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Integration of high and low field ¹H NMR to analyse the effects of bovine dietary regime on milk metabolomics and protein-bound moisture characterisation of the resulting mozzarella cheeses during ripening

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3	cheeses during ripening
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ABSTRACT

30	The influence of dairy cow feeding regime was investigated using ¹ H nuclear magnetic resonance
31	(NMR). Two different NMR analytical systems were deployed: high field ¹ 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 41	cheeses.
42	

43 1. Introduction

44

Milk production in temperate climates such as New Zealand (O'Brien et al., 1999) and 45 Ireland relies substantially on grass growth as the principle bovine dietary source. Ideally matched 46 to ruminant digestion needs, pasture grazing is valued at many levels such as animal welfare 47 (Verkerk, 2003) and the promotion of natural animal foraging behaviour (Charlton, Rutter, East, & 48 Sinclair, 2011; Legrand, von Keyserlingk, & Weary, 2009). However, this system is not practical in 49 all other countries because of climatic extremes. Hence, an alternative milk production system 50 widely practiced in mainland Europe utilises year-round indoor housing in which the dairy cows' 51 dietary needs are addressed using a total mixed ration (TMR). It has been pointed out that in this 52 system animal welfare presents some concern due to incidences of lameness and mastitis that 53 diminish animal comfort and reduce milk output (Fregonesi, Veira, Von Keyserlingk, & Weary, 54 55 2007; Haskell, Rennie, Bowell, Bell, & Lawrence, 2006). On the other hand, this system protects animals from extreme climatic conditions (heat, cold and wetness) and allows better management of 56 57 animal nutrition formulations. The influence of different feeding systems on the milk production has been extensively 58 studied over the years (Couvreur, Hurtaud, Lopez, Delaby, & Peyraud, 2006; Lee, Theobald, 59 Tweed, Winters, & Scollan, 2009; White et al., 2001). A more recent study (O'Callaghan et al., 60 2016b) is of particular interest as it compared the influence of three different feeding regimes: TMR 61 with outdoor grazing of perennial ryegrass (Lolium perenne L.; GRO), and outdoor grazing of 62 perennial ryegrass/white clover (Trifolium repens L.; GRC) on milk raw composition and quality 63 during lactation. The results show that, in general, the GRO feeding system increased milk quality, 64 particularly higher concentrations of protein and fat, and improved the nutritional profile of milk 65 fat. In a related study (O'Callaghan et al., 2016a), butters produced from GRO and GRC milks had 66 a higher nutritional value and improved sensory scores compared with TMR butter. 67

However, while the milk composition from Irish dairy farming and its influence on the dairy 68 processing has been extensively investigated (Kelly, O'Keeffe, Keogh, & Phelan, 1982; Keogh, 69 Kelly, O'Keeffe, & Phelan, 1982; Phelan, O'Keeffe, Keogh, & Kelly, 1982), the influence that 70 feeding has on metabolomics and on industrial processes such as the cheese making remain open. 71 Increasingly, more advanced analytical instrumentation is being applied to probe in greater detail 72 compositional differences and other markers in milks produced as a result of different feeding 73 regimes, e.g., HR ¹H NMR spectroscopy has been already used successfully to investigate the 74 influence of breeding on milk composition (Sundekilde, Frederiksen, Clausen, Larsen, & Bertram, 75 2011), milk somatic cells, usually correlate with mastitis, with the changes in metabolomics profile 76 77 (Sundekilde, Poulsen, Larsen, & Bertram, 2013b). In this study, a 600 MHz NMR installed with a cryoprobe was used to obtain spectra of milk 78 ultrafiltrates from the already cited three feeding regimes. At the same time, a LF NMR was used to 79 80 investigate the relaxation time of the proton of water present within the microstructure of mozzarella cheese prepared from the aforementioned milks, since it is known that LF-NMR is able 81 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).

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2. Materials and methods

- 86
- 87 2.1. Milk sampling
- 88

Milk sampling was conducted in mid-May (15 May 2016) from the afternoon lactation, at
the period of maximum production. Sixty spring-calving Friesian cows were allocated to three
groups (n = 20) at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark,
Fermoy, Co. Cork, Ireland. Groups were randomised based on milk yield, milk solids yield, calving
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 mg mL^{-1}) to prevent bacterial growing and stored at 4 °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,

which were feed on perennial ryegrass (GRO), perennial ryegrass and white clover (GRC) or total mix ration (TMR) on four separate occasions/days (trials), between October 10 and November 5, based on each of the three dietary treatments. Between 800 and 1000 kg of milk from the three different herds (GRO, GRC and TMR) was collected in three different tanks from both morning and afternoon milkings. Milk was then standardised to a protein:fat ratio of 1.2. After overnight storage at 4 °C the milk was pasteurised (72 °C for 15 s) to reduce bacterial load before cheesemaking.

118

119 2.2. ¹H Nuclear magnetic resonance

121	Prior to ¹ H NMR analysis, sufficient D_2O was added to all filtered samples to produce a
122	solution of composition 90:10 H_2O/D_2O (v/v). TSP (sodium 3-trimethylsilyl-2,2,3,3-tetra- ² H-
123	propanoate) was employed as an internal chemical shift reference for each solution. ¹ 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

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

The chemical shift region between 0.5 and 10 ppm (excluding residual water signal at 4.7–5 ppm)
was subdivided into 0.01-ppm integral regions and integrated, reducing each spectrum into 950
separate variables in the region. Spectra were scaled using the Pareto scaling and meanscale.
Principal component analysis (PCA) was used to provide a transformation of the original variables
(NMR resonances) into a substantially reduced set of uncorrelated variables, the principal

120

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 (Q2) 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.

Samples were prepared for analysis by collecting a cylindrical plug of 1.5 cm of diameter and 5 cm of height from a cheese block. The samples were all collected using a cork borer from the bulk part of the cheese. Three samples were collected from any cheese block. The cheese samples were inserted in a NMR tube (diameter 1.5 cm) and closed with Parafilm-M to avoid dehydration during analysis.

Before commencing spectra collection, the temperature of the sample was equilibrated at 25
 °C in a thermostatically-controlled unit (Techne Dri Block DB3) for 1 h. The analysis was

171 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 $^{\circ}$ 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 (T2). 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 (τ) value of 420µs. 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 T4, T3, T2 and T1
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. T4 relating to expressible
185	serum showed a relaxation time close to that of free water. T3 was associated with the fat present in
186	the cheese, while T2 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. T1 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

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

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199 2.7. Moisture and fat content

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201	(Grated cheese samples were analysed in duplicate at one day (post production) for protein,
202	moistur	e, fat, salt and pH. For the purpose of this paper, only selected results relating to moisture
203	obtained	d by oven drying at 102 °C for 5 h (IDF, 1982) and fat determined by Röse-Gottlieb method
204	(IDF, 19	996) were shown. Additional details on production and composition of these cheeses are
205	outlined	l in Gulati et al. (2018).
206 207 208	2.8.	Expressible serum
200	1	Expressible serum of cheese was investigated by grating 120 g of cheese and centrifuging it
210	at 12,50	$00 \times g$ for 75 min at 25 °C as described by Guo and Kindstedt (1995). The expressible serum
211	was ind	icated as the weight of serum expressed for 100 g of cheese.
212		
213	3.	Results
214		
215	<i>3.1. 1</i>	NMR-based milk metabolomics
216		
217	r	The influence of feeding system was investigated using spectra generated during ¹ H NMR
218	spectros	scopy of milk sera, an example of which is illustrated in Fig. 1. The spectrometer was fitted
219	with a c	ryo-probe to improve the signal to noise ratio. Spectra obtained from this analysis were in
220	accorda	nce with those reported in a previous studies (Sundekilde, Larsen, & Bertram, 2013a) and
221	metabol	lomics 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

those of a previous study (Klein et al., 2010).

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

total sample (Table 2).

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

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

250

251 *3.2. Cheese microstructure*

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

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

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

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

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

278

279 **4. Discussion**

280

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

289

290 4.1. Metabolomics

291

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

Further investigation of the metabolomics data was explored using PCA and PLS-DA
approaches. It was clear since the first PCA analysis (Fig. 2) that cows fed with GRO showed a

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

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

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

Both carnitine and choline are of importance nutritionally to milk (Campoy et al., 1998; Zeisel, Mar, Howe, & Holden, 2003). Choline is an important structural component in the formation of phospholipids, cell membrane signalling and lipid transporter. Its biosynthesis is insufficient to meet bodily needs, hence a reliance on supplementation from dietary sources. Carnitine plays an important role in the catabolism of long chain fatty acids, except that its biosynthesis during early life is less efficient. Hence carnitine deficiency may in extreme cases lead to infant mortality (Crill & 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.
336 337 338	4.2. Cheese microstructure
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.

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

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

360

361 **5.** Conclusion

362

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

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

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

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1 Figure legends

2

Fig. 1. Solvent suppressed, 600 MHz ¹H NMR spectrum in 90:10 H₂O–D₂O of a milk sample.

- Fig. 2. Results from the principal component analysis (PCA) obtained by comparing the three 5 different feeding systems: (a) scores plot for the first and second principal component; (b) loading 6 line plot of the first principal component of Pareto-scaled nuclear magnetic resonance (NMR) data 7 8 (n = 56); (c) loading line plot of the second principal component of Pareto-scaled nuclear magnetic resonance (NMR) data (n = 56). Sample markers are coloured continuously according to their 9 feeding regime: green, cows fed outdoor with grazing of perennial ryegrass (Lolium perenne L.); 10 red, cows fed outdoor with grazing of perennial ryegrass/white clover (Trifolium repens L.); blue, 11 cows fed indoor housing using a total mixed ration. 12
- 13

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

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

27

Fig. 5. Variation of the proton relaxation time of mozzarella cheese obtained from clover feeding
during ripening up to 50 days (---, day 1; ---, day 10; ---, day 20;, day 30; ---, day 50).
Inset on the top left shows the zoom area within 100 and 3000 ms. The positioning of the different
water populations (T₁...T₄) according to signal intensity is indicated.

Fig. 6. Variation of (left column) moisture population detectable in the ¹H relaxation spectra during the ripening of mozzarella cheese obtained from three different feeding systems and of (right column) the relaxation time belonging to the different moisture populations: $-\blacksquare$, GRO – cows fed outdoor by grazing of perennial ryegrass (*Lolium perenne* L.); ... \bullet ..., GRC – cows fed outdoor by grazing of perennial ryegrass/white clover (*Trifolium repens* L.); - \blacktriangle - -, TMR – cows fed indoor housing using a total mixed ration.

Table 1

List of chemical shift value and metabolomics detected in the ¹H NMR spectra of ultrafiltered permeate of milk.

Metabolomic	Chemical shift	Metabolomic	Chemical shift	Metabolomic	Chemical shift	Metabolomic	Chemical shift
	(ppm)		(ppm)		(ppm)		(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 a	3.81	Lactose (total)	3.94	Orotate	6.2
Alanine	3.79	Galactose a	4.07	Lactose (total)	4.45	Phosphocholine	3.03
Betaine	3.26	Galactose ^β	3.49	Lactose a	3.59	Phosphocholine	3.18
Butyrate	0.9	Galactose ^β	4.57	Lactose a	3.66	Phosphocholine	3.58
Carnitine	2.44	Galactose-1-phosphate	5.38	Lactose a	3.84	Phosphocholine	3.93
Carnitine	3.21	Glucose	5.1	Lactose a	3.88	Phosphocholine	4.16
Carnitine	3.43	Glucose-1-phosphate	5.51	Lactose a	3.96	Taurine	3.27
Carnitine	4.57	Glutamate	2.36	Lactose a	5.23	Taurine	3.43
Choline	3.18	Glycerophosphocholine	3.65	Lactose B	3.29	Triethylamine-N-oxide	3.27
Choline	3.51	Glycerophosphocholine	4.32	Lactose B	3.6	Urea	5.79
Choline	4.06	Glycine	3.57	Lactose B	3.66	Valine	1.05
cis-Aconitate	3.15	Hippurate	7.54	Lactose B	3.81	β-Hydroxybutyrate	1.2
Citrate	2.52	Hippurate	7.64	Lactose B	3.84		
Citrate	2.72			Lactose B	3.96		
		Y		Lactose ^β	4.67		

Table 2

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

Sample	Urea (mg L ⁻¹)	Lactose (g L ⁻¹)
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.^a

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

^a 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.











