production  
370 conditions. On the other hand, a lower citrate concentration in milk would contribute to an increase  
371 in storage module ( $G'$ ) during chymosin-induced gelation to the benefit of curd formation during  
372 cheesemaking. These differences in metabolomics for now are attributable to the effects of different  
373 feeding systems. However, it may be opportune in future work to combine NMR metabolomics  
374 with the potential of mid-infrared (MIR) spectroscopy because of its capability to monitor ruminant  
375 de novo fatty acid synthesis (Woolpert et al., 2016) provides additional information on the health  
376 status of the lactating cow and its impact on milk composition.

377 In relation to cheese ripening process, GRO-cheese shows that it needs a shorter ripening  
378 time to reach its final water distribution as defined by relaxometry. These findings add to advances  
379 in pastoral-based dietary regimes where applicable in dairy producing countries.

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381

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385

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1 **Figure legends**

2

3 **Fig. 1.** Solvent suppressed, 600 MHz  $^1\text{H}$  NMR spectrum in 90:10  $\text{H}_2\text{O}$ - $\text{D}_2\text{O}$  of a milk sample.

4

5 **Fig. 2.** Results from the principal component analysis (PCA) obtained by comparing the three  
6 different feeding systems: (a) scores plot for the first and second principal component; (b) loading  
7 line plot of the first principal component of Pareto-scaled nuclear magnetic resonance (NMR) data  
8 ( $n = 56$ ); (c) loading line plot of the second principal component of Pareto-scaled nuclear magnetic  
9 resonance (NMR) data ( $n = 56$ ). Sample markers are coloured continuously according to their  
10 feeding regime: green, cows fed outdoor with grazing of perennial ryegrass (*Lolium perenne* L.);  
11 red, cows fed outdoor with grazing of perennial ryegrass/white clover (*Trifolium repens* L.); blue,  
12 cows fed indoor housing using a total mixed ration.

13

14 **Fig. 3.** Correlation coefficient and prediction graphic of the partial least squares discrimination  
15 analysis (PLS-DA) of the proton nuclear magnetic resonance ( $^1\text{H}$  NMR) metabolite profile of milk  
16 from different feeding regimes. Sample markers are coloured continuously according to their  
17 individual feeding regime: green, cows fed outdoor by grazing of perennial ryegrass (*Lolium*  
18 *perenne* L.); red, cows fed outdoor by grazing of perennial ryegrass/white clover (*Trifolium repens*  
19 L.); blue, cows fed indoor housing using a total mixed ration.

20

21 **Fig. 4.** Partial least squares discrimination analysis (PLS-DA) coefficient plots of the unit variance-  
22 scaled model back-transformed: (a) score of the comparison between grass feeding (GRS) and grass  
23 plus clover feeding (CLV) regimes – higher scores indicate higher concentrations within the CLV  
24 samples; (b) score of the comparison between the outdoor (GRS and CLV) and indoor feeding  
25 regimes (total mix ration, TMR) – numbers indicate different metabolomic signals: 1, hippurate; 2,  
26 orotate; 3, urea; 4, creatinine; 5, lactate; 6, choline; 7, citrate.

27

28 **Fig. 5.** Variation of the proton relaxation time of mozzarella cheese obtained from clover feeding  
29 during ripening up to 50 days (—, day 1; - - -, day 10; - - - -, day 20; ·····, day 30; —, day 50).  
30 Inset on the top left shows the zoom area within 100 and 3000 ms. The positioning of the different  
31 water populations ( $T_1 \dots T_4$ ) according to signal intensity is indicated.

32

33 **Fig. 6.** Variation of (left column) moisture population detectable in the  $^1\text{H}$  relaxation spectra during  
34 the ripening of mozzarella cheese obtained from three different feeding systems and of (right  
35 column) the relaxation time belonging to the different moisture populations: —■—, GRO – cows  
36 fed outdoor by grazing of perennial ryegrass (*Lolium perenne* L.); ···●···, GRC – cows fed outdoor  
37 by grazing of perennial ryegrass/white clover (*Trifolium repens* L.); - -▲- -, TMR – cows fed  
38 indoor housing using a total mixed ration.

**Table 1**List of chemical shift value and metabolomics detected in the  $^1\text{H}$  NMR spectra of ultrafiltered permeate of milk.

Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)	Metabolomic	Chemical shift (ppm)
3-Methylhistidine	2.25	Creatine	2.88	Hippurate	7.84	Lecithin	3.12
3-Methylhistidine	3.74	Creatine	3.79	Isobutyrate	1.16	Lecithin	3.75
3-Methylhistidine	3.97	Creatinine	3.05	Isoleucine	0.93	Lecithin	3.83
3-Methylhistidine	7.14	Creatinine	4.06	Lactate	1.33	Lecithin	4.18
3-Methylhistidine	8.09	Ethanolamine	3.15	Lactate	4.11	Lecithin	4.22
Acetate	1.92	Ethanolamine	3.83	Lactose (total)	3.54	Malonic acid	3.11
Acetone	2.24	Formate	8.45	Lactose (total)	3.67	Methionine	2.15
Adenine	8.12	Fucose	1.25	Lactose (total)	3.73	N-Acetylcarbohydrates	2.06
Adenine	8.13	Fumarate	6.52	Lactose (total)	3.78	Ornithine	1.8
Alanine	1.48	Galactose $\alpha$	3.81	Lactose (total)	3.94	Orotate	6.2
Alanine	3.79	Galactose $\alpha$	4.07	Lactose (total)	4.45	Phosphocholine	3.03
Betaine	3.26	Galactose $\beta$	3.49	Lactose $\alpha$	3.59	Phosphocholine	3.18
Butyrate	0.9	Galactose $\beta$	4.57	Lactose $\alpha$	3.66	Phosphocholine	3.58
Carnitine	2.44	Galactose-1-phosphate	5.38	Lactose $\alpha$	3.84	Phosphocholine	3.93
Carnitine	3.21	Glucose	5.1	Lactose $\alpha$	3.88	Phosphocholine	4.16
Carnitine	3.43	Glucose-1-phosphate	5.51	Lactose $\alpha$	3.96	Taurine	3.27
Carnitine	4.57	Glutamate	2.36	Lactose $\alpha$	5.23	Taurine	3.43
Choline	3.18	Glycerophosphocholine	3.65	Lactose $\beta$	3.29	Triethylamine-N-oxide	3.27
Choline	3.51	Glycerophosphocholine	4.32	Lactose $\beta$	3.6	Urea	5.79
Choline	4.06	Glycine	3.57	Lactose $\beta$	3.66	Valine	1.05
cis-Aconitate	3.15	Hippurate	7.54	Lactose $\beta$	3.81	$\beta$ -Hydroxybutyrate	1.2
Citrate	2.52	Hippurate	7.64	Lactose $\beta$	3.84		
Citrate	2.72			Lactose $\beta$	3.96		
				Lactose $\beta$	4.67		

**Table 2**

Average value of urea and lactose detected from the three different feeding regimes milk samples obtained from Milkoscan analysis.

Sample	Urea (mg L <sup>-1</sup> )	Lactose (g L <sup>-1</sup> )
Grass - clover	29 ± 4	4.8 ± 0.1
Grass	19 ± 3	4.7 ± 0.4
TMR	28 ± 2	4.8 ± 0.1

**Table 3**

Moisture content and expressible serum parameters of cheese made from milk from spring calved herds on 3 different feeding systems in late-lactation. <sup>a</sup>

Parameter	Ripening time (days)	Feeding system		
		Grass	Grass-clover	TMR
Moisture (% w/w)		48.41 <sup>a</sup>	47.81 <sup>a</sup>	47.12 <sup>a</sup>
Expressible serum (% TM)	1	19.88 <sup>a</sup>	17.98 <sup>a</sup>	18.42 <sup>a</sup>
	10	0.41 <sup>a</sup>	6.77 <sup>a</sup>	9.14 <sup>a</sup>
	20	0	0	0
	30	0	0	0
	50	0	0	0

<sup>a</sup> Data are the mean values of four replicate trials in late-lactation (each made on a separate occasion); values within a row relating to Grass, Grass-clover or TMR diets and not sharing a common lowercase superscript letter differ significantly ( $P < 0.05$ ) for the effect of feeding system. TM, total moisture.

