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Relationship between Grazing Lamb Growth Rate and Blood Plasma Analytes as Profiled by Gas Chromatography with Time-of-Flight Mass Spectrometry (GC-TOF/MS).

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There can be considerable variation in the performance of individual lambs grazing on the same pasture. Gas chromatography with time-of-flight mass spectrometry (GC-TOF/MS) was used to profile the relative abundances of metabolites in plasma from growing lambs to determine any correlation effects between plasma metabolites and liveweight gain. Analysis of relative abundance of 336 analyte clusters and liveweight gain revealed that the growth rates of female lambs were significantly positively correlated with 5 analyte clusters and negatively correlated with 5 other analyte clusters. Growth rates of male lambs were likewise significantly positively correlated with 9 analyte clusters and negatively with 5 analyte clusters. Analytes identified as being associated with lamb growth rate included the amino acids valine, methionine, phenylalanine, cystine and asparagine, and oxalic acid, phenylacetic acid, and phosphoric acid. A number of currently unidentified analytes were significantly correlated with growth rate. Stepwise regression of the analytes on lamb growth rate yielded relationships that accounted for 48% and 58% of the variation in female and male lamb growth rates, respectively. This study demonstrated that by using GC-TOF/MS in combination with multivariate statistical techniques it is possible to correlate the presence of specific analytes in sheep plasma with growth rate.

KEYWORDS: Management regime; diet composition; GC-TOF/MS; lamb nutrition; metabolite markers

INTRODUCTION

Previous studies have shown that grazing two or more animal species together can result in improved performance of one or more of the species, leading to a higher total output per unit area (*1*, *2*). In particular, sequential grazing systems involving different animal species grazing an area in succession have shown to improve productivity (*2*,*3*). In the case of combining cattle with sheep, this improvement is thought to be due to cattle and sheep having different preferences for both plant species and plant parts. A recent study directly compared mixed and sequential grazing systems for sheep and cattle in the uplands and found that both resulted in improvements in lamb performance without compromising cattle productivity (*4*). While this study showed the grazing regime had a significant effect on lamb growth rate, considerable variation was detected in the growth rates of individual animals that could not be attributed to differences in the management regime. Compared to animal production in lowland areas, productivity of stock grazing upland swards tends to be low, partly because of the small breed types that are typically used in the uplands, and partly because of the quality of the sward even on improved areas of land (*5*). However, among genetically similar animals (i.e., of the same breed or cross-breed) some individuals apparently thrive and grow well, while others grow relatively poorly, even when given access to the same food resource and allowed the same diet selection opportunities. Slow growth rates and poor quality carcasses lead to a reduction in farmer income, lowering the economic sustainability of lamb production in upland regions. Therefore, it would be of great benefit to understand the factors that cause variation in growth rates within a group of similar animals. Increased plasma concentrations of primary metabolites, e.g., amino acids, could indicate increased food intake, while increased concentrations of plant secondary metabolites may indicate the consumption of inappropriate (e.g., mildly toxic) plants.

We have previously demonstrated the use of gas chromatography with time-of-flight mass spectrometry (GC-TOF/MS) combined with chemometric analytical techniques to discriminate between samples from animals on similar but distinct diets comprising moorland plant species (*6*). We have shown that GC-TOF/MS metabolite profiles of sheep plasma and feces correlate with the consumption of specific plants in the diet, and have demonstrated the validity of this technique to discriminate between similar diets. In the present study, we hypothesized that there are significant differences in the chemical composition of sheep blood plasma, detectable at a single time point during the

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postweaning phase of lamb growth, which are associated with differences in animal growth rate and with management regime. The objectives of the present study, therefore, were to use GC-TOF/MS to profile metabolites in blood plasma collected from lambs at approximately two months postweaning and to use these data to correlate the abundance of specific analyte clusters in the plasma samples with animal performance.

MATERIALS AND METHODS

All procedures involving animals in this experiment were licensed and regulated by the UK Home Office under the Animals (Scientific Procedures) Act of 1986, and were authorized by the Institute of Biological, Environmental and Rural Sciences' Local Ethical Review committee.

Management. The experimental design and sampling procedures for the main experiment have been reported in detail elsewhere (*4*). Briefly, groups of growing lambs were grazed on one of four grazing regime treatments at the Bronydd Mawr Research Station (Trecastle, Brecon, UK). These compared the effect of grazing one or more animal species together or sequentially: (1) sheep only during the grazing season from May to October (S/S); (2) cattle only from May to July followed by sheep only from August to October (C/S); (3) cattle and sheep from May to July, sheep only for the rest of the grazing season (C + S/S); and (4) cattle and sheep for the whole grazing season of May to October (C + S/C + S). Individual plot sizes were 2.0 ha (treatments 3 and 4), 1.0 ha (treatment 2), and 0.5 ha (treatment 1), and each treatment plot was replicated three times (i.e., there were 12 plots in total). From weaning in July until termination of grazing, lambs only were used as they were considered to be more responsive than ewes to changes in sward composition, particularly in white clover content and herbage nutritive value. The numbers of core animals on the individual plots of the four treatments were as follows: S/S, 8 ewes + 11 lambs until end of July; 8 lambs thereafter; C/S, 4 steers until end of July, 16 lambs thereafter; C + S/S, 4 steers and 16 ewes + 22 lambs until end of July, 32 lambs thereafter; and C + S/C + S, 4 steers and 16 ewes + 22 lambs until end of July; 4 steers and 16 lambs thereafter. During the preweaning period, ewes and lambs were allocated to treatment on the basis of lamb liveweight, ensuring roughly equivalent total weight turned out onto each of the plots, and also by gender and litter size, in order to achieve a 50:50 sex ratio and a lamb/ewe ratio of 1.4:1. At weaning, stock were pooled and reallocated to plots and treatments. Core animals remained on their designated replicate plot for the duration of the experimental period, i.e., during the preweaning and postweaning periods. To maintain sward heights at a target sward surface height of 6 cm, a put-and-take stocking system was used, with nonexperimental animals added or removed on a weekly basis as required. Core experimental sheep consisted of Beulah Speckled Face ewes and their Suffolk cross lambs born in mid-March. The mean live weight of the lambs at turnout following weaning was 29.4 kg, 29.4 kg, 30.9 kg, and 29.4 kg on the S/S, C/S, C+S/S, and C+S/C+S treatments, respectively (sed 0.65 kg; not significant (NS)). The corresponding weights at removal from the plots were 37.2 kg, 37.8 kg, 39.0 kg, and 39.4 kg, respectively (sed 0.75 kg; $P < 0.05$). The cattle that had grazed the plots were Charolais cross steers, born in the spring of 2002, with a mean turnout weight of 370 kg.

Samples for the current study were collected during the postweaning grazing period in 2003, the third of the three years that the main grazing experiment ran for. Following weaning, lambs had been allocated to plots on the basis of live weight and sex in order to achieve a similar mean initial live weight. Male lambs had been castrated soon after birth. All lambs were drenched with anthelmintic and dosed with a mineral bolus prior to turnout, and received further drenches of anthelmintic at pasture (*4*). The live weights of the core experimental lambs were recorded every three weeks from turnout postweaning on the 21 July until grazing ceased on 13 October. Blood samples were collected on 15 September by jugular venepuncture. This meant that the lambs had been grazing their treatment swards for 8 weeks during which time pasture availability was not limited by sward growth. It was anticipated that much beyond this time herbage growth rates would start to decline and may have required core animals to be removed from the treatment plots in order to maintain target sward heights (*4*). One sample per animal was collected, with approximately 10 mL of blood collected onto ice into evacuated blood sampling tubes (Vacuette; Greiner Bio-One Ltd., Gloucester, UK) containing sodium fluoride/EDTA K3. Plasma and blood cells were separated by centrifugation at approximately 1700*g* for 20 min at 4 °C, and the resultant plasma was decanted into microcentrifuge tubes before being frozen at −20 °C until analysis.

Although 216 core lambs were used in the postweaning phase of the experiment, it was not possible to collect blood samples from four of these animals. There was also limited analytical capacity on the GC/MS machine, which meant that only approximately two-thirds of the plasma samples could be analyzed, allowing for duplicate analysis of 20% of the samples. Therefore, to achieve a maximum spread of growth rates, lambs were ranked in order of their growth rates over the whole postweaning period, and, irrespective of sex or treatment regime, two out of every three samples were randomly selected for plasma analysis.

During the postweaning period, the lambs on all four treatments grazed on improved permanent pastures that had been reseeded at least 10 years previously. These were dominated by perennial ryegrass (*Lolium perenne*) (50−60% of sward content) with a white clover (*Trifolium repens*) content of between 5−10%. Unsown grasses were mainly bents (*Agrostis* spp.), meadow grasses (*Poa* spp.), crested dogstail (*Alopecurus pratensis*), sweet vernal (*Anthoxanthum odoratum*), and Yorkshire fog (*Holcus lanatus*).

Sample Preparation and Analysis. Thawed blood plasma (200 μL) was extracted using methanol/chloroform, and the polar fraction was prepared by liquid partitioning into water and derivatized to the corresponding methoxime/trimethylsilyl (TMS) derivative (*7*). Twenty percent of the samples were analyzed in duplicate, from extraction to analysis. Gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry (GC/EI-TOF-MS) was performed using an Agilent 6890N24 gas chromatograph with splitless injection connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH) (*7*). Metabolites were quantified using peak apex height after baseline subtraction by ChromaTOF software version 1.00, Pegasus driver 1.61 (LECO Inc., St Joseph, USA) and TagFinder processing (*8*) of at least three mass fragments. Metabolites were identified using NIST08 software (http://www.nist.gov/srd/mslist.htm) and the mass spectral and retention time index (RI) collection of the Golm metabolome database (*9*, *10*). Mass spectral matching was manually checked and accepted with thresholds of match > 650 (maximum 1000) and RI < 1.0% (*11*). Retention indices represented van den Dool indices calculated from additions of internal standards of C12, C15, C19, C22, C32, and C36 *n*-alkanes. Peak abundances of mass fragments occurring as multiple peaks or that had similar properties in the chromatogram were combined to give a total of 392 analyte clusters, some of which were removed as chromatographic noise to give 360 distinct clusters. Peak heights of each of the analyte clusters were normalized to the total ion current to give relative abundance values for each cluster (the total sum of which was 1).

Data Description. Data from 119 individual animal plasma samples were used for statistical analysis. These animals covered the full range of growth rates found across all treatments and each sex. However, as a result of the random nature of sample selection (with respect to lamb sex, treatment, and plot replicates) from all available samples, no plasma samples were analyzed from female lambs on treatment S/S. The average daily increase in weight over the 8-week postweaning period until the blood sample was taken was calculated for each lamb by linear regression. These are shown in the dot-plots in **Figure 1**. Analyte cluster values below the limit of detection were recorded as zero. About 80% of the matrix had nonzero values, and clusters were further investigated when an analyte cluster was present in more than 80% of the samples analyzed, which left 336 analyte clusters for analysis.

Data Analysis. Statistical analyses were carried out using Matlab (Matlab Inc., Cambridge, UK). There are known biological differences between the growth of male and female lambs, and because this could be a reflection of their grazing behavior, separate statistical analyses were carried out for each sex. Lamb growth data were previously analyzed to look for differences between the four treatments or between the plots of land on which they grazed (*4*), and analysis in this study concentrated on the correlation of growth rates with blood composition. Pearson correlation coefficients of the relative abundances of the 336 analyte clusters on lamb growth were calculated and scrutinized carefully in order to discover which, if any, of the analyte clusters were significantly (defined as $P < 0.05$) associated with lamb growth. A subset of analyte clusters,

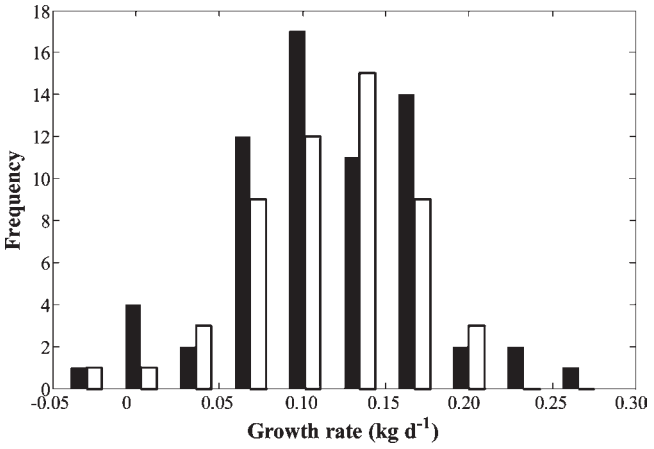

Figure 1. Distribution of male (solid bars) and female (open bars) average daily growth rates over the 8-week postweaning period.

one subset for each sex, was further analyzed by stepwise regression (using the stepwisefit procedure of Matlab) to investigate the amount of variation in lamb growth rate that could be accounted for by the relative abundances of one or more of those analyte clusters significantly correlated with the lamb growth rate. Since the 336 analyte clusters were highly intercorrelated, principal component analysis was used to reduce the dimensions with the aim of identifying important components. Subsets of those analyte clusters identified as being significantly correlated with lamb growth rate were used in further principal component analyses, and cluster analysis was used to produce dendrograms in a search for any groupings. Scores on the first few components of the principal component analysis of clusters significantly correlated with lamb growth were used to explore regression models for predicting lamb growth rates.

RESULTS AND DISCUSSION

In general, a positive association between certain plasma primary metabolites (particularly amino acids) and lamb growth rate would be expected because the intake of nutrient metabolites required for growth, their presence in blood, and growth rates are related (*12*). Some metabolites, such as essential amino acids that are generally regarded as limiting for growth (principally methionine, but also lysine, threonine, histidine, and phenylalanine (*13*)) might be expected to be present in high concentrations in plasma in fast growing lambs. However, by definition, their availability limits growth, and thus, they would be taken out of blood by the growing tissues, and therefore, their plasma concentrations might not be related to the growth rate. However, should an animal consume plants or plant parts containing antinutritive secondary metabolites, they may consume less food, and thus grow less quickly. Results from the main grazing experiment showed that both mixed and sequential grazing of cattle and sheep resulted in improvements in lamb growth performance, without compromising cattle performance (*4*). However, although that study showed that the grazing regime had a significant effect on lamb growth, considerable variation was detected in the growth rates of individual animals grazing together on the same plot, even though they had the same diet selection opportunities and had similar genetic backgrounds, and this was observed with animals on all four management regimes.

While the food source for animals such as carnivores can be characterized as being patchily distributed and highly nutritious, that of a herbivore will be typified by the nutrients being at a comparatively low density (*13*) and being more evenly distributed (*14*). However, despite the relative uniformity in food distribution for the herbivore, variation exists in the type and quantity of nutrients supplied by different plant types and species, and selective feeding by animals such as sheep offers an opportunity to consume a diet that has a higher nutritional value than the average for the vegetation it is feeding upon (*14*−*16*). This generally results in sheep selecting grass species in preference to shrub species and leaf in preference to stem, but considerable between-animal variation has been reported in the contribution of particular plant species to the overall diet (*15*, *16*), which will in turn have implications for nutrient supply.

Table 1. List of Analyte Clusters, Together with Tentative Identities Where Known, Whose Relative Abundances in Blood Plasma Were Significantly Correlated with Lamb Growth ($P < 0.05$)[a]

tentative identity	male lambs	female lambs
adenosine 5′-monophosphate	−	
asparagine	+	
cystine		+
lysine	+	
methionine	+	
phenylalanine	+	
valine	+	
oxalic acid		−
phenylacetic acid	−	
phosphoric acid		−
A115002-101[b]	−	−
A116007-101		+
A226001-101	+	
A257009-101	+	+
A259002-101	+	+
A278004-101	+	+
NA1[c]	−	
NA2		−
NA3	−	
NA4		−

[a] + = positive correlation; − = negative correlation; no symbol = no significant correlation. [b] A-numbers indicate currently unidentified mass fragments present in the Golm mass fragment library (i.e. they have been found before). [c] NA-numbers indicate currently unidentified mass fragments not present in the Golm mass fragment library.

The metabolites present in blood plasma of ruminants originate from dietary origins and from within-animal (rumen and animal tissue) biosynthesis. Many common metabolites such as amino acids, sugars, and organic acids can be synthesized by the grazing animal or its gut microbial population (*17*), or are derived directly from the diet, where they constitute primary plant metabolites. Primary metabolites arise as a consequence of basic metabolism, i.e., the biochemical processes necessary for survival, growth, and reproduction of the plant. Primary metabolites are generally ubiquitous throughout the plant and often present at relatively low concentrations, although there are major exceptions (e.g., storage carbohydrates). Many plants also accumulate secondary metabolites. These are generally the result of more complex biosynthetic pathways that are not present in animals and often offer the plant protection against herbivory, microbial pathogen invasion, invertebrate pests, and environment stresses (*17*). Secondary metabolites frequently accumulate in specific parts and tissues of the plant, often represent the end products of nonreversible biosynthetic pathways, and may accumulate to high concentrations. Flavonoids, terpenes, alkaloids, and condensed and hydrolyzable tannins are common examples of plant secondary metabolites. Many plant secondary metabolites have antinutritional properties, for example, sorghum contains a number of secondary metabolites that depress forage digestibility in mammals (*18*, *19*).

Using Pearson's correlation coefficients, a number of metabolites represented by analyte clusters in the plasma samples were found to be significantly correlated with lamb growth rate (**Table 1**). Plasma concentrations of several amino acids (valine,

methionine, phenylalanine, cystine, and asparagine) were positively associated with lamb growth; however, none of these is necessarily derived from the diet. These are common metabolites that are also biosynthesized in ruminants. The reason why most of the amino acids were positively correlated with male lamb growth but not female lamb growth is unknown. Other plasma analyte clusters that showed an association with animal performance but were not characterized by mass spectral and retention index libraries may be plasma derivatives of compounds biosynthesized in ruminants or plant secondary metabolites not biosynthesized in ruminants. This second group of analyte clusters fell into subgroups: (i) those that were present in the Golm mass spectral library (*10*) but which are not positively identified (e.g., analyte A259002-101) and (b) those that were not present in the library (listed in **Table 1** as NA followed by a number, e.g., NA1).

Principal component analyses, using all 336 analyte clusters, showed that for each sex, many components were needed to account for most of the variation in lamb growth rate. To account for about 85% of the total variation, at least 18 components were needed for female lambs and 22 for male lambs. None of the first few component scores was significantly correlated with lamb growth of either sex. For the female lambs, 5 of the 336 analyte clusters had significant ($P < 0.05$) positive correlations with lamb growth, while 5 analyte clusters had significant negative correlations. For male lambs, 9 analyte clusters were significantly ($P < 0.05$) positively correlated with male lamb growth rate, and 5 were negatively correlated, although not all were the same as those found with the female lambs (**Table 1**). These subsets of analyte clusters were used in a revised principal component analysis. The output now showed that only 4 (female) and 6 (male) components were needed to account for at least 85% of the variation in lamb growth rates, and the first principal component was highly correlated ($P < 0.001$) with lamb growth of both sexes.

Hierarchical cluster analysis of the analyte clusters significantly correlated with lamb growth showed differences between male and female lambs. For female lambs, little discernible clustering was evident (**Figure 2A**), other than phosphoric acid being distinct from other (largely unidentified) analyte clusters. However, there were two major clusters evident for male lambs (**Figure 2B**), with one cluster largely comprising amino acids and the other largely comprising unidentified analyte clusters.

Stepwise regression of female lamb growth rate on those clusters that were significantly correlated with it yielded the following equation ($r^2 = 0.48$):

$$\begin{aligned}\text{Growth rate (kg d}^{-1}) &= 0.085 + 13.07 \\ &\times \text{A259002-101} - 101.22 \\ &\times \text{NA2} + 75.24 \times \text{A115002-101}\end{aligned}$$

Where A2590002-101 and A115002-101 are unidentified analytes present in the Golm database, and NA2 is an unidentified analyte not present in the database. The units for these analyte clusters are relative abundance.

Similarly, the regression equation of male lamb growth rate on significantly correlated analyte clusters was ($r^2 = 0.58$):

$$\begin{aligned}\text{Growth rate (kg d}^{-1}) &= 0.120 + 17.60 \times \text{A259002-101} + 18.34 \\ &\times \text{methionine} - 17.14 \times \text{NA3} - 12.51 \\ &\times \text{adenosine 5}'\text{-monophosphate} - 24.24 \\ &\times \text{A115002-101}\end{aligned}$$

The units for the analyte clusters are relative abundance.

It is noteworthy that although the relative abundances of several amino acids were identified as being positively correlated with male lamb growth, only methionine (an essential amino acid) was significant in the regression equation and also that only two analyte clusters were common to the regression equations of both male and female lambs. As these regression equations stand, they are not particularly useful for predicting lamb growth rates from other data sets because they are based on relative abundances of analyte clusters in the current data set and also because some of the analytes are unidentified. However, they demonstrate the principle that growth rates can be reasonably well predicted by relative concentrations of plasma metabolites and that some analytes positively influence growth rate while others negatively influence growth rate. Further work to characterize currently unidentified analytes would increase the practical value of this type of regression.

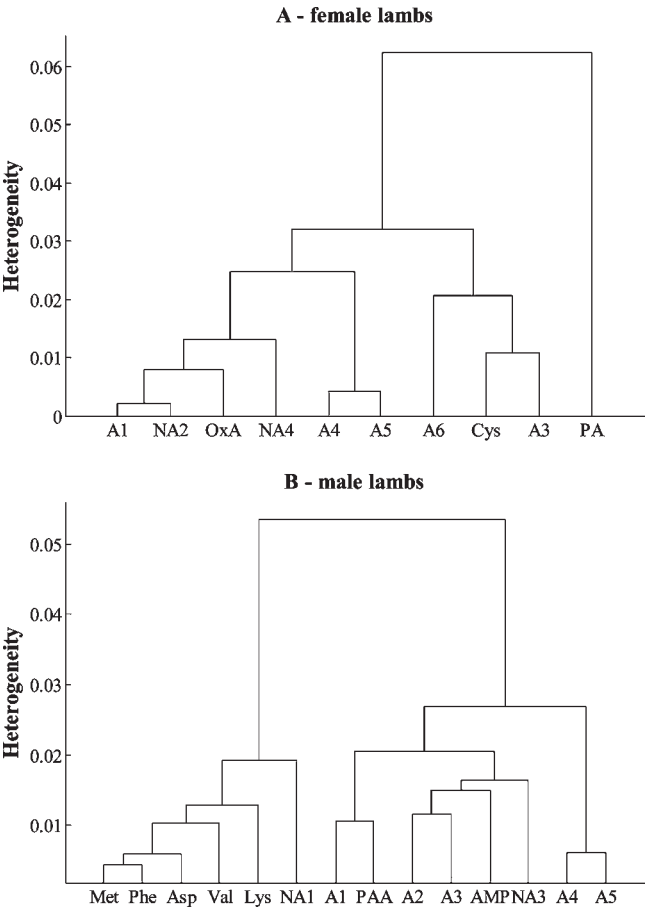

Figure 2. Dendrogram of hierarchical association of analyte clusters with Ward linkage and heterogeneity distance for (**A**) female lambs and (**B**) male lambs. Key: A1−A6 are unidentified analytes present in the Golm database: A1 = A115002-101; A2 = A226001-101; A3 = A257009-101; A4 = A259002-101; A5 = A278004-101; A6 = A116007-101; NA1, NA3, and NA4 are unknown analytes not present in the Golm database; OxA = oxalic acid; PA = phosphoric acid; PAA = phenylacetic acid; AMP = adenosine 5′-monophosphate.

The first 10 principal component scores of significantly correlated analyte clusters were also used in a stepwise regression analysis on lamb growth. Only 2 principal components (components 1 and 3) were statistically significant in the regression equation for female lamb growth, and the amount of variation accounted for by this regression was less than that using the relative abundance values of specific clusters ($r^2 = 0.38$). Likewise, 5 components were significant in the regression equation for male lambs (components 1, 2, 3, 6, and 7), but again, the amount of variation accounted for ($r^2 = 0.54$) was less than that accounted for using specific analyte clusters.

This study was based on a hypothesis that there are significant differences in the chemical composition of sheep blood plasma, detectable at a single time point during the postweaning phase of lamb growth, that are associated with differences in animal growth rate. We have demonstrated that by using GC-TOF/MS combined with multivariate statistical techniques we can correlate the presence of specific analytes in sheep plasma with animal live weight gain. Furthermore, this study demonstrates the potential of metabolomic strategies for the identification of biomarkers related to specific diet components, e.g., the presence of analytes in plasma analyzed by GC-TOF/MS that may be found only in a specific plant species would indicate the consumption of that particular plant. Reasonable regression models for predicting male and female lamb growth were generated by regressing lamb growth on the relative abundance of a small number of analytes. Thus, the study has confirmed the validity of the novel approach to identifying factors that affect growth rates that was adopted. Given the somewhat speculative nature of the work, it was deemed appropriate to limit the associated blood sampling. Further work is now required to expand this approach and identify those currently unidentified analyte clusters related to lamb growth, and to explore variation in analyte concentrations over time. This should include an exploration of diurnal variation in the plasma concentrations of potential markers. Further studies on plasma profiles of ruminants on defined diets would clarify the relationship between animal intake and animal performance. Analyte identification could be achieved using a combination of further GC/MS experimentation, with better separation and clarification of specific analyte peaks, potentially coupled with fractionation and nuclear magnetic resonance experiments.

These findings could potentially be developed to allow the growth rates of animals to be predicted from a simple blood test. Such a tool would be of particular benefit where animals are extensively grazed on poorer quality pastures and could both improve the economic viability of sheep enterprises and safeguard animal welfare.

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