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Comparison of lipid profiles in the faeces of beef cattle fed three common temperate grass silage diets and their relevance to dietary composition

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ABSTRACT. Faecal lipidome signatures may vary depending on diet. Analyzing 17 different lipidome compounds and calculating ratios between them, we analysed the composition of faecal lipidomes (fatty alcohols, stanols, and archaeol) of beef cattle fed different diets. In this study, we measured the faecal lipidome profiles of beef cattle fed three types of grass silage representative of United Kingdom grasslands by gas chromatography-mass spectrometry. The forage consisted of 1) permanent pasture (sown perennial ryegrass mixed with unsown species); 2) reseeded perennial ryegrass monoculture; and 3) reseeded mixture of perennial ryegrass and white clover (ca 80:20 fresh weight). The contents of three forages varied significantly in water-soluble carbohydrates, acid detergent fibre, neutral detergent fibre, modified acid detergent fibre, crude protein, metabolizable energy, and crude ash. Diet significantly affected the composition of the faecal lipidome. Apart from stigmasterol, sex and the diet-by-sex interaction did not affect the faecal lipidome. Further, the ratios of lipidome compounds in faeces were validated as biomarkers of diet composition. The 24-ethyl coprostanol, 5-stigmastanol, campesterol, and even chain fatty alcohols such as C18-OH (3-hydroxy stearoyl carnitine), C22-OH – alcohol fraction with 22 carbon residues, C24-OH – alcohol fraction with 24 carbon residues, C26-OH – alcohol fraction with 26 carbon residues, and various lipidome ratios differed significantly between diets on a univariate basis. Based on an analysis of the composition and ratios of faecal lipidomes, this study provides a means for predicting the diet composition of agricultural livestock and wild herbivores.

Introduction

Faecal lipidome profiling has been proposed as a biomarker for various parameters that are difficult to measure (e.g., diet composition and feeding pattern of grassland animals) (Lamichhane et al., 2018). Lipidomics, a distinct branch of metabolomics, enables the study of lipid metabolism in the gastrointestinal tract and microbiome and their interactions (Bull et al., 2002; Gill et al., 2011). Presently available techniques, e.g., gas chromatography-mass spectrometry (GC-MS), offers a comprehensive perception of the configuration and activity of particular lipidome components

(Derrien et al., 2011; Ferreira et al., 2012). Herbivore faeces comprise lipids from distinctive classes that are derived from host and microbial metabolism and these lipids can potentially interact; hence, the concentration may change according to diet, animal, and environment (Lin et al., 2009; 2012; Heublein et al., 2017). Understanding the relationship between diet composition and faecal lipidome not only offers an improved understanding of lipid metabolism but also has significant implications for the identification of novel biomarkers for parameters that are difficult to measure; e.g., diet preferences and feeding habits of grassland livestock and wild (Ali et al., 2004; Gomez et al., 2015; Lopez et al., 2015). Researchers have investigated faecal lipidomes for identifying dietary composition, diet selection, and species identification based on sterols, stanols, bile acids, archaeol, and long-chain fatty acids (Bell et al., 2016; Prost et al., 2017; Harrault et al., 2019).

The animal's diet, host, and microbiome metabolism and their interactions predominantly control the bovine faecal steroid profile (Lopez et al., 2015). Dietary influence on faecal lipidome composition may be based on dietary intake (primary sterol consumption) or based on animals' capability to synthesise sterols (derived sterols). In the rumen, the ruminal microbes hydrogenate sterols into stanols (Bull et al., 2002). The sterols and 5α-stanols are naturally found in the environment while, 5β-stanols, 5β-stanones and epi-5β-stanols are direct indicators of animal excreta (Derrien et al., 2011). Coprostanol and epicoprostanol, 5β-stanols derived from cholesterol, are found in excessive amounts in the excreta of omnivores, whereas, β-sitosterol or phytosterol (sterol of plant origin)-derived 5β-stanols (24-ethylcoprostanol and 24-ethylepicoprostanol) are present in excessive amounts in herbivore faeces (Harrault et al., 2019). The C_{29} sterols, mainly stigmasterol and sitosterol dominated the faecal lipidome since they are common compounds in plant lipid membranes and waxes (Heublein et al., 2017).

Ruminants ferment fibrous diets in the rumen to produce volatile fatty acids, H_2, and CO_2 and methanogenesis removes H_2 from the rumen and thus acts as an 'H_2 sink' (Gill et al., 2011; Białek and Czauderna, 2021). Dietary supplementation of cholesterol also affects faecal cholesterol levels (Marounek et al., 2012). For instance, hydrophobic amidated pectins drastically changed the homeostasis of cholesterol in rats (Marounek et al., 2010). The stool level of cholesterol considerably increased with pectinamide, either at a lower or greater degree of replacement, from 18.5 and 17.3 µmol/g in controls to 31.8 and 28.0 µmol/g, respectively. Accordingly, coprostanol concentrations dropped, pointing to hydrophobic amidated pectins as clinically useful hypocholesterolemic agents (Marounek et al., 2007). Archaeol (2,3 diphthanyl-O-sn glycerol) is the unique lipid present in the archaeal membrane and most of the methanogens in the rumen belong to the domain *Archaea*, making it the exclusive source of faecal archaeol (Gill et al., 2011). Faecal fatty alcohols, which are plant epicuticular wax compounds are also derived from the diet (Heublein et al., 2017). The content and distribution of the faecal lipidome (fatty alcohols, sterols, stanols, stanones, and archaeol) from different animals vary due to changes in the diet composition (Lin et al., 2012). An increase in the fibre content of the diet (ADF) increases the proportion of these sterols in the faeces (Derrien et al., 2011). Notably, the biohydrogenation products of these sterols, 5β-C_{29} stanols differentiate between the different perennial ryegrass cultivars (Heublein et al., 2017).

Dietary composition is also used as a proxy to assess enteric methane emissions from ruminant livestock at a farm level and under practical conditions (Meo-Filho et al., 2023). Moreover, faecal analysis is specifically convenient for the identification of biomarkers, as it can be collected with a less invasive procedure than other sampling techniques (Bull et al., 2002). Some faecal lipid compounds are not derived from the diet but released through microbial degradation; e.g., archaeol is a unique membrane lipid component of methanogenic archaea. Thus, faecal lipidome profiling enables comprehension of complex host and microbiome metabolism in addition to their interactions (Gill et al., 2011). The use of multiple faecal lipidome compounds or their ratios predicts dietary composition and species specificity as individual lipidome compounds define individual origins of deviation in dietary composition (Gill et al., 2011). Therefore, each compound in lipidome ratios corrects for insufficiencies compared to using individual lipidome biomarkers (Heublein et al., 2017). This indicates that identifying distinct faecal lipidome compounds in a single assay could contribute to the identification of faecal steroid profiles that could serve as diet composition markers (Lin et al., 2009).

In UK lowland temperate grassland systems, the most common pastures are 1) permanent pasture – defined as grassland which has not been reseeded for at least 10 years and is a mixture of sown and unsown species; 2) monoculture re-sown grassland – mainly consisting of perennial ryegrass (*Lolium perenne*) and *Agrostis stolonifera* species, e.g., high sugar grasses, and 3) perennial ryegrass and white clover (*Trifolium repens*) mixtures typically sown to

reduce inorganic N inputs (Pilgrim et al., 2010; Lee et al., 2001; Wu et al., 2022). Previous studies have tested the relationship between faecal lipidome and diet composition, predominantly faecal fatty alcohols and acids on contrasting diets (Lin et al., 2009; Ferreira et al., 2012).

Since the faecal steroid profile changes with diet and gut microbial activity, abundance, and diversity, steroid ratios (rather than absolute individual steroid concentration) enhance biomarker efficiency (Bull et al., 2002; Derrien et al., 2011; Harrault et al., 2019). Studies have also attempted to evaluate the faecal microbiome signature as a dietary marker (Maixner, 2019; Reese et al., 2019).

There is, however, no detailed investigation of the effect of dietary composition on the composition of faecal lipidome and its ratio of it in cattle-fed temperate grassland diets. We hypothesized that faecal lipidome profiling in beef cattle could differentiate between the three most common temperate grassland diets. The aim of the current study was to identify faecal lipidome signatures using faeces lipidome components and their ratios. Additionally, a study was conducted to determine the relationship between diet type and diet quality, which was determined by the nutrients in the forage.

Material and methods

Animals, experimental design, and diet

All experimental techniques performed were approved by the Animal Welfare and Ethical Review Board of Rothamsted Research under the auspices of the Animals (Scientific Procedures) Act 1986, and its amendments of 2013 as directed in project licence P592D2677 issued by the Home Office.

Ten Charolais × Hereford-Friesian beef cattle (average live weight 337 ± 62.2 kg; average age 9.3 ± 0.1 months) were selected from each of the three forage systems (n = 10, five males and five females in each farming system) at the North Wyke Farm Platform (NWFP) located at Devon, UK to obtain three groups similar in sex composition (Takahashi et al., 2018). The NWFP comprises three different sward types each occupying about 21 ha under the following pasture management plans: 1) permanent pasture (PP), of predominately *Agrostis stolonifera* L. and *Lolium perenne* L. (unknown variety); 2) reseeded grassland with a high-sugar perennial ryegrass cv. AberMagic; perennial ryegrass (PRG), and 3) reseeded pasture consisting of a mix of perennial ryegrass (cv. AberMagic) and white clover (cv. AberHerald) (PRG-WC). The botanical composition of the three swards was as follows: PP was dominated by *A. stolonifera* L. (50.4%) and *L. perenne* L. (33.2%) and comprised other minor species such as *Holcus lanatus*, *Trifolium repens, Taraxacum officinale, Alopecurus geniculatus, Rumex obtusifolius,* and *Poa trivialis*, accounting for less than 17% of the covered soil; in PRG the major species were *L. perenne* (73.4%), *Alopecurus geniculatus* (<4%), and *P. annua* (<4%), with other minor species such as *Ranunculus repens*, *A. stolonifera*, *H. lanatus, Poa trivialis* and *T. repens* making up the rest of the sward; in PRG-WC the major species were *L. perenne* (58.8%) and *T. repens* (14.7%), and comprised other minor species such as *A. stolonifera* (<4%), *H. lanatus* (<4%), *P. annua* (<4%), *R. repens* (<4%) and *A. geniculatus* (<4%). The first cut silage from these swards was fed during the winter housing period. A small amount of concentrate was also fed (approximately 1 kg/animal/day) during the winter housing period as a supplement in the GreenFeed emissions monitoring system (Meo-Filho et al., 2023).

Forage analysis

From housing (early November 2017) until faeces sampling time (mid-January 2018), forage samples were collected randomly from each farmlet at weekly intervals (1 kg fresh weight (FW); bulked) as fed. The samples collected from November 2017 until faeces sampling (n = 7) were freeze-dried, ground, and stored at −20 °C until analysis. Fibre, water-soluble carbohydrates (WSC), and total N (as an estimator of crude protein (CP)) were subsequently measured. In short, the various fractions of fibre composition were measured using an Auto Fiber Analysis System (Fibertec 8000, FOSS, Cheshire, United Kingdom). Modified acid-detergent fibre (MADF) enables concurrent estimation of acid-detergent lignin and thus there is a better correlation between MADF and digestible dry matter or digestible organic matter (Devendra and Lewis, 1973). MADF is the most commonly used procedure for assessing silage digestibility in the UK (Givens et al., 1995). Metabolizable energy (ME) was estimated from MADF fractions using UK-specific equations for silages: ME (MJ/kg DM) = 15.0 − 0.0140 (MADF) (Alderman and Cottrill, 1996). Dry matter (DM) was assessed as the difference between fresh weight before and dry weight after freeze-drying. Crude ash was determined as the remaining mineral material following the furnacing of plant matter at 400 °C. An elemental analyser and isotope ratio mass spectrometer was used to measure the total N contents of the feed and CP was estimated by applying a standard conversion coefficient of 6.25.

Forage WSC was extracted by adding 20 ml of milli-Q water (MerckMillipore, Watford, United Kingdom) to 200 mg of a given sample and shaking for an hour at room temperature. Next, extracts were filtered and stored at −20 °C until being analysed using an Agilent 1260 Infinity HPLC system (Agilent Technologies LDA Limited, Cheshire, United Kingdom) consisting of a quaternary pump, degasser, autosampler, heated column oven, and evaporative light scattering detector (ELSD). The instrument was controlled and analysed using Agilent Openlab software and the ELSD settings were: evaporator = 90 °C; nebuliser = 50 °C; Flow rate = 1.1 SLM (standard l per min).

Faecal lipidome profiling

Faecal samples were collected from 5 males and 5 females in each farming system (n = 10 per farmlet) 73 days after housing. Around 100 g fresh weight of faecal matter was collected from each animal by anal faecal grab sampling and subsequently freeze-dried and milled to a 0.8 mm sieve size for lipidome profiling using GC-MS (McCartney et al., 2013).

An altered monophasic extraction technique was employed to extract total lipid extract (TLE) (McCartney et al., 2013). The solvents used were of HPLC grade (Fisher Scientific, Loughborough, United Kingdom)) and aqueous solutions were prepared with Milli-Q water, pre-extracted 3 times with dichloromethane (DCM). To prepare buffered water, potassium dihydrogen phosphate solution (0.05 M) was used. The pH was adjusted to 7.2 with sodium hydroxide pellets and extracted with 3 × 50 ml of DCM. Buffered water, methanol, and chloroform in a ratio of 4:10:5 were used to prepare the monophasic solvent. Before extraction, an internal standard (100 μg 2-hexadecanol, Sigma Aldrich, Gillingham, United Kingdom)) was added to 300 mg of the faecal sample, 2 ml of monophasic solvent was subsequently added to the faeces and ultrasonicated for 15 min. The mix was then centrifuged for 5 min at 1268 *g*. This extraction procedure was done 3 additional times. Next, 2 ml of chloroform and 2 ml of buffered water were added to the extract, mixed, and centrifuged for 5 min at 1268 *g*. The aqueous phase was then separated with 2 ml of chloroform twice, then mixed and evaporated under a moderate stream of N_2.

Acid methanolysis was carried out for the elimination of polar head groups. For this, 5% hydrochloric acid in methanol (5 ml) was added to the TLE. The mix was heated in a securely closed boiling tube at 100 °C for 3 h. 2 ml of Milli-Q water and 2 ml of chloroform was then added, and mixed and the organic and aqueous layers were separated. The bottom organic layer was taken and washed using chloroform twice. The mixed contents were evaporated using a moderate stream of N_2. To acquire the alcohol fraction, the extract was fractionated with an activated silica column (0.5 g silica gel 60 Å particle size, activated at 125 °C, Agilent Technologies LDA Limited, Cheshire, United Kingdom). To condition the column, 2 ml DCM was used. The sample was transferred to the column in 1 ml of DCM. Next, the apolar fraction was eluted using 4 ml DCM and the alcohol fraction with 5 ml of DCM: methanol (1:1). The solvent was evaporated with a moderate stream of N_2.

The alcohol fraction was reconstituted in 400 μl DCM and 100 μl was transferred to a vial evaporated and derivatised by the addition of 50 μl of N, O-bis (trimethylsilyl) trifluoroacetamide, sealed and heated at 70 °C for 1 h. The processed contents were suspended in 100 μl hexane before analysis by GC-MS (Agilent 6890N/5973N GC-MS, Agilent Technologies, Santa Clara, CA, USA) fitted with a capillary column HP-5MS (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies LDA Limited, Cheshire, United Kingdom). The sample (1 μl), was injected using an Agilent 5863 auto-injector (Agilent Technologies LDA Limited, Cheshire, United Kingdom) through a spitless injection port at 300 °C. The oven programme was held at 50 °C for 2 min, raise to 320 °C at 10 °C/min, and hold for 11 min. The helium carrier gas flow was a constant 1 ml/min. The temperature of the transfer line to the MS was at 280 °C and the ion source was at 230 °C. The instrument was monitored using Agilent Chemstation software (Agilent Technologies LDA Limited, Cheshire, United Kingdom). Archaeol was recognised based on its distinctive mass spectrum (significant analytic ions were m/z 130, 278, 284, and 426 and retention time of archaeol standard). The alcohols and sterols were recognised by their characteristic mass spectrum, retention times, and reference to the National Institute of Standards and Technology (NIST, Gaithersburg, United Kingdom) library.

Quantification was undertaken by comparing the target peak area with that of the internal standard peak area. The variation in response was taken into account by creating a standardisation curve prepared by adding 100 ng/μl of internal standard (2-hexadecanol; Mstd) along with 5, 25, 50, 150, and 250 ng/μl of standard compound (1,2-diO-phytanyl-sn-glycerol; Mx). The highest areas of the internal standard (A std) alongside the archaeol standard (Ax) were determined. The ratio of the standard in areas (Ax/Astd) was charted alongside

the ng (Mx/Mstd), and derived the following regression equation – Mx/Mstd = (Ax/Astd*0.7716 + 0.0658). Internal standard (100 µg) was added to each sample; therefore, Mx = (Ax/Astd*0.7716 + 0.0658) × 100. A similar calibration curve to that above was used to quantify the sterols/stanols but using 5α-Cholestan-3β-ol to create the curve instead of archaeol: Mx/Mstd = (Ax/Astd*0.9214 + 0.0018). Internal standard (100 µg) was added to each sample; therefore, Mx = (Ax/Astd*0.9214 + 0.0018) × 100. The alcohols were quantified by direct comparison of peak area with the internal standard, as the difference in response factor was negligible (Figure 1).

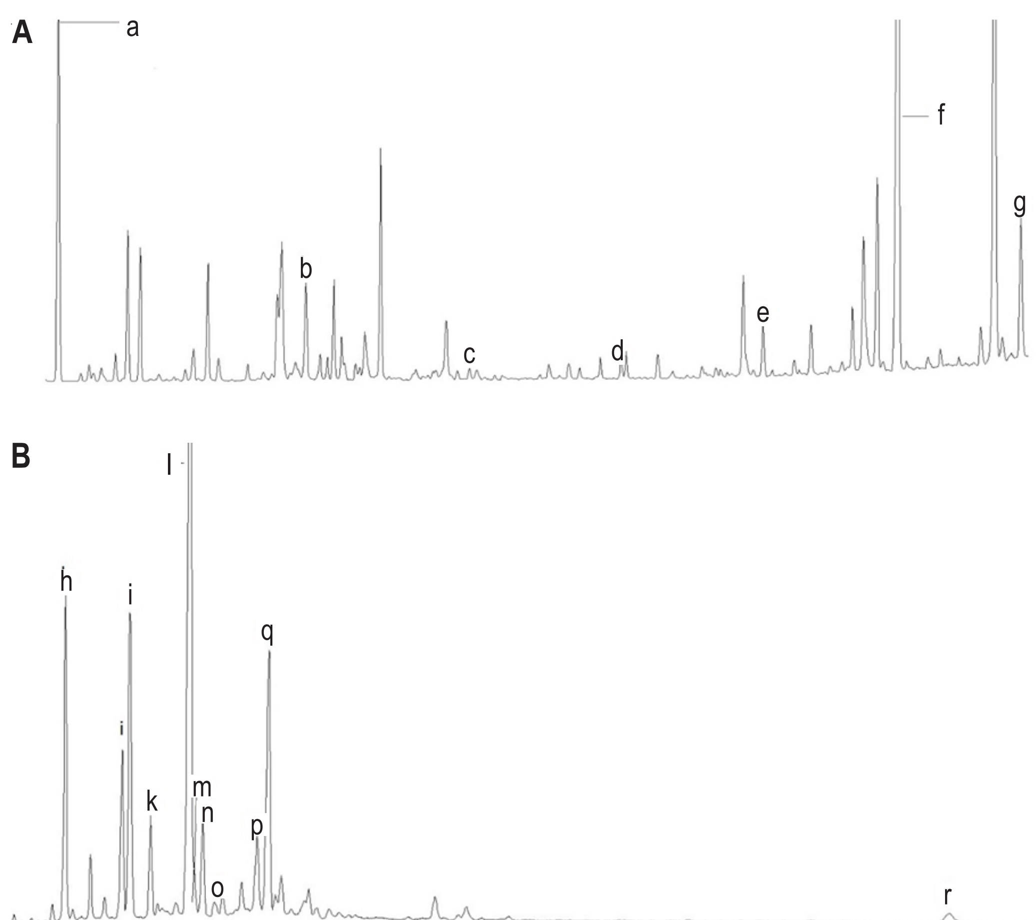

Figure 1. Chromatogram (intensity of the compounds vs time in minutes) displaying the trimethylsilyl alcohol derivatives in bovine faeces (A), and chromatogram illustrating the trimethylsilyl stanol/sterol and archaeol derivatives in bovine faeces (B)

a – 2-hexadecanol (internal standard), b –3-hydroxystearoylcarnitine (C18-OH), c – alcohol fraction with 20 carbon residues (C20-OH), d – alcohol fraction with 22 carbon residues (C22-OH), e – alcohol fraction with 24 carbon residues (C24-OH), f – alcohol fraction with 26 carbon residues (C26-OH), and g – alcohol fraction with 28 carbon residues (C28-OH), h – coprostanol/epicoprostanol, i – cholesterol, j – 5β-campestanol/5α-cholestenol, k – 24-ethyl-coprostanol, l – 5β-stigmastanol/5β-epistigmastanol, m – campesterol, n – campestanol, o – stigmasterol, p – β-sitosterol, q – 5α-stigmastanol, r – archaeol

Statistical analysis

The objective was to identify faecal biomarkers that demonstrate the dietary differences associated with metabolic changes between diet and sex groups. Univariate analyses of the differences in a range of silage nutritional composition variables between the three diets (PP, PRG, PRG-WC) were obtained through ANOVA of the data collected for samples on seven weekly occasions, allowing also for differences between the seven occasions as a blocking factor. Similarly, univariate analyses of the differences in faecal lipidome compound variables (metabolites) and ratios of these variables due to the different diets, between male and female animals, and the interaction between diet and sex, were obtained through ANOVA of the data collected from the 10 animals in each diet regimen, considering the animals to provide replication of the different diet-sex combinations. Correlation analyses were done using Pearson parametric correlation test.

For both sets of variables (silage nutritional composition, faecal lipidome compounds) residual plots did not show any major deviations from the underlying assumptions of homogeneity of variance and normality and so transformations were not required before analysis. All analyses were performed in R v3.3.2, using the base function *aov* and associated functions for ANOVA (R Core Team., 2017).

Results

Diet quality

The correlation between different nutrient components is given in Table 1. The correlation was significant between Ash and CP, MADF, ADF and CP, ADF and ME and ME and CP. There was a positive correlation between DM and ME, DM and CP, MADF and ADF and ME and CP.

Table 1. Correlation matrix showing the correlation between different nutrient components in the forage

	DM	Ash	MADF	ADF	ME	CP
DM		−0.08	−0.22	−0.17	0.22	0.08
Ash	0.72		0.17	0.13	−0.17	0.59
MADF	0.29	0.42		0.54	−1	−0.64
ADF	0.42	0.53	0.001		−0.54	−0.32
ME	0.29	0.42	0.001	0.001		0.64
CP	0.71	0.002	0.001	0.12	0.001	

DM – dry matter, MADF – modified acid detergent fibre, ADF – acid detergent fibre, ME – metabolizable energy, CP – crude protein; correlation coefficients are shown above the diagonal and corresponding *P*-values are given below the diagonal

The nutrient composition of silage (averaged from housing to faecal sampling) and concentrate are presented in Table 2. The WSC content was higher in PRG-WC than in the other two pasture types, and ME was only higher in PRG-WC than in PP ($P < 0.05$; Table 2). The CP content was lower in PRG-WC than in the other pastures, whereas NDF, ADF, MADF, CP, and DM were higher in PP ($P < 0.05$; Table 2).

Faecal lipidome

A chromatogram showing different faecal lipidome compounds is shown in Figure 1. A total of 17 faecal lipidome compounds were identified. The correlation plot showing the correlation between different faecal lipidome components is shown in Figure 2. In general, a strong, positive and significant correlation was obtained between fatty alcohols and sterols.

Table 2. Mean chemical composition of the silage and concentrated feed fed to the animals before collecting faecal samples

Composition	PP	PRG	PRG-WC	SED	*P*-value
DM, %	28.27	27.19	28.23	0.688	0.248
Crude ash, % of DM	9.09	9.86	9.09	0.384	0.159
WSC, % of DM	2.49[a]	1.83[a]	7.36[b]	0.312	<0.001
NDF, % of DM	45.81[a]	42.42[a]	40.82[b]	0.879	<0.001
MADF, % of DM	30.31[a]	29.05[a]	28.41[b]	0.428	0.003
ADF, % of DM	27.19[a]	25.91[a]	25.41[b]	0.575	0.025
ME, MJ/kg DM	10.756[a]	10.934[b]	11.023[b]	0.06	0.003
CP, % of DM	14.771[a]	14.327[a]	11.306[b]	0.222	<0.001

seven silage samples were tested per pasture type; pasture types: 1) permanent pasture (PP), predominately perennial ryegrass of unknown cultivar, 2) reseeded grassland consisting of a high-sugar perennial ryegrass cv. AberMagic (perennial ryegrass, PRG), 3) reseeded pasture with a mix of perennial ryegrass (cv. AberMagic) as well as white clover (grass and white clover, PRG-WC), n = 21, residual df = 12; nutritional components: DM – dry matter, WSC – water-soluble carbohydrate, NDF – neutral detergent fibre, MADF – modified acid detergent fibre, ADF – acid detergent fibre, ME – metabolizable energy, CP – crude protein; the nutrient composition of the concentrate given was DM (%) – 88.2, crude ash (% of DM) – 8.91, CP (% of DM) – 18.4, oil (% of DM) – 5.21, crude fibre (% of DM) – 14.5, ME (MJ/kg DM) – 12.7 and NDF (% of DM) – 42.3; the feed manufacturer provided concentrate composition; the SED – denotes standard errors of difference of means; [ab] – different superscripts denote significant differences between treatments

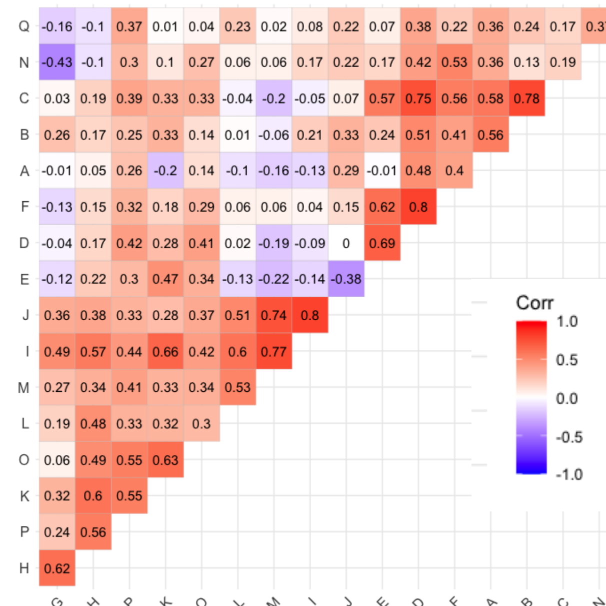

Figure 2. Correlation plot showing the correlation between different lipidome components

A – 3-hydroxystearoylcarnitine (C18-OH), B – alcohol fraction with 20 carbon residues (C20-OH), C – alcohol fraction with 22 carbon residues (C22-OH), D – alcohol fraction with 24 carbon residues (C24-OH), E – alcohol fraction with 26 carbon residues (C26-OH), F – alcohol fraction with 28 carbon residues (C28-OH), G – 5β-campastanol, H – 24-ethyl coprostanol, I – 5β-stigmastanol, J – coprostanol, K – campastanol, L – campesterol, M – 5α-stigmastanol, N – cholesterol, O – stigmasterol, P – β-sitosterol, Q – archaeol

There was a significant negative correlation between C26-OH and campestanol and between coprostanol and cholesterol (Figure 2). The correlation of archaeol with that of cholesterol and coprostanol was negative. A dietary influence was observed for faecal lipidome compounds C18-OH, C22-OH, C24-OH, C26-OH, 24-ethyl coprostanol, 5β-stigmastanol, campesterol, and various lipidome ratios ($P < 0.05$; Table 3). Nine lipidome compo-und ratios (R1 – coprostanol: 5β-stigmastanol; R2 – archaeol: archaeol + 5β-stigmastanol; R3 – 24-ethyl coprostanol: coprostanol; R4 – archaeol: archaeol + 5β-stigmastanol + 24-ethyl coprostanol; R5 – archaeol: archaeol + 5β-stigmastanol + 24-ethyl coprostanol + campesterol; R6 – archaeol: 5β-stigmastanol; R7 – 5β-stigmastanol: 24-ethyl coprostanol; R8 – coprostanol: (coprostanol + 5β-stigmastanol) × 100; R9 – archaeol: 5α-stigmastanol + 5β-stigmastanol) differed between diets ($P < 0.05$; Table 3). Except for stigmasterol, the influence of sex and diet × sex interaction on faecal lipidome and their ratios were not significant (Table 3).

Table 3. Composition and ratio of faecal lipidome components according to forage type and sex of the cattle

Compound, mg/g DM	PRG-WC (Mean)	PP (Mean)	PRG (Mean)	SED (Diet)	*P*-value		
					Diet (D)	Sex (S)	D x S
Fatty alcohols							
C18-OH	0.011[a]	0.072[b]	0.070[c]	0.008	<0.01	0.840	0.752
C22-OH	0.023[a]	0.024[a]	0.017[b]	0.003	0.017	0.920	0.934
C24-OH	0.044[a]	0.046[a]	0.035[b]	0.005	0.035	0.361	0.712
C26-OH	1.092[a]	2.054[b]	1.178[c]	0.196	<0.01	0.717	0.645
C20-OH	0.011	0.010	0.018	0.001	0.658	0.404	0.601
C28-OH	0.113	0.115	0.110	0.013	0.935	0.434	0.368
Stenols/stanols							
5β-campastanol	0.843	0.846	0.955	0.055	0.085	0.529	0.232
24-ethyl coprostanol	0.273[a]	0.213[b]	0.273[a]	0.019	0.006	0.663	0.362
5β-stigmastanol	2.408[a]	3.016[b]	2.619[c]	0.159	0.003	0.475	0.422
coprostanol	0.713	0.722	0.761	0.067	0.751	0.255	0.909
campastanol	0.068	0.065	0.079	0.009	0.227	0.985	0.445
campesterol	0.265[a]	0.241[b]	0.322	0.024[c]	0.008	0.595	0.075
5α-stigmastanol	0.836	0.870	0.726	0.097	0.317	0.782	0.054
Sterols							
cholesterol	0.451	0.495	0.071	0.037	0.524	0.301	0.250
stigmasterol	0.045	0.041	0.040	0.005	0.218	0.035	0.149
β-sitosterol	0.275	0.303	0.253	0.026	0.163	0.915	0.322
Archaeol	0.05	0.043	0.043	0.004	0.163	0.160	0.466
Steroid ratios							
R1	0.303[a]	0.238[b]	0.29[c]	0.023	0.027	0.122	0.809
R2	0.021[a]	0.014[b]	0.016[b]	0.002	0.002	0.577	0.522
R3	0.392[a]	0.3[b]	0.368[c]	0.031	0.019	0.201	0.674
R4	0.0187[a]	0.013[b]	0.015[b]	0.002	0.004	0.555	0.498
R5	0.017[a]	0.012[b]	0.014[b]	0.001	0.005	0.562	0.406
R6	0.022[a]	0.014[b]	0.016[b]	0.002	0.002	0.581	0.523
R7	8.88[a]	14.22[b]	9.61[c]	0.296	<0.001	0.369	0.293
R8	23.06[a]	19.2[b]	22.36[c]	1.399	0.025	0.122	0.786
R9	0.016[a]	0.012[b]	0.013[b]	0.001	0.003	0.496	0.260

pasture types: 1) permanent pasture (PP), predominately perennial ryegrass of unknown cultivar, 2) re-seeded grassland consisting of a high-sugar perennial ryegrass cv. AberMagic (PRG), 3) reseeded pas-ture with a mix of perennial ryegrass (cv. AberMagic) as well as white clover (PRG-WC); R1 – coprostanol: 5β-stigmastanol; R2 – archaeol: archaeol + 5β-stigmastanol; R3 – 24-ethyl coprostanol: coprostanol; R4 – archaeol: archaeol + 5β-stigmastanol + 24-ethyl coprostanol; R5 – archaeol: archaeol + 5β-stigmastanol + 24-ethyl coprostanol+campesterol; R6 – archaeol: 5β-stigmastanol; R7 – 5β-stigmastanol: 24-ethyl co-prostanol; R8 – coprostanol: (coprostanol + 5β-stigmastanol)*100; R9 – archaeol: 5α-stigmastanol + 5β-stigmastanol; with n = 30, the residual df associated with the SED is 18; *P*-values associated with diet, sex, and interaction effects have df values of 27, 28, and 24, respectively; a total of ten animals per diet regimen were used to collect data on faecal lipidomes; the SED denotes standard errors of difference of means; DM – dry matter [abc] – means within a row with different superscripts are significantly different at $P < 0.05$

Discussion

This study is the first of its kind to develop and describe a faecal lipidome data set on beef cattle fed typical temperate perennial ryegrass and ryegrass and clover mixed silage diets. Faecal fatty alcohols, stanols, and nine faecal steroid ratios were identified as diet composition markers within these diets. The results suggest that subtle dietary composition differences could be predicted from multiple faecal lipidome markers and their ratios, where lipidome components or their combinations explain fermentation and lipid metabolism pathways and their interactions (Bull et al., 2002). The combinations of two or more steroids or their ratios reflected better dietary composition differences since individual steroids delineated independent sources of deviation in dietary composition and one steroid could correct for insufficiencies in the other (s) (Derrien et al., 2011).

Identification of dietary composition in grassland as well as in wild animals is of considerable interest in pasture management and wild herbivore conservation (Lopez et al., 2015). Faecal lipidome compounds and their ratios are increasingly used as biomarkers to identify diet composition in domestic and wild animals. Altered faecal lipidome reflects the gross outcome of nutrient intake, digestion, and absorption by both the gut microbiome and the animal gastrointestinal tract, providing means to study animal-microbiota interactions (Suchecka et al., 2017). Faecal lipidome profile also shows changes in lipid metabolism, a hallmark characteristic of diet-induced metabolic alterations and hence there is considerable interest in its use as a biomarker to predict dietary composition (Ahvenjarvi et al., 2004).

In the present study, PP had the highest NDF content. The PRG-WC contained the highest level of WSC and lowest levels of ADF, NDF and MADF. Both traditional and improved varieties of perennial ryegrass are the most common grass in the temperate grasslands and these varieties primarily differ in their WSC content wherein the differences in intake and fermentation properties of various ryegrass varieties are attributed to the differences in WSC content (Rivero et al., 2019a). These variations in NDF, ADF, MADF, WSC, CP, and ME between the three diets may have led to differential faecal lipidome fingerprints (Ferreira et al., 2012). Hence, some fatty alcohols and steroid ratios differed between PRG vs PRG-WC. Although all diets were based on perennial ryegrass, PP was an unknown cultivar from leys at least 20 years old, whereas, for both PRG-WC and PRG, they were based on the same AberMagic sown cultivar, sown within the last 5 years (Rivero et al., 2019b).

Specifically, differences in WSC content in different varieties may have led to variations in palatability, which may lead to differences in intake (Rivero et al., 2019a). Another mechanism is differences in ruminal pH caused by differential WSC content in the diet that may alter rumen fermentation subsequently leading to changes in the faecal lipidome profile (Rivero et al., 2021a). Grasses with higher WSC may contain less fibre as the increased WSC content has to come at the expense of either protein or fibre fractions (Rivero et al., 2019b). Changes in fibre fractions (NDF and ADF) may also lead to changes in fermentation since fibrolytic bacteria mainly digest these fractions (Taweel et al., 2005). During fermentation, easily digestible soluble carbohydrates are digested in the first phase while fibre digestion takes place in the second phase. The delay between the first and the second phase (lag time) varies with the forage fibre content and WSC composition (Lee et al., 2003; 2021).

In ruminants, microbes synthesise fatty acids in the gut and endogenous fatty acids are synthesised in the gut from denuded epithelial cells (Lee et al., 2003). The present study demonstrates that faecal lipidome profiling gives an overview of the net loss and gain of dietary fatty acids in the rumen. A previous study at the NWFP showed that there is no significant change in the fatty acid composition of forages between farmlets (PP, PRG, or PRG-WC) or sampling periods (McAuliffe et al., 2018). Accordingly, using absolute quantities of a single faecal steroid as a diet quality marker may be questionable due to the low concentrations of candidate faecal lipidome biomarkers in the faeces (Davies et al., 2022).

Also, faecal steroids may undergo degradation during the storage and processing of faeces, thus illustrating varied dependencies of individual faecal steroids as diet quality markers (Derrien et al., 2011). To overcome this, ratios of different steroids or combinations of various steroid species are used as proxies instead of the absolute concentration of steroids Lopez et al., 2015). Compared to a single faecal lipidome compound, ratios and combinations could correct deficiencies and explain variations due to dietary changes resulting from rumen fermentation changes (Harrault et al., 2019). In the present study, nine ratios showed significant variations between dietary clusters for PP vs PRG or PRG-WC.

Additionally, diet quality affects microbiome turnover in the digestive tract by modifying the

passage speed of contents through the gastrointestinal tract and therefore also affects the length of interaction time of microorganisms with digesta (Goopy et al., 2014). Generally, grass silage has lower concentrations of DM, nitrogen, and total fatty acids and higher concentrations of fibre and ether extract than clover silages (Rivero et al., 2019a). However, rumen fermentation features were alike for grass, clover, and grass and clover mixed diets except for n-butyric acid levels, suggesting the possibility for changes in the faecal lipidome in animals fed clover silages (Kara, 2021).

Moreover, the absorption coefficient of fatty acids in the ruminant gut is different for different diets; e.g., absorption coefficients of some fatty acids are lower on clover silage diets compared to grass silage which is due to the differential duodenal flow rate of ingesta or due to action of saponins in white clover that reduces intestinal absorption of dietary fat (Taweel et al., 2005). Dietary variations in rumen fermentation are mediated through the dynamic activity in volatile fatty acid synthesis, carbohydrate metabolism, and methanogenesis. Rumen fermentation is a dynamic activity, which is caused by interspecies electron transfer among methanogens and other rumen microbes, and hence, archaea and other microbes form microbial structures that function as an organ in the rumen (Rozbicka-Wieczorek et al., 2016; Białek and Czauderna M., 2021; Rivero et al., 2021a).

At the host level, endocrine cells in the digest-ive system distinguish gastrointestinal luminal constituents and synthesise hormones, which in turn control host metabolism (O'Callaghan et al., 2016). Similarly, 'molecular cross-talk genes' regulate host and microbiome interaction in the gut and, thus, host metabolic efficiency is a function of host and microbiome interaction (Henderson et al., 2015). Polyphenols in PRG-WC affect carbohydrate fermentation, protein degradation, and lipid metabolism in the rumen modulating biohydrogenation of unsaturated fatty acids and suppressing gram-positive fibrolytic bacteria and ciliate protozoa causing a reduction of volatile fatty acid production (Rivero et al., 2020; Cooke et al., 2022).

Thus, taken together, diet-mediated changes in the gut cause a profound effect on the faecal lipidome (Wilkinson et al., 2020; Rivero et al., 2021b). However, in the present study, the presence of white clover, which may have influenced rumen microbial fermentation, did not differentiate strongly the lipidome to act as a biomarker. Differences in fibre content in the diet may cause faecal lipidome changes in the PP diet over the more digestible high-sugar cultivar AberMagic, which constituted PRG and PRG-WC (Orr et al., 2016; 2019).

Conclusions

This study demonstrated that the lipidome components and their ratios in the faeces of beef cattle fed three distinct temperate grassland forages could serve as biomarkers for the quality of the forage. The substances found in the faeces lipidome and their ratios in this study might be seen as the first step in developing a promising technique to find faecal markers for diet quality in grassland livestock and wild herbivores. Biomarkers that show the feed mix of domesticated and wild herbivores may be developed in the future.

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Conflicts of interest

The Authors declare that there is no conflict of interest.

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