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Influence of herd diet on the metabolome of Maasdam cheeses

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Abstract

The untargeted metabolic profiles of ripened Maasdam cheese samples prepared from milk derived from three herd groups, fed: (1) indoors on total mixed ration (TMR), or outdoors on (2) grass only pasture (GRA) or (3) grass and white clover pasture (CLO) were studied using high resolution nuclear magnetic resonance (^{1}H NMR), high resolution magic angle spinning nuclear magnetic resonance (^{1}H HRMAS NMR) and headspace (HS) gas chromatography mass spectrometry (GC-MS). A total of 31 compounds were identified using ^{1}H NMR and 32 volatile compounds including 7 acids, 5 esters, 4 alcohols, 4 ketones, 4 sulphur compounds, 2 aldehydes, 3 hydrocarbons, 2 terpenes and a lactone were identified using GC-MS in Maasdam cheeses ripened for 97-d. On comparing the ^{1}H NMR metabolic profiles, TMR-derived cheese had higher levels of citrate compared to GRA-derived cheese.. The toluene content of cheese was significantly higher in GRA or CLO compared to TMR cheeses and dimethyl sulfide was identified only in CLO-derived cheese samples as detected using HS GC-MS. These compounds are proposed as indicator compounds for Maasdam cheese derived from pasture-fed milk. Clear differences between outdoor or indoor feeding systems in terms of cheese metabolites were detected in the lipid phase, as indicated by principal component analysis (PCA) from ^{1}H HRMAS NMR spectra, although differences based on PCA of all ^{1}H NMR spectra and HS-GC-MS were less clear. Overall, this study presented the metabolite profile and identified specific compounds which may be useful for discriminating between ripened Maasdam cheese and related cheese varieties manufactured from indoor or outdoor herd-feeding systems.

Key words: herd diet, feeding system, Maasdam cheese, cheese metabolic profile, ^{1}H NMR, ^{1}H HRMAS NMR, cheese volatile compounds

1. Introduction

Maasdam cheese is manufactured with mesophilic starter cultures and thermophilic adjunct cultures along with propionic acid bacteria (PAB) as a secondary culture. The latter contributes to the development of associated sensory properties (e.g., sweet and nutty flavor), along with the formation of eyes. Generally, in comparison to milk, cheeses are more complex biological systems because of the metabolism of fat, protein and lactose to organic acids, amino acids, peptides, and the subsequent formation of a large number of volatiles or non-volatiles compounds (McSweeney, 2004).

Untargeted high resolution proton nuclear magnetic resonance (^{1}H NMR) of cheese extracts (liquid-state) has been used to characterize the metabolic profiles of different cheese types with established *protected designation of origin* (PDO) related to their geographical origin of manufacture, e.g., Parmigiano-Reggiano (Consonni & Cagliani, 2008) or buffalo Mozzarella cheese (Brescia, Monfreda, Buccolieri, & Carrino, 2005). This approach has also been used to determine changes associated with bacterial cultures (Rodrigues, Santos, Rocha-Santos, Gomes, Goodfellow, & Freitas, 2011) and adjunct cultures, e.g., in Fiore Sardo from ovine milk (Piras et al., 2013). Moreover, ^{1}H-NMR has been used to differentiate cheese based on stage of ripening (Consonni & Cagliani, 2008; Piras et al., 2013; Rodrigues et al., 2011) or when brining conditions have been altered (Ruyssen et al., 2013), as indicated by changes in metabolite concentration. Similar interest has been shown for high resolution magic angle spinning (^{1}H HRMAS) NMR in which a solid sample (solid-state) is directly analyzed without necessarily extracting compounds from cheese samples into a liquid phase, as in ^{1}H NMR methods (Lamichhane et al., 2015; Mazzei & Piccolo, 2012; Shintu & Caldarelli, 2005).

Previously, O'Callaghan et al. (2016) reported TMR feeding systems yield significantly higher average daily milk yield (27.71 L/d), milk solids (2.24 kg/d), protein (0.94 kg/d), fat (1.31 kg/d) and lactose (1.32 kg/d) compared to milk produced under pasture-based feeding systems, although the concentrations of fat and protein tend to be lower in TMR derived milks due to the higher milk volume. Recently, interest has focused on the ability to differentiate between milk or milk products derived from cows fed on pasture or on total mixed rations (TMR) composed of silage, concentrate with added minerals and vitamins (Kilcawley, Faulkner, Clarke, O'Sullivan, & Kerry, 2018). Demand for pasture-based dairy products has increased over those produced from controlled supplementation of silage and concentrate (Getter, Behe, Howard, Conner, & Spaniolo, 2015), further highlighting the importance of determining marker compounds which can differentiate products from milk derived from different diets. The feeding regime of a herd partly contributes to the sensory properties of dairy products, depending on the concentration and odor threshold of compounds therein (Kilcawley et al., 2018). O'Callaghan et al. (2017) reported that full-fat Cheddar cheese made from milk derived from pasture-fed cows had a higher concentration of toluene and lower concentration of 2,3-butanediol compared to cheese produced from TMR feeding systems. Likewise, investigation of volatiles profiles in cheese samples using gas chromatography mass spectrometry (GC-MS) also facilitates comparison of samples between treatments due to the presence of specific volatile compounds (Engels, Dekker, de Jong, Neeter, and Visser, 1997; Suzzi et al., 2015; Lamichhane et al., 2018b). Identification of compounds that could differentiate cheeses based on herd feeding system are of particular interest where a grass-based feeding system is predominant. Profiling of volatiles and non-volatiles compounds of the Maasdam cheese metabolome using ^{1}H NMR or ^{1}H HRMAS NMR and GC-MS could identify specific compounds related to the feeding practices of cows.

The primary objective of this research was to apply ^{1}H NMR spectroscopy and GC-MS to establish the metabolic profiles of Maasdam cheeses produced from milks of cows kept indoors and fed TMR or cows kept outdoors and grazed in pastures of either grass only (GRA) or grass and white clover (CLO). A second objective of the present study was to generate a chemical fingerprint of Maasdam cheese samples using ^{1}H NMR and ^{1}H HRMAS NMR spectroscopy, to illustrate the differences in chemical fingerprints between liquid-state and solid-state NMR.

2. Materials and Methods

2.1. Experimental Design

The experimental design of the present study was described by O'Callaghan et al. (2016). Briefly, a spring-calved herd of Friesian cows at Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Ireland, was separated into three cohorts, in which group 1 (18 cows) was kept indoors and fed a TMR diet, group 2 (18 cows) was kept outdoors and grazed a grass only (perennial ryegrass; *Lolium perenne* L.) pasture (GRA), and group 3 (18 cows) was also kept outdoors and grazed a grass and white clover (*Trifolium repens* L.) pasture (CLO). The GRA and CLO received no feed supplementations during the period in which milk were collected for this experiment. Selection of the experimental cows was randomized based on milk and milk solids yield for the first two weeks post-calving, parity number, and calving date. During the current study, the experimental cows were at mid-lactation. The composition and nutritional quality of each feed was as described by O'Callaghan et al. (2016). To facilitate neutralization of the ruminal pH, 0.7% (wt/wt) of acid buffer prepared from red marine algae (Celtic Sea Minerals, Co, Cork, Ireland) was incorporated in the TMR.

2.2. Milk collection and preparation

Milk from the morning and afternoon milking from each group of cows over 2 days was collected on 3 separate occasions over a 3-week period. Approximately 600 L of milk were collected on each occasion and standardized to a protein-to-fat ratio (PFR) of 1.17 and stored overnight at 4°C. As milk from GRA-fed cows contained a greater quantity of solids than that from TMR- or CLO-fed cows, protein and fat levels in those milks were standardized by combining skim milk, cream, and ultrafiltration (UF) permeate and retentate produced from the respective raw milks (Supplementary Fig. S1). Ultrafiltration of skim milk was carried out as described by Kelly, Kelly, Mehra, Oldfield, Raggett, and O'Kennedy (2000) using a spiral-wound membrane plant (Memtech Ltd, Swansea, UK, max surface area 144 m^2, molecular weight cut-off = 10 KDa) at 45°C to achieve a milk protein level of ~ 5%.

2.3. Maasdam cheese-making process

Maasdam cheese was manufactured from milk produced from the three different feeding systems, in triplicate trials at pilot-scale (Moorepark Technology Ltd., Moorepark, Fermoy, Co. Cork, Ireland). The cheesemaking process was as described by Lamichhane, Kelly, and Sheehan (2018a) using standardized and pasteurized milk in stainless steel jacketed cheese vats equipped with automated variable-speed-cutting and stirring facilities (APV Schweiz AG, Worb, Switzerland). Briefly, cheese milk (380 kg, 32°C) in each vat was inoculated with frozen pellets (Chr. Hansen Ltd, Denmark) of mesophilic *(*C950*; Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris* and *Leuconostoc)*, thermophilic (LH-B01; *Lactobacillus helveticus)* and propionic acid bacteria (PS-60; PAB) at the rate of 18 mg/kg of milk, 4.8 mg/kg of milk, and 7 mg/kg of milk, respectively. This was followed by the addition of calcium chloride (34% wt/vol) at level of 3.19 ml/kg and rennet (0.18 ml/ kg of milk, Chymax plus, 200 IMCU/ml; Chr. Hansen, Denmark) after diluting (~1:10) in deionized water. Cutting, stirring, cooking, curd

washing, pre-pressing and brining was as described by Lamichhane et al. (2018a). Cheeses wheels (~12 kg) were wrapped under vacuum and pre-ripened at 8°C for 10 days, transferred to a warm room at ~23°C for 21 days and finally ripened under refrigeration (2-4°C) up to 150 d from the manufacture date.

2.4. Sample preparation for NMR

Cheeses were sampled at 97 and 150 days of ripening, grated, and freeze dried (~30 g) for 24 hours and stored at -20°C. These sampling points were chosen to represent the typical age of Maasdam-type cheese as sold in retail markets. Cheese samples were freeze-dried to minimize metabolic activity prior to analysis when transporting samples internationally. For analysis, samples were thawed in a cold room, and then ground using a mortar and pestle to make a fine powder of uniform particle size.

2.4.1. Sample extraction for ^{1}H NMR

Cheese powder (50 mg) samples were weighed in 1.5 ml Eppendorf tubes in which a methanol-chloroform-water (4:3:3) extraction was carried out by the method of Folch, Lees, and Sloane-Stanley (1957). The particles were thoroughly mixed with extraction solvents using a vortex and held over-night for phase separation, and subsequent centrifugation (1400 × g) for 30 min at 4°C was performed. The supernatant (methanol –water-soluble phase) was transferred into a new Eppendorf tube and vacuum-dried in an Eppendorf Concentrator Plus at 30°C, stored at -80°C and analysed within a week. The soluble extract was dissolved with 700 µl Deuterium oxide (D_2O) containing 0.025% sodium trimethylsilyl[2,2,3,3-$^{2}H_4$]-1-propionate (TSP; Sigma-Aldrich A/S, Copenhagen, Denmark) as an internal chemical shift reference. The dissolved and

vortexed (~30 s) extract (700 µl) was transferred into 5-mm NMR tubes and capped. Samples were prepared in duplicate from a total of 18 cheese samples (n = 36).

2.5. ^{1}H NMR Spectroscopy

The instrumental conditions were as described by Sundekilde, Poulsen, Larsen, and Bertram (2013b), using Proton (^{1}H) NMR spectroscopy at 298 K (25°C) on a Bruker Avance III 600 spectrometer equipped with a TXI-probe (Bruker Biospin, Rheinstetten, Germany). Briefly, operating conditions were: ^{1}H frequency 600.13 MHz, 5 s relaxation delay, 0.1 s mixing time, 64 scans, 32 K data point and 12.15 ppm spectral width. Acquisition time was 2.25 s and standard 1D spectra were acquired using a NOESY sequence 90° pulse with water suppression by pre-saturation during relaxation delay and mixing time (Bruker pulse: noesypr1d). The Free Induction Decay (FID) data were multiplied by 0.3-Hz line-broadening function before Fourier transformation. Topspin 3.0 (Bruker BioSpin) was used for phase and baseline correction of ^{1}H NMR spectra. Spectra were referenced using a TSP signal. NMR signals were identified in accordance with the literature (Sundekilde, Larsen, & Bertram, 2013a), the Human Metabolome Database (www.HMDB.ca) and the Chenomx NMR suite (Chenomx Inc., Alberta, Canada).

2.6. ^{1}H HRMAS NMR spectroscopy (solid state NMR)

The methods followed for ^{1}H HRMAS NMR analysis was as described by Lamichhane et al. (2015). Briefly, fine powder (7 ±3 mg) of freeze-dried cheese samples was weighed (n=18) into 30 µL inserts (Bruker Biospin, Germany) in which 10 µl of D_2O (containing TSP for chemical shift reference) was added and centrifuged for 15 s to enable mixing of D_2O with the sample. The inserts were placed into a 4-mm Zirconium rotor (Bruker Biospin, Germany). Bruker Avance III 600 MHz was used for HRMAS in using $^{1}H/^{13}C/^{31}P$ MAS Probe with gradient

(Bruker Biospin, Germany). Analysis was performed at ^{1}H frequency 600.13 MHz, 5 KHz spin rate, 273 K, 17.36 ppm spectral width. Spectra were recorded in Carr-Purcell-Meiboom-Gill (CPMG) (Bruker pulse: cpmgpr1d) or Nuclear Overhauser Effect Spectroscopy (NOESY) pulse sequence. The number of scans was 128, relaxation delay of 3 s, T2 echo time was 400 ms, the acquisition time was 1.57 s and the collected data points were 32 K. The FID was multiplying with 0.3 Hz of line broadening prior to Fourier Transform. The phase and base-line was correcting using Topspin 3.0 software.

2.7. Volatile profiles by HS GC-MS

For profiling of volatile compounds in the cheese, grated samples (4 g) were weighed into a magnetic screw-capped amber HS vials (20 ml) with a silicone/PTTE septum (Apex Scientific, Maynooth, Ireland). The temperature of vials was raised to 40°C for 10 min under the condition of pulsed agitation (5 s on and 2 s off at 500 rpm). Agitation was performed by rotation of the module under controlled conditions as outlined. Volatile extraction was performed using a single 50/30 um Carboxen /divinylbenzene/polydimethylsiloxane (DVB/CAR/PDMS) solid-phase micro-extraction (SPME) fibre (Agilent Technologies Ireland Ltd, Cork, Ireland), which was exposed to the HS for 20 min at a depth of 54 mm at 40°C using a Shimadzu AOC-5000 injection system (Mason Technology, Dublin Ireland). The fibre was injected to a Shimadzu 2010 plus GC (Mason Technology) *via* a merlin microseal (Sigma-Aldrich A/S) at 250°C for 2 min in split-less mode. The column was an Agilent DB-5ms column (Agilent Technologies Ireland Ltd) and the oven temperature was set at 30°C for 30 min, increased to 230°C (6.5°C min^{-1}), then increased to 320°C (15°C min^{-1}) over 41.5 min, with helium as the carrier gas at a uniform pressure of 1.58 bar. The column oven temperature was increased to 320°C so that any potential less volatile material was removed before the next analysis. The

detector was a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer (Mason Technology) in single quadrupole mode. The temperature of ion source and interface were set at 220°C and 280°C, respectively. Electronic ionization (70 v) with the mass range between 35 and 250 amu were selected for MS mode. Total ion chromatograms were processed using TargetView (Markes International Ltd, Llantrisant, UK), and identification was carried out using mass spectra comparisons to the NIST 2014 mass spectral library, the Shimadzu flavor and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created in GC-MS Solutions software (Shimadzu, Japan) with target and qualifier ions. Kovat retention indices were also used aid identification as described by van Den Dool and Dec. Kratz (1963) and matched against peer-reviewed publications where possible to confirm compound identification. An auto-tune of the GC-MS was carried out prior to the analysis to ensure optimal GC-MS performance. A set of external standards was run at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection were performing within specification.

2.8. Multivariate data analysis

Proton NMR spectra were aligned based on TSP using icoshift (Savorani, Tomasi, & Engelsen, 2010). NMR spectra were normalized excluding residual water and extraction solvent peak in the chemical shift range 10 to 0.5 ppm. In addition, resonance signals from water, urea and filtering procedures were removed using Matlab 7.13 (Mathworks, Inc., Natick, Ma, USA) generating variables (n = 928) for multivariate analysis. Firstly, principal component analysis (PCA) was applied to the unit variance-scaled data for identifying clustering based on feeding system on a total of 36 samples of Maasdam cheese (18 from 97 d and 18 from 150 d). To discriminate cheese samples, an orthogonal partial least square discriminant analysis (OPLS-DA)

approach was exploited, which removes the variables that are systematically uncorrelated to the model, improving the model predictability (Bylesjö, Rantalainen, Cloarec, Nicholson, Holmes, & Trygg, 2006; Sundekilde et al., 2013b). Cross-validation of the OPLS-DA model was performed by dividing the samples into 4 groups and randomly leaving 1 group out of the model. Sample duplicates were left out together. Integrals were calculated for peak area of the identified compounds.

2.9. Statistical analysis

A one-way ANOVA was performed on the peak area of compounds identified from the NMR spectra and volatile compounds from GC-MS spectra using PROC ANOVA function with Tukey's multiple comparison in SAS (version 9.3, SAS Institute Inc., Cary, NC) to determine the effect of GRA, CLO or TMR feeding system at 5% significance level. Principal component analysis (PCA) of volatile compounds was carried out using minitab 17 (Minitab Inc.).

3. Results and discussion

3.1. High resolution ^{1}H NMR spectroscopic analysis of Maasdam cheese samples

The representative ^{1}H NMR spectra (δ 0.5 to 4.5, δ 6.7 to 8.5) for Maasdam cheese prepared from milk from cows fed on (A) GRA (B) CLO or (C) TMR is shown in Fig. 1. The 31 assigned compounds are summarized in Table 1 with code numbers. The resonance signals from organic acids (lactate, propionate and acetate), and amino acids dominated the ^{1}H NMR spectra in the Maasdam cheese. The spectra also showed resonance signals from other low molecular weight compounds present in cheese samples; however, these could not be assigned. The resonance signals from lactose were absent in the spectra, as lactose was converted to lactic acid

during ripening. Between the chemical shift from 0.5 to 4.5 ppm of the ^{1}H NMR spectrum, the signals from aliphatic group of organic acids, amino acids and alcohol (ethanol and 2,3 butanediol) were predominant, whereas resonances from aromatic group of amino acids were predominant between 6.9 to 8.5 ppm.

The Chenomx NMR suite databases were used for the comparison of the chemical shift values and the values were largely in accordance with those published previously (Sundekilde et al., 2013a). By comparing the spectra, it was shown that metabolic profiles of the ripened cheese samples from GRA, CLO or TMR feeding systems were not largely different, apart from the differences in intensities of some compounds.

3.1.1. Comparison of different feeding systems (^{1}H NMR spectra)

The ^{1}H NMR metabolomic data from 36 cheese samples (18 from 97 days and 18 from 150 days of ripening) was first treated with an unsupervised PCA to investigate the clustering of the cheese samples based on the feeding systems of the cows. However, PCA with dependent variables of GRA, CLO and TMR, did not show any clustering of the cheese samples, indicating that the source of variation in the spectrum was largely unexplained by the feeding system (Supplementary Fig. S2). The OPLS-DA score plots showed the separation of cheese samples based on feeding regimes of cows (Fig. 2 A, B C); however, variance within the samples was low, as indicated by smaller values of predictive components and first orthogonal components. It is worth noting that the coefficients of determination and Q^2 values of the models were satisfactory for predictive reliability of the OPLS-DA model.

For better interpretability, OPLS-DA coefficients were back-transformed from the unit variance scale and color-coded based on OPLS-DA loading weight (Cloarec et al., 2005).

Primarily, it was found that the resonance of organic acids had high correlation (lactate, acetate, and propionate), which minimized the impact of compounds that are present in lower concentration (Supplementary Fig. S3). Therefore, the resonance signals of organic acids (e.g., propionate, lactate, acetate) were removed from the spectra and a reduced model for OPLS-DA coefficient plots was generated. The corresponding coefficients plot of the OPLS-DA model shows the compounds that are responsible for discrimination between cheese samples (Fig. 2A, B, C).

3.1.2. Relative quantification of ^{1}H NMR signals

The integrals of resonance signals of compounds identified by ^{1}H NMR were calculated (Table 2). The peak areas of acetate, citrate, carnitine, ornithine, and tyrosine were found to be highly influenced in cheeses derived from the different feeding practices. However, large differences were not observed in the OPLS-DA coefficient for these metabolites, except for citrate levels (Fig. 2A). The statistical analysis as shown in Table 2 was univariate, whereas multivariate analysis was used in the OPLS-DA model. The effect of covariance in the latter analytical method may explain why significant differences observed in metabolites in cheese samples using univariate analysis were not observed using OPLS-DA.

O'Callaghan et al. (2017) reported higher levels of 2,3-butanediol in Cheddar cheese derived from a TMR feeding system than from pasture grazing. In contrast, no significant differences in 2,3-butanediol content was observed between the experimental cheese samples, although a slightly higher level was observed in GRA cheese samples compared to CLO or TMR. The discrepancy could be due to citrate metabolism by starter or non-starter bacteria present in cheese milk that favors the metabolism of citric acid partly into 2,3-butandiol.

Mesophilic cultures, e.g., *Lactococcus* spp and *Leuconostoc* spp. added into milk in the present study are known to catalyze citrate (McSweeney, 2004). Interestingly, the citric acid level was significantly higher in TMR compared to GRA cheeses and this was also positively correlated with cheeses derived from TMR feeding systems in the OPLS-DA model (Fig. 2A). O'Callaghan et al. (2018) observed a greater level of citric acid in TMR-fed milk than in GRA- or CLO-fed milk. Results of the present study indicate that a higher citric acid level in TMR cheese could be due to a higher proportion of citrate in milk, possibly as a result of herd diet and de-novo-fatty-acid sysnthesis (Garnsworthy et al., 2006). However, citrate as a single marker may not be sufficient to distinguish cheeses by feed type, but it could be a component of a number of markers to distinguish cheeses derived from different feeding systems. This requires further investigation.

3.2. Proton high resolution MAS NMR spectroscopic analysis of Maasdam cheese samples

The representative ^{1}H HRMAS NMR (CPMG pulse sequence) spectra of GRA, CLO and TMR cheese samples between chemical shifts 0 to 8.5 ppm are shown in Supplementary Fig. S4. As expected, spectra with CPMG pulse sequence suppressed the signals from fatty acids and resonance signals from organic acids and amino acids dominated. The identified compounds are also presented in Table 1. Spectra with NOESY pulse sequence of same sample were dominated by fatty acids, which superimposed the signals of organic acids and amino acids (Supplementary Fig. S5). The CPMG or NOESY ^{1}H HRMAS spectra obtained were largely similar to previously reported spectra for ripened cheese (Lamichhane et al., 2015; Shintu & Caldarelli, 2006). Cheese samples produced from GRA, CLO and TMR feeding systems appeared to have a similar resonance peak (in both CPMG or NOESY pulse sequence), indicating similar metabolic profile of all cheese samples despite the variation in feeding systems. The results of ^{1}H HRMAS NMR

are consistent with the results of ^{1}H NMR in that feeding system did not show large effects on the spectral resonance peak of experimental Maasdam cheese samples.

3.2.1. ^{1}H HRMAS NMR spectral analysis of Maasdam cheese

The unsupervised PCA of HRMAS NMR resonance signal from 18 cheeses (9 from 97 days and 9 from 150 days) revealed a tendency towards grouping in spectra from CPMG pulse sequence and clear grouping in NOESY pulse sequence between outdoor (GRA and CLO) and indoor (TMR) feeding systems (Fig. 3A, B). The PCA plot also showed that ^{1}H HRMAS data with CPMG pulse sequence was less variable (PC2, 12.9%; PC3 10.6%), this was in agreement with the result of ^{1}H NMR in which compounds were analyzed in extracted samples. However, the variability of ^{1}H HRMAS data with NOESY pulse sequence was PC1 78.0%, PC2, 8.47%. The spectral signal from CPMG pulse sequence largely suppressed signals from lipids, which led the appearance of resonance signals from amino acids and organic acids (Supplementary Fig. S4) whereas, in spectra with NOESY pulse sequence, lipid signals masked the signals of small molecular weight compounds (supplementary Fig. S5). This indicates that Maasdam cheeses prepared from milk of herds kept indoors or outdoors on separate feeding systems largely differed due to lipid profile. It is suggested that to identify more subtle differences in the fatty acid profile arising due to herd diet, analysis of fatty acid methyl esters would be desirable. Maasdam cheese prepared from milk derived from cows fed GRA or CLO could not be separated, indicating that metabolome were largely similar in the final cheeses. O'Callaghan et al. (2017) also reported less variability between GRA or CLO feeding systems in Cheddar cheese.

The advantage of NOESY HRMAS data over ^{1}HNMR in the present study is that it can highlight possible differences caused by lipids, which indeed appeared between cheese samples

prepared from milk of outdoor or indoor feeding systems. The lipid content in cheese has been reported to be highly influenced by the feeding system of cows (O'Callaghan et al., 2017). O'Callaghan et al. (2017) reported that cheese prepared using milk from a TMR feeding system had higher levels of saturated fatty acids compared to cheeses made using milk from a GRA or CLO feeding system. Our results suggest that most discrimination based on different feeding systems for Maasdam cheeses is related to lipids or metabolites soluble in lipids, since most of the unbound water-soluble metabolites may also be removed along with whey during cheesemaking, as indicated by low variation of ^{1}H NMR spectra during multivariate data analysis. Differences observed in citrate levels in cheee samples produced from different feeding system could be due to its entrapment in casein micelles and in protein network. For detecting lower molecular weight compounds that are masked by lipids and amino acids, a specific sample extraction (e.g., exclusion of lipids) may be required.

3.3. Volatile compounds in cheese

The composition of herd feeds supplied and their volatiles profiles have been reported elsewhere (Faulkner et al., 2018; O'Callaghan et al., 2016). The profile of volatile compounds in GRA, CLO or TMR Maasdam cheeses ripenend after 97 days are shown in Table 3. Headspace GC-MS analysis of experimental cheese samples detected a total of 32 compounds, including 7 acids, 5 esters, 4 alcohols, 4 ketones, 4 sulphur compounds, 2 aldehydes, 3 hydrocarbons, 2 terpenes and a lactone. The contents of the majority of volatile compounds were similar between experimental cheeses, except for the levels of toluene and dimethyl sulfide.

Principal component analysis was carried out on the volatile profiles with respect to GRA, CLO and TMR cheeses and PCA showed clustering of the samples between feeding

systems, particularly between indoor or outdoor feeding systems (Supplementary Fig. S6; however, a trial effect was also apparent. The first, second and third principal components (PC) explained the variance of 27%, 21%, and 12%, respectively. Compounds that were positively correlated with PC1, PC2 and PC3 were not significantly different between cheese samples, indicating cheese samples were largely similar in terms of volatile compounds.

The profile of volatile compounds has a significant role on the flavour properties of cheeses and these are primarly produced as a consequence of breakdown of citric acid, lactic acid, amino acids and fatty acids during cheese ripening, due to the action of enzymes associated with microorganisms or coagulant (McSweeney, 2004). Maasdam cheeses are effectively intermediate between Gouda and Emmental, in which mesophilic cutures (*Lactococcus and Leuconostoc*) are added along with thermophilic cultures and PAB (Lammichhane et al., 2018) and their combined action results in the formation of a wide range of volatile compounds. It is generally known that PAB primarily convert lactate to volatile compounds such as propionate and acetate, along with H_2O and CO_2. Propionic acid bacteria are known for accelarating lipolysis and catabolism of amino acids such as isoleucine during ripening (Thierry, Maillard, Richoux, Kerjean, & Lortal, 2005) and are the major contributors to flavour formation in Swiss-type cheeses (Kilcawley, 2017). A high abundance of acetic acid and propionic acids were detected in GRA, CLO or TMR cheeses. Butanoic acid (butyric acid) was also detected, which could possibly originate from the butyrate-fermenting *Clostridia* bacteria (Lamichhane et al., 2018b) or lipolysis by enzymes. Branched short-chain fatty acids such as 2-methylbutanoic acid is believed to originate from the breakdown of branched amino acids such as leucine and isoleucine (Urbach, 1997) mainly by PAB in Swiss-type cheeses (Thierry et al., 2005). The presence of free fatty acids (hexanoic and octanoic acids) in all three types of cheeses was

attributed to the lipolytic activity of cheese microbiota (Collins, McSweeney, & Wilkinson, 2003).

Moreover, some compounds either present in the feed of cows, or produced in the rumen during digestion, may pass into milk and subsequenly accumulate in cheese, forming a basis for differentiation according to feeding systems (Martin, Verdier-Metz, Buchin, Hurtaud, & Coulon, 2007). β-carotene in feed decomposes to toluene in the rumen and is transferred to milk (Coppa, Martin, Pradel, Leotta, Priolo, & Vasta, 2011; Faulkner et al., 2018). In the present study, GRA or CLO cheeses had a signficantly higher concentration of toluene compared to TMR cheeses, and a higher level of toluene was observed in CLO than in GRA cheeses. O'Callaghan et al. (2017) reported that Cheddar cheese derived from GRA or CLO feeding systems had more than two times the β-carotene content compared to cheese derived from a TMR feeding system. Since fresh forage is likely to have higher level of β-carotene compared to mixed rations, cheeses prepared from pasture-derived milk may contain higher toluene levels after the decomposition of β-carotene (Kilcawley, et al., 2018). The level of β-carotene in Maasdam cheese was not determined in the current study. Nevertheless, the impact of toluene on sensory perception is minimal because of a high odor threshold (Faulkner et al., 2018). The presence of *p*-xylene and 1-octene were also noted in all three cheese types. Xylene in milk has been reported to originate from feed samples (Faulkner et al., 2018); its level in cheese depends on the feed botanical diversity (Buchin, Martin, Dupont, Bornard, & Achilleos, 1999).

Alcohols such as 1-propanol, 3-methyl 1-butanol, 2-methyl-1-butanol, 2,3-butanediol were detected but their level was not significantly different between cheese samples. Thierry et al. (2005) reported that alcohols could be produced through a heterofermentative pathway by lactic acid bacteria. However, O'Callaghan et al.(2017) observed higher levels of 2,3-butanediol

in TMR cheeses compared to GRA or CLO Cheddar cheese, although such differences were not observed in the present study using both ^{1}HNMR and GC-MS techniques. The level of 2,3-butanediol could have been influenced through citrate metabolism by the mesophilic starter culture (McSweeney, 2004).

Esters of propionate are unique compounds present in Swiss-type cheeses and impart a sweet and fruity odor to the cheese (Liu, Holland, & Crow, 2004). Coppa et al. (2011) reported higher levels of esters in pasture-derived milk compared to hay-derived milk. Esterases released from lactic acid bacteria or PAB probably lead to the formation of ethyl groups, along with free fatty acids, during ripening, influencing the ester content of cheese (Liu et al., 2004). The presence of primary alcohols and carboxylic acids in cheese has been reported to be critical factor for esterification rather than concentration of enzymes (Kilcawley et al., 2018). Ethyl isobutyrate, ethyl butanoate, ethyl hexanoate and ethyl octatnoate were also detected in all three types of cheese, indicating esterification in the presence of esterases.

Four volatile sulfur compounds, namely carbon disulfide, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide were detected in the cheese samples. However, dimethyl sulfide was only detected in CLO cheeses. In contrast, O'Callaghan et al. (2017) reported similar levels of dimethyl sulfide levels in Cheddar cheese prepared from indoor or outdoor feeding systems. Differences observed between the cheeses could be attributed to differences in oxidation-reduction potential influenced by the manufacturing protocol and starter cultures (Caldeo and McSweeney, 2012). Dimethyl disulfide and dimetyl trisulfide are known to influence cheese flavour (Landaud, Helinck, & Bonnarme, 2008); however, their levels (e.g., dimethyl trisulfide) are probably influenced by the presence of PAB (Thierry et al., 2005). Although dimethyl

sulfone was previously highly correlated with pasture-based products (Kilcawley et al., 2018), it was not detected in this study, presumably due to the proteolytic activity of cheese microbiota.

Ketones, particularly methyl ketones, are commonly found in cheese and are important for aroma (Urbach, 1997). Acetoin (3-hydroxy 2-butanone), 2-butanone, 2-heptanone, and 2-nonanone were detected in all three types of cheese. The levels of acetoin was higher in GRA or CLO compared to TMR cheese samples (albeit not signficantly different). Acetoin is generally identified in high abandance in Maasdam cheeses mainly due to citrate metabolism by *Lactococcus* spp. and *Leuconostoc* spp. (Engels et al., 1997; Lamichhane et al., 2018b) or due to conversion of aspartate by *L. lactic* (Le Bars & Yvon, 2008). The influence of PAB on acetoin production is unclear (Thierry et al., 2005), which indicates that acetoin most probably resulted from the metabolism of citrate by the added mesophilic lactic culture (McSweeney, 2004); however, this could possibly be reduced to butanone (Engels et al., 1997).

Aldehydes are highly abundant volatile compounds present in different cheese varieties, including Maasdam cheeses (Engels et al., 1997). Aldehydes are produced from α-keto acids, which are derived from transaminase reactions of amino acids (McSweeney, 2004). These are reported as an intermediate product, which eventually lead to the formation of alcohol or carboxylic acids (Singh, Drake, & Cadwallader, 2003). Only two aldehyde compounds, benzaldehyde and benzeneacetaldehyde, were identified. Carpino et al. (2004) observed the presence of benzeneacetaldehyde in pasture-derived cheeses and Aprea et al. (2016) showed higher levels of benzaldehyde in cheese made from milk derived from cows grazed on pasture containing poor levels of nutrients, and its level could be influenced by the processing steps such as milk pasteurization (Faulkner et al. 2018).

The levels of terpenes in milk and milk products are believed to be influenced by the feed supplied to cows and offers an opportunity to discriminate based on botanical composition of the feed. D-limonene and α-pinene were indentified in the experimental cheese samples and were not influenced by feeding systems, in agreement with the findings of O'Callaghan et al. (2017). However, O'Callaghan et al. (2017) suggested a possible metabolism of terpene compounds by cheese microbiota.

In summary, volatile analysis of Maasdam cheese suggests that the influence of cheese bacterial (starter culture and secondary culture) metabolic pathways may be greater than the influences of differences in the constituents of the milks used for cheese manufacture, except for toluene and dimethyl sulfide.

4. Conclusions

An untargeted metabolomics approach was utilized to investigate the metabolome (volatiles and non-volatiles) present in Maasdam cheese using one-dimensional ^{1}H NMR, ^{1}H HRMAS NMR, and HS-GC-MS. Discriminant (OPLS-DA) analysis of ^{1}H NMR data revealed that Maasdam cheese prepared from TMR-based feeding systems correlated most with citrate compared to cheese made from a grass-based feeding system. Cheese prepared from GRA- or CLO-fed milk was not largely different in terms of metabolic profile. When cheese samples were analyzed using ^{1}H HR-MAS NMR, clear separation in PCA was observed between TMR cheeses and those from pasture-fed cheese, which is likely to be due to difference in the lipid content in those samples. Levels of toluene as determined by HS-GC-MS and citrate by ^{1}H NMR in Maasdam cheese samples were identified as a basis for separating cheeses from outdoor or indoor feeding practices. The presence of dimethyl sulfide in cheese is likely to be linked to the

presence of white clover in the pasture. Overall, feed-induced variation in metabolite content of Maasdam cheese was low, suggesting that metabolites produced by starter cultures and secondary PAB cultures have a much greater role and can dominate the majority of compounds derived from milk/feed.

Ethical approval

The animal welfare body is a legal requirement of Article 26 of Directive 2010/63/EU and Regulation 50 of S.I. No. 543 of 2012. Teagasc has both an animal ethics committee and animal welfare body. The Health Products Regulatory Authority License number for this project is AE19132/P019.

Conflict of Interest

Declarations of interest: none.

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Figure legends

Fig. 1. Representative ^{1}H NMR spectra of Maasdam cheese samples after 97 days of ripening. Spectrum A, B and C represents cheese made from cow milk fed on grass only pasture (GRA), fed on grass and clover (CLO) pasture and fed total mixed ration (TMR), respectively. The chemical shift between 0.5 and 4.5 ppm represents the aliphatic region. The aromatic region (6.7 and 8.8 ppm) is vertically expanded (2x). The name of assigned compounds is shown in Table 1; numbers are nominal and nd refers to not identified.

Fig. 2. Orthogonal projection to latent squares discriminant analysis (OPLS-DA) scores plots and corresponding back-transformed coefficients plots of a cross-validated model of the unit variance scaled data; of cheese prepared from cow milk fed on (A) grass only in pasture (GRA) versus total mixed ration (TMR), (B) grass and clover in pasture (CLO) versus TMR, and (C) GRA versus CLO. The resonances from lactate, propionate, and acetate were excluded. Each variable has been colored according to the OPLS-DA loadings (correlation between NMR variable and feeding systems). The list of compounds with code number is shown in Table 1. Online PDF contains the color version.

Fig. 3. Principal component analysis of ^{1}H HRMAS NMR spectra with different pulse sequence, (A) CPMG or (B) NOESY, for spectra of ripened cheeses made from TMR-fed cow milk and combined data generated from outdoor feeding of grass only (GRA) and grass with clover (CLO). Circles indicate the grouping of compounds. Numbers in A are sample label.

Table 1 [1]HNMR chemical shift assignment for Maasdam type cheese

SN	Code	Chemical shift (multiplicity)			
1	Leucine	0.95 (d)	1.7 (m)	0.96(d)	3.72(m)
2	2,3-Butanediol	1.14 (d)			
3	Alanine	1.47 (d)	3.76(m)		
4	Butyrate	1.54 (m)		0.87(t)	
5	Acetate	1.91(s)			
6	Propionate	2.19(m)	1.04(t)		
7	4-Aminobutyrate	2.30(m)	2.28(t)	1.88(m)	
8	Glutamate	2.33(m)			
9	Isobutyrate	1.03(d)			
10	Succinate	2.43 (s)			
11	Citrate	2.70 (d)	2.53(d)		
12	Lysine	3.74 (t)	1.72(m)	3.00 (t)	
13	Carnitine	3.22 (s)			
14	Tyramine	7.22 (d)	6.91(d)	2.95 (t)	
15	Proline	3.41 (m)	2.00(m)		
16	Glycine	3.54 (s)			
17	Choline	3.51(m)	3.19(s)		
18	Valine	3.60 (d)	2.26(m)	1.02(d)	0.98(d)
19	Ethanol	1.18(t)			
20	Isoleucine	3.66(d)	1.27(m)	1.00(d)	0.92(t)
21	Glutamine	3.77 (t)	2.46(m)		
22	Ornithine	3.05(t)			
23	Methionine	3.85(m)	2.64(t)		2.12 (s)
24	Arginine	3.24 (t)			
25	Betaine	3.89 (s)	3.25 (s)		
26	Asparagine	2.95(m)	2.84(m)	4.0(m)	
27	Lactic acid	4.11 (m)	1.31 (d)		
28	Tryptophan	7.73 (d)	7.53 (d)		
29	Tyrosine	7.19 (m)	6.89 (m)		
30	Phenylalanine	7.40 (m)	3.98 (t)	3.27(m)	3.10(m)
31	Formate	8.44 (s)			

S = singlet, d = duplet, t=triplet, m=multiplate

Table 2 Peak area of the identified metabolites in [1]HNMR spectra.

		Experimental cheese groups (Peak area)			
	Metabolites	GRA	CLO	TMR	*P*
1	Leucine (x10^8)	3.2[a]	3.1[a]	3.2[a]	0.94
2	2,3-butanediol (x10^7)	2.3[a]	1.7[a]	1.8[a]	0.12
3	Alanine (x10^7)	5.5[a]	5.2[a]	5.8[a]	0.67
4	Butyrate (x10^6)	2.8[a]	2.5[a]	2.1[a]	0.08
5	Acetate (x10^8)	2.8[a]	2.1[b]	2.7[a]	<0.01
6	Propionate (x10^8)	1.9[a]	1.5[a]	1.9[a]	0.08
7	4 Aminobutyrate (x10^7)	2.0[a]	1.9[a]	1.9[a]	0.88
8	Glutamate (x107)	2.0[a]	1.7[a]	2.3[a]	0.36
9	Isobutyrate (x107)	3.2[a]	3.2[a]	3.5[a]	0.75
10	Succinate (x10^7)	6.6[a]	5.6[a]	7.9[a]	0.02
11	Citrate (x10^6)	1.1[b]	2.0[b]	3.2[a]	<0.01
13	Carnitine (x10^6)	5.7[ab]	4.6[b]	7.9[a]	0.01
14	Tyramine (x10^7)	1.1[a]	0.8[a]	1.4[a]	0.12
16	Glycine (x10^7)	1.1[a]	1.0[a]	1.1[a]	0.96
17	Choline (x10^7)	1.2[a]	1.1[a]	1.4[a]	0.15
18	Valine (x10^7)	2.0[a]	1.8[a]	2.0[a]	0.74
19	Ethanol (x10^6)	3.2[a]	2.5[a]	3.4[a]	0.95
20	Isoleucine (x10^6)	8.5[a]	8.3[a]	11[a]	0.43
21	Glutamine (x10^6)	1.8[a]	1.8[a]	2.1[a]	0.64
22	Ornithine (x10^6)	5.2[a]	2.8[b]	4.6[a]	<0.01
23	Methionine (x10^7)	1.2[a]	1.1[a]	1.2[a]	0.6
24	AAGLULEUAL (x10^8)	1.2[a]	1.0[a]	1.2[a]	0.34
25	Betain (x10^5)	4.9[a]	4.9[a]	4.3[a]	0.86
26	Asparagine (x10^7)	2.0[a]	2.0[a]	1.6[a]	0.09
27	Lactate (x10^8)	3.6[a]	4.9[a]	2.5[a]	0.02
28	Tryptophan (x10^5)	5.1[a]	5.1[a]	6.5[a]	0.14
29	Tyrosine (x10^6)	2.2[ab]	1.5[b]	2.7[a]	<0.01
30	Phenylalanine (x10^8)	1.3[a]	1.2[a]	1.3[a]	0.4
31	Formate (x10^6)	9.2[a]	7.8[ab]	7.3[b]	0.03

[a-b] means with same superscript letter were not different ($\alpha = 0.05$).

AAGLULEUAL = peak area from arginine, glutamate, leucine, and alanine.

Table 3. Effect of feeding systems[1] on volatile compounds in Maasdam cheese at 97 day of ripening.

Volatile compounds	Std	RI	Experimental cheese groups (peak area)						
			GRA	SE	CLO	SE	TMR	SE	*P*
Acid									
Acetic acid	629	680	4.3*10^7[a]	2.1*10^6	3.9*10^7[a]	1.3*10^6	4.0*10^7[a]	3.9*10^6	0.45
Propanoic acid	725	720	4.1*10^7[a]	1.0*10^7	3.2*10^7[a]	1.1*10^7	3.5*10^7[a]	8.4*10^6	0.79
Butanoic acid	787	809	1.4*10^7[a]	9.9*10^5	8.8*10^6[a]	2.7*10^6	1.6*10^7[a]	2.2*10^6	0.11
3-Methylbutanoic acid	838	847	1.7*10^6[a]	2.8*10^5	1.6*10^6[a]	5.8*10^5	2.9*10^6[a]	7.9*10^5	0.28
2-Methylbutanoic acid	831	853	2.2*10^6[a]	2.3*10^5	2.1*10^6[a]	1.0*10^6	3.9*10^6[a]	9.3*10^5	0.28
Hexanoic acid	1005	980	8.0*10^6[a]	4.0*10^6	3.2*10^6[a]	3.0*10^6	1.0*10^7[a]	1.0*10^7	0.74
Octanoic acid	1163	1164	1.4*10^6[a]	1.9*10^5	1.2*10^6[a]	2.2*10^5	2.6*10^6[a]	8.5*10^5	0.22
Alcohols									
1-Propanol	549	537	4.5*10^5[a]	1.2*10^5	1.1*10^5[a]	1.1*10^5	3.4*10^4[a]	3.4*10^4	0.05
3-Methyl-1-butanol	731	740	6.1*10^5[a]	2.4*10^5	4.5*10^5[a]	1.3*10^5	4.9*10^5[a]	1.8*10^5	0.83
2-Methyl-1-butanol	743	747	6.3*10^5[a]	1.5*10^4	1.9*10^6[a]	8.3*10^5	8.6*10^5[a]	4.8*10^5	0.28
2,3-Butanediol	802	814	4.1*10^6[a]	6.4*10^5	3.5*10^6[a]	1.0*10^6	4.2*10^6[a]	3.1*10^5	0.75
Aldehydes									
Benzaldehyde	997	971	2.9*10^6[a]	1.4*10^6	1.7*10^6[a]	7.6*10^5	2.1*10^6[a]	1.8*10^6	0.83
Benzene acetaldehyde	1048	1053	2.7*10^4[a]	8.5*10^3	2.3*10^4[a]	3.2*10^3	2.8*10^4[a]	6.6*10^3	0.85
Esters									
Ethyl propanoate	714	712	1.2*10^6[a]	1.6*10^5	8.9*10^5[a]	5.7*10^4	1.1*10^6[a]	1.9*10^5	0.42
Ethyl isobutyrate	767	770	7.6*10^4[a]	7.6*10^4	3.7*10^4[a]	3.7*10^4	5.5*10^4[a]	5.5*10^4	0.89
Ethyl butanoate	799	802	1.5*10^6[a]	5.7*10^5	1.7*10^6[a]	6.5*10^5	7.9*10^5[a]	8.9*10^4	0.44
Ethyl hexanoate	1002	998	1.6*10^5[a]	2.9*10^4	1.5*10^5[a]	3.4*10^4	2.4*10^5[a]	4.2*10^4	0.22

Ethyl octanoate	1198	1194	7.5*10^4 [a]	2.1*10^4	6.7*10^4 [a]	2.2*10^4	1.3*10^5 [a]	3.3*10^4	0.23
Hydrocarbons									
Toluene	723	759	9.4*10^5 [a]	2.7*10^5	1.7*10^6 [a]	2.7*10^5	1.5*10^5 [b]	1.6*10^4	<0.01
o-Xylene	872	875	5.8*10^4 [a]	1.3*10^4	5.2*10^4 [a]	1.5*10^4	4.7*10^4 [a]	1.8*10^4	0.89

Continued Table 3. Effect of feeding systems[1] on volatile compounds in Maasdam cheese at 97 day of ripening.

		Experimental cheese groups (peak area)							
	Std	RI	GRA	SE	CLO	SE	TMR	SE	*P*
1-Octene	788	799	3.4*10^5 [a]	1.9*10^5	6.6*10^5 [a]	3.6*10^5	1.7*10^5 [a]	8.8*10^4	0.4
Ketones									
2-Butanone	599	576	6.1*10^7 [a]	1.3*10^7	4.3*10^7 [a]	5.5*10^6	3.5*10^7 [a]	4.5*10^6	0.16
Acetoin	738	744	1.1*10^7 [a]	4.3*10^6	1.0*10^7 [a]	5.2*10^6	7.9*10^6 [a]	2.5*10^6	0.87
2-Heptanone	891	892	1.9*10^6 [a]	1.1*10^5	1.7*10^6 [a]	1.3*10^5	2.1*10^6 [a]	3.1*10^5	0.42
2-Nonanone	1094	1092	2.8*10^5 [a]	3.4*10^4	2.5*10^5 [a]	2.4*10^4	2.9*10^5 [a]	4.6*10^4	0.79
Lactones									
δ-Decalactone	1504	1504	1.5*10^4 [a]	8.0*10^3	2.5*10^4 [a]	4.6*10^3	3.5*10^4 [a]	5.8*10^3	0.16
Sulfur compounds									
Dimethyl disulfide	754	756	1.3*10^5 [a]	3.5*10^4	6.7*10^4 [a]	2.1*10^4	9.2*10^4 [a]	1.2*10^4	0.28
Carbon disulfide	536	530	1.5*10^5 [a]	1.2*10^4	1.3*10^5 [a]	2.1*10^3	1.1*10^5 [a]	8.5*10^3	0.06
Dimethyl sulfide	497	515	0 [b]	0.	5.4*10^4 [a]	1.2*10^4	0 [b]	0.	0.002
Dimethyl trisulfide	983	980	3.6*10^4 [a]	1.3*10^4	1.4*10^4 [a]	5.8*10^3	1.8*10^4 [a]	4.1*10^3	0.23
Terpenes									
D-Limonene	1033	1037	3.0*10^4 [a]	2.6*10^3	3.2*10^4 [a]	7.3*10^2	3.0*10^4 [a]	2.6*10^3	0.6
α-Pinene	940	941	8.4*10^3 [a]	4.5*10^3	9.9*10^3 [a]	5.0*10^3	6.4*10^3 [a]	3.3*10^3	0.85

Volatile compounds with $P < 0.05$ signficiantly differ between each treatment. Data presented are the means from 3 replicate trials.

[1]GRA = Grass only, CLO = Grass with clover, TMR = total mixed ration, SE = standard error.

Highlights

- Milk of indoor-fed cows led to higher levels of citrate in cheese.
- Milk of pasture-based cows led to higher levels of toluene in cheese.
- Feeding white clover to cows increased dimethyl sulfide levels in cheese.