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Effect of selected plant species within biodiverse pasture on *in vitro* fatty acid biohydrogenation and tissue fatty acid composition of lamb

K. E. Kliem[1], A. L. Thomson[1], L. A. Crompton[1], D. I. Givens[2]

[1]Animal Dairy and Food Chain Sciences, School of Agriculture, Policy and Development, University of Reading, Reading, RG6 6AR, United Kingdom.

[2]Institute for Food Nutrition and Health, University of Reading, Reading, RG6 6AR, United Kingdom.

Corresponding author: K. E. Kliem Email: k.e.kliem@reading.ac.uk

Short title: Fatty acid profiles of biodiverse forage species

Abstract

The effect of botanical diversity on supply of polyunsaturated fatty acids (PUFA) to ruminants in vitro, and the fatty acid (FA) composition of muscle in lambs was investigated. Six plant species, commonly grown as part of UK herbal ley mixtures (*Trifolium pratense*, *Lotus corniculatus*, *Achillea millefolium*, *Centaurea nigra*, *Plantago lanceolata* and *Prunella vulgaris*), were assessed for FA profile, and *in vitro* biohydrogenation of constituent PUFA, to estimate intestinal supply of PUFA available for absorption by ruminants. Modelling the *in vitro* data suggested that *L. Corniculatus* and *P. Vulgaris* had the greatest potential to increase 18:3 n-3 supply to ruminants, having the highest amounts escaping *in vitro* biohydrogenation . Biodiverse pastures were established using the six selected species, under-sown in a perennial ryegrass-

based sward. Lambs were grazed (~50 days) on biodiverse or control pastures and the effects on the FA composition of *m. longissimus thoracis* (lean and subcutaneous fat) and *m. semimembranosus* (lean) were determined. Biodiverse pasture increased 18:2 n-6 and 18:3 n-3 contents of *m. semimembranosus* (+14.8 and +7.2 mg/100g tissue respectively) and the subcutaneous fat of *m. l. thoracis* (+158 and +166 mg/100g tissue respectively) relative to feeding a perennial ryegrass pasture. However, there was no effect on total concentrations of saturated FA in the tissues studied. It was concluded that enhancing biodiversity had a positive impact on muscle FA profile reflected by increased levels of total PUFA.

Keywords: Biodiversity; multispecies swards; fatty acids; biohydrogenation; lamb muscle

Implications

The improvement of muscle fatty acid (FA) profile in lambs through increased polyunsaturated fatty acid (PUFA) concentration achieved in the present study adds to the growing body of evidence supporting the replacement of monoculture pasture with biodiverse mixtures. By including a greater proportion of species that were found to promote PUFA supply to ruminant tissues, such as selfheal and birdsfoot trefoil, biodiverse seed mixtures could be formulated to optimise the FA profile of resulting ruminant food products. However, the seemingly low persistence of these species within a competitive mixed sward remains a challenge to commercial uptake.

Introduction

There is increasing interest in low-input biodiverse pasture as a sustainable forage for grass-based ruminant production systems (Luescher *et al.,* 2014). However, to-date, little work has focused on the ability of individual plants within biodiverse pastures to beneficially modify the fatty acid (FA) profile of ruminant food products, with the aim of increasing mono- and polyunsaturated fatty acids (MUFA, PUFA) and decreasing saturated fatty acid (SFA) concentrations (Elgersma, 2015). Certain plant species (Asteraceae, Apiaceae, Rosaceae, Cyperaceae) have been positively correlated with PUFA in milk (Collomb *et al*., 2002). In ruminant muscle, biodiverse systems have been associated with enhanced PUFA concentrations in lambs (Whittington *et al*., 2006; Campidonico *et al*., 2016) and Lourenco *et al*. (2007) reported increased concentrations of docosahexaenoic acid (DHA; a very long chain n-3 PUFA) in the intramuscular fat of lambs grazing a biodiverse pasture compared with an intensive ryegrass pasture. However, few studies have attempted to relate species composition within a biodiverse sward to the FA supplied by the plants and their potential to alter the composition of ruminant food products. Identifying which species have the most potential for improving the resulting FA composition in ruminant products could aid in designing targetted seed mixtures for this purpose. For example, red clover (*Trifolium pratense*) and certain other perennial forage species contain enhanced levels of polyphenol oxidase, which can prevent lipolysis and subsequent rumen biohydrogenation of plant PUFA (Dewhurst *et al*., 2006; Lee *et al.*, 2014). In addition, plants containing condensed tannins may protect PUFA from biohydrogenation, and thus enhance PUFA concentration in ruminant products (Campidonico *et al.*, 2016; Girard *et al.*, 2016a). However, the degree of PUFA protection can be influenced by the concentration, chemical structure, and degree of polymerisation of the condensed tannins, which can vary both within and between plant species (Azuhnwi *et al.*, 2013).

This may explain why certain tannin containing species appear to affect FA profile to a greater extent than others. Girard *et al*. (2016b) observed that sainfoin (*Onobrychis viciifolia*) raised PUFA concentration in cheese to a greater extent than birdsfoot trefoil (*Lotus corniculatus*), with both providing similar amounts of alpha-linolenic acid (18:3 n-3) to the animal. Therefore, the objectives of the present study, were to (i) determine the impact of a range of species on PUFA biohydrogenation *in vitro*, and (ii) determine the impact of increasing pasture botanical biodiversity by including selected plant species, on the FA profile of lamb meat.

Material and methods

Experiment 1: In vitro *biohydrogenation of selected species*

Sample collection. Six "candidate" plant species - birdsfoot trefoil (*Lotus corniculatus*), knapweed (*Centaurea nigra*), ribwort plantain (*Plantago lanceolate*), red clover (*Trifolium pratense*), selfheal (*Prunella vulgaris*) and yarrow (*Achillea millefolium*) - were selected from a larger group of species, due to containing relatively high concentrations of 18:2 n-6 and 18:3 n-3, and being relatively easy to establish (Kliem *et al.,* 2006). The entire above-ground plant material of each plant was collected from one of several already established plots on four separate occasions during the growing season (Kliem *et al*., 2006), mixed well and transported to the laboratory. Samples were stored at -20°C before being lyophilised and milled (<1 mm).

In vitro biohydrogenation. To Wheaton flasks (capacity 125 ml), 1.0 g (+/- 0.01 g) of each freeze-dried and milled sample was accurately weighed in triplicate, and 90 ml of a reduced anaerobic buffer (Theodorou *et al*., 1994) added. Flasks were warmed to 39ºC prior to inoculation with 10 ml strained bovine rumen fluid collected approximately

2 h post-feeding from two lactating dairy cows receiving a total mixed diet comprising 50:50 forage:concentrate (DM basis), with the forage portion being predominantly maize silage. Flasks were loosely stoppered and vented via a needle. Flasks were incubated at 39ºC with regular mixing by agitation of the bottles. Three flasks per plant species were removed following 0, 3, 6, 9, 12, 24 and 48 h incubation, flask contents were frozen at -20ºC and then lyophilised. Lyophilised residue was mixed and stored at -20ºC before subsequent FA analysis.

Fatty acid analysis. FA analysis of the whole plant material prior to *in vitro* biohydrogenation was performed on triplicate sub-samples of freeze-dried plant material, using a method based on Sukhija and Palmquist (1988) with toluene for extraction and 2% (v/v) sulphuric acid in methanol for methylation. Resulting FA methyl esters (FAME) were analysed on a Varian 3400 CX Gas Chromatograph equipped with a flame-ionization detector, using a temperature programme (Shingfield *et al*., 2003). Identification of FAME peaks was completed using a known external standard (GLC463, Nu-Check Prep., MN, USA). Individual FA concentrations were normalised according to the total lipid content, determined as ether extract (MAFF, 1986). The contents of individual FA were reported on an oven DM basis following measurement of the residual DM content of the freeze-dried samples (after oven drying at 100ºC for 18 h).

Biohydrogenation residues were analysed for FA composition using a method based on Folch *et al*. (1957) and methylated using a bi-methylation method (base-catalysed then acid-catalysed) derived from Kramer and Zhou (2001). A known amount of internal standard (Heneicosanoic acid methyl ester, H3265, Sigma-Aldrich, UK) was

added prior to methylation in order to quantify FAME. Extracted FAME were analysed as described above. The FA profiles were expressed as total mg/flask. Results for 18:2 n-6 and 18:3 n-3 were used to calculated the extent of *in vitro* biohydrogenation for each plant based on the disappearance of 18:2 n-6 and 18:3 n-3.

Data analysis. Flask contents of selected fatty acids were analysed for effects of plant, time and their interaction by means of the Mixed model in SAS (v9.4, SAS Institute, Cary, NC, US), which included within sampling time comparison of plant least squares means (analysed using the PDIFF function). Results were considered significantly different when $P<0.05$. Curves (constructed using the mean of three flasks over the entire incubation period from 0 to 48 h) describing the rate and extent of *in vitro* biohydrogenation (disappearance of 18:2 n-6 and 18:3 n-3) were fitted to the exponential model of Ørskov and McDonald (1979) using SigmaPlot (Systat Software Inc., London). Hydrogenation of FA was described by the equation $P_t = x + ye^{-zt}$, where P_t is the amount (mg) of FA present at incubation time t, x is the non-hydrogenated FA fraction (mg), y is the hydrogenated fraction (mg) and z is the fractional rate of disappearance of y (/h). Curve parameters were compared as in Boufaïed *et al*. (2003); effective disappearance (*ED*) and rumen bypass (*BP*) of 18:2 n-6 and 18:3 n-3 were calculated using a rumen fractional passage rate (k) of 0.03/h (Alcaide *et al*., 2000). This rate describes the passage of small particulate matter in sheep.

Experiment 2: Fatty acid profile of lamb

Plant species and establishment of biodiverse pastures. The same six species assessed *in vitro* in experiment 1 were established within a permanent, perennial ryegrass-based sward at the University of Reading. In the previous five years the sward

had been used to graze sheep and had received approximately 100 kg fertiliser nitrogen/ha/year. The site was divided into ten plots (5 x 2 arrangement; each 60 m x 29 m) allocated in a paired block design to either the biodiverse or a control (no additional species sown) treatment. Blocking was completed to account for potential variation in background conditions. The biodiverse plots were power harrowed prior to under-sowing at ~5 kg seed/ha, twice the recommended seed rate (DEFRA, 2004). The weight of each species within the seed mixture was as follows: birdsfoot trefoil (19 %), knapweed (24 %), ribwort plantain (32 %), red clover (13 %), selfheal (10 %) and yarrow (2 %; Emorsgate Seeds, Norfolk, UK). These proportions were used so that the same number of seeds per g was included of each species. Establishment of the six 'sown' species was completed using 0.75 and 0.25 of the total seed amount (5 kg) in spring and autumn, respectively. Owing to poor establishment of the biodiverse pastures a further ~5 kg seed/ha was applied in late autumn. After sowing the biodiverse plots were rolled and then left undisturbed for at least six weeks. The control pastures received 100 kg fertiliser nitrogen/ha in the first year but no additional fertiliser was applied to the biodiverse pastures.

Immediately prior to the start of the grazing study in the following spring, both the biodiverse and control plots were assessed for species richness as determined by the number and abundance of different sown and unsown plant species, and assessment of contribution to the overall biomass. This was achieved by estimating the number of different species and percentage ground cover of vascular plant species in 12 randomly positioned 50 x 50 cm quadrats within each plot (areas within 1 m of the fences were excluded from the sampling). Simultaneuosly, ten random samples per plot were obtained by harvesting the above-ground plant matter that were pooled within

176 plots, frozen (-20ºC), freeze-dried and milled, and stored at -20ºC and subsequently
177 analysed for fatty acid analysis, as per the process described for whole plants in
178 Experiment 1.
179
180 *Experimental animals and the grazing study.* Fifty greyface mule x Texel castrated
181 male lambs from an early lambing flock were weaned in April of the grazing year and
182 given a forage-based diet until the start of the grazing study in mid-May. The lambs
183 were weighed prior to the study (mean weight ±SEM 26.8 kg ± 0.39), and five lambs
184 were randomly allocated to each plot to ensure a similar mean live-weight within each
185 plot and across the two treatments (26.8 and 26.7 kg for biodiverse and control
186 pastures, respectively). Lambs had access to water *ad libitum*, and were weighed
187 weekly, with live-weights recorded. The grazing period continued for a minimum of 50
188 d (mean ± s.e.m. control 64.7 ± 0.93 days, biodiverse 64.3 ± 0.93 days) after which
189 time animals reaching the target weight of 45 kg or attaining optimum body condition
190 score by palpation of the loin area were selected for slaughter. A total of three lambs
191 from each plot were slaughtered. Animals were transported to the University of Bristol
192 for slaughter, which occurred according to European Union Welfare guidelines. On
193 arrival animals were stunned by captive bolt followed by abrupt exsanguination.
194 Carcases were prepared and graded, and and tissue samples were taken for study
195 from *musculus longissimus thoracis* and *musculus semimembranosus,* and
196 subcutaneous fat from above *m. l. thoracis.* Samples were stored frozen at -20ºC until
197 required for FA analysis.
198
199 *Fatty acid analysis*. Prior to analysis tissue samples were partially defrosted at room
200 temperature for approximately 30 minutes and prepared by cutting into ~ 1 cm^3 pieces,

and blended to a homogeneous paste in a food processor within 2 minutes. Subsequently, FA in samples were extracted using the Folch *et al*. (1957) method followed by a base-catalysed methylation as described for *in vitro* samples in Experiment 1. For FA extraction, 2.0 g of each tissue (in duplicate) were homogenised in chloroform/methanol (2:1, v/v) using an IKA® Ultra-Turrax dispersal tool (IKA®-Werke GmbH & Co.. Staufen, Germany). After washing the extract with saline solution, the solvent was removed under vacuum at 40^{o}C using a rotary evaporator and the remaining lipid extract was re-suspended in hexane. FAME were analysed as outlined previously, FA contents and profiles were obtained for each sample, and were expressed as mg/100 g fresh tissue.

Data analysis. Live-weight was analysed using the Mixed procedure of SAS (SAS version 9.4; SAS Institute), with a model that included fixed effects of time, treatment and time by treatment interaction (including time as a repeated measurement), and random effects of plot and lamb within plot. Pasture total lipid, FA content and species richness were analysed using a two-way ANOVA, with fixed effects of treatment and block. Tissue FA were analysed using the Mixed procedure of SAS with a model including fixed effects of treatment, block, and treatment by block interaction. Results were considered significantly different where $P<0.05$, and tendencies were reported where P was between 0.05 and 0.1.

Results

Experiment 1

Of the six plant species, selfheal contained the highest amount of total FA, and ribwort plantain the least (Table 1). Yarrow was particularly high in 18:2 n-6, and Selfheal

contained the greatest quantity of 18:3 n-3 (Table 1). The effect of the six selected plant species on *in vitro* flask contents of selected FA are reported in Table 2. There were effects (P<0.001) of plant, time and plant by time interaction for all FA presented in Table 2. At 0 h incubation, all flasks contained similar amounts of 18:0 (*P*=0.124), but over time flask contents increased (*P*<0.001). The interaction between plant and time for 18:0 reflected a lag in 18:0 accumulation for knapweed, and the greatest (*P*<0.05) 18:0 accumulation at 48 hours for selfheal and birdsfoot trefoil. For *cis*-9 18:1, at 0 h incubation there was a difference (*P*<0.001) between plants, most probably due to the high content in selfheal (Table 2). Over time this decreased (*P*<0.001) for all flasks, but again the rate of disappearance varied between plants, with this being lowest for red clover after 3 hours of incubation. Flask contents of *trans*-11 18:1 were similar across all plants at time 0 (*P*=0.542), but over time contents increased (*P*<0.001) to a peak between 6 and 12 hours before decreasing again. The greatest amount of *trans*-11 18:1 was measured in flasks containing birdsfoot trefoil.

There were differences (*P*<0.001) between plants for both 18:2 n-6 and 18:3 n-3, at 0 h incubation.Over time both decreased (*P*<0.001) but at different rates. According to the disappearance curves, knapweed contained the highest amount of non-hydrogenatable 18:2 n-6, and selfheal the lowest (Table 3). The effective disappearance of the hydrogenatable fraction was highest for yarrow, with yarrow and knapweed containing the highest amount of rumen bypass 18:2 n-6. Selfheal contained the highest amount of hydrogenatable 18:3 n-3, but had the lowest rate of 18:3 n-3 disappearance of all plants. Due to this the ruminal bypass 18:3 n-3 was highest for selfheal.

Experiment 2

Pasture botanical composition and lamb performance. The number of different species (both sown and unsown) was higher in the biodiverse than the control pastures when expressed per quadrat (P<0.016) and per plot (P<0.019). However, birdsfoot trefoil was not recorded in any of the biodiverse pastures. The mean contribution of the sown and un-sown (non-grass species) plant species to the overall biomass was 25.4% in the biodiverse pastures. This contribution was largely comprised of ribwort plantain.The total lipid and FA composition of the conventional and biodiverse pastures immediately prior to start of the lamb grazing study is presented in Table 4. There were no statistically significant (P>0.05) differences in the individual FA contents of the two pasture types. The predominant FA was 18:3 n-3 and accounted for approximately 50% of the total FA.

Live-weight change of the lambs grazing the conventional and biodiverse pastures is summarised in Figure 1 and demonstrates an effect of time (P<0.001) but no effect (P=0.717) of treatment, with no interaction (P=0.773). Overall mean live-weight gains were 10.2 and 10.0 kg (±1.91 SEM) over the grazing period for conventional and biodiverse groups, respectively.

*Fatty acid composition of tissues.*The summary of the amounts of key FA groups in all three tissues analysed are reported in Table 5 (for full details of FA content, see Supplementary tables S1, S2 and S3). The total FA content of *m. l. thoracis* was similar for the lambs grazing the biodiverse and control pastures: mean 1573 and 1648 mg/100 g tissue respectively. The amount of 18:2 n-6 tended to be higher (P=0.060) in *m. l. thoracis* from the lambs grazing the biodiverse pasture, however, other

differences in FA content and profile were small. A significant block and block x treatment effect ($P < 0.05$) was recorded for 22:2 *cis*-13, *cis*-16, due to the higher level of this FA in one of the blocks.

The total FA content was numerically higher in the *m. semimembranosus* than in the *m. l. thoracis* tissue (Table 5). A lower ($P<0.04$) content of *trans*-11 18:1 was found in *m. semimembranosus* from lambs grazing the biodiverse pasture (Table 5). At the same time 18:2 n-6 and 18:3 n-3 concentrations were higher (both $P<0.02$), resulting in a higher total n-3 and n-6 PUFA content in tissue from lambs grazing biodiverse pasture. Block x treatment effects were recorded for a number of FA, mainly due to some blocks having different mean values from the remaining blocks, which magnified any subtle treatment differences.

Subcutaneous fat contained 47,261 and 46,723 mg total FA/100 g tissue from lambs grazed control and biodiverse pastures respectively (Table 5). The content of *trans*-10 18:1, *trans*-12 18:1, 19:0, 18:2 *cis*-9, *cis*-12, 18:3 n-3, 20:3 n-6, 24:0/20:5 n-3, 22:5 n-3 and total n-3, n-6, and very long chain n-3 PUFA were all higher ($P<0.05$) in subcutaneous fat from lambs grazed on biodiverse pasture compared with control pasture (Supplementary table S3).

Discussion

In vitro biohydrogenation

It has been suggested (Dewhurst *et al*., 2001) that the proportion of leaf in the whole plant DM is an important determinant of FA concentration due to forage lipids being predominantly of leaf origin (Harfoot, 1981). Differences in leaf:stem ratios between

plant species may explain some of the differences in plant FA contents observed. The *in vitro* biohydrogenation characteristics were similar for the six plant species studied. Selfheal and birdsfoot trefoil displayed the greatest accumulation of 18:0 but these plants contained the greatest initial amounts of total PUFA. Rate of disappearance of 18:2 n-6 was similar for all plants apart from knapweed (mean of plants excluding knapweed, 0.12 mg/h, knapweed 0.09 mg/h). This may indicate that knapweed exerts some other effect on rumen microbes and/or their enzymes, either by inhibiting the initial lipolysis or biohydrogenation itself. Indeed, knapweed resulted in a lower accumulation of both *trans*-11 18:1 and 18:0 which suggests less biohydrogenation. Kumarasamy *et al*. (2003) found that serotonin conjugates extracted from the seeds of knapweed had antimicrobial activity. These conjugates may also be present in other fractions of knapweed and therefore conferring potential antimicrobial effects.

For 18:3 n-3, both selfheal and birdsfoot trefoil displayed similar high values for ED compared with the other plants, and yet the accumulation of 18:0 for birdsfoot trefoil did not increase at the same rate as that of selfheal. This may be due to birdsfoot trefoil inhibiting the intermediary pathways of biohydrogenation, through, for example, the presence of condensed tannins. After ingestion some condensed tannins from birdsfoot trefoil remain free and unbound that may inhibit the extracellular enzyme action of certain bacteria (Barry and Manley, 1986). Min *et al*. (2002) found that including birdsfoot trefoil in the diet of sheep decreased the population of the rumen bacteria *Butyrivibrio proteoclasticus*, which is one of the few bacterial species that conducts the final step of rumen biohydrogenation of 18:3 n-3 (converting *trans*-11 18:1 to 18:0). However, if condensed tannins affect bacterial biohydrogenation in this way, no reduction in the initial rate of disappearance of both 18:2 n-6 and 18:3 n-3 was

observed in the present study. No estimate of the tannin content or that of other polyphenols was completed in the present study but it is highlighted as an area of future study. When a rumen passage rate of 0.03/h (rate at which small particles leave the rumen of sheep) was applied, the amount of 18:3 n-3 by-passing hydrogenation was numerically higher for selfheal and birdsfoot trefoil. However, this observation is likely to reflect the higher initial concentration of 18:3 n-3 in these plants.

Compared with previous *in vitro* research, red clover did not appear to perform better than other species in terms of effective disappearance and by-pass of 18:2 n-6 and 18:3 n-3. Van Ranst *et al*. (2013) reported lower lipolysis and biohydrogenation of 18:3 n-3 and 18:2 n-6 with silages containing increasing amounts of red clover, which may have been due to the presence of polyphenol oxidase (PPO) within the red clover. In the present study however, red clover was being compared not with ryegrass but with other species which may have exerted similar biohydrogenation-inhibiting effects. An *in vivo* study reported higher concentrations of 18:3 n-3 in rumen fluid following the feeding of a 50:50 grass:red clover silage to lambs, compared with a 100% grass silage, suggesting PPO as a possible reason (Campidonico *et al*., 2016). However there was no difference between the grass:red clover silage and grass:sainfoin silage treatments, with sainfoin suggested as having a different mechanism of action for inhibiting biohydrogenation.

Using biodiverse pasture to alter the fatty acid profile of tissues

To accelerate the assembly of a species-rich community within grassland, deliberate under-sowing permanent pasture with selected plant species is required. Establishment of the biodiverse pastures over approximately 12 months significantly

increased the number of different ‘sown’ and unsown species present in the experimental plots as compared with the control pastures. On average the biodiverse pastures were shown to contain an average of 16 different plant species per plot, compared with 9 for control pastures. However, despite the higher species richness, the most abundant species, and concomitant contributor to the overall plant biomass, was ribwort plantain. The abundance of the remaining species was low and therefore made a substantially smaller contribution to the available biomass available for grazing. Other studies carried out with the aim of introducing different species to create biodiverse pastures have been, for example, four years in duration (e.g. Hopkins *et al*., 1999; Pywell *et al*., 2002). Therefore a longer period of establishment is required to enable some slower growing species to proliferate following initial sowing in order to create a truly biodiverse pasture.

The *m. semimembranosus* total FA concentrations were lower than those measured by Whittington *et al*. (2006) comparing different biodiverse systems with a control pasture. The *m. l. thoracis* total FA concentration was similar to that observed by Lourenço *et al.* (2007) comparing an intensive ryegrass pasture with an established biodiverse pasture. There were few differences in the FA profile between the pasture treatments for *m. l. thoracis*. Lourenço *et al*. (2007) observed a number of differences in this tissue between animals grazing biodiverse or intensive *lolium perenne*-based pastures, including a higher 18:2 n-6 resulting in an increased n-6:n-3 ratio for the biodiverse treatment. In the study of Lourenço *et al*. (2007) the lambs grazed the biodiverse pastures for a period of 12 weeks (84 days) compared to 50 days in the present study. This shorter grazing period may reflect some of the differences in the results recorded although a minimum of 50 days grazing is generally recommended in

376 order to detect differences in muscle phospholipids (Wood, personal communication).
377 Another reason for the lack of effect in the present study is low establishment of
378 biodiverse species.
379
380 *M. semimembranosus* contains a higher amount of phospholipids, which have a higher
381 PUFA content (De Smet *et al*., 2004). The differences in n-6 PUFA content observed
382 in the present study for the lambs grazing biodiverse pastures are similar to those
383 observed by Whittington *et al*. (2006), however these authors did not observe
384 increases in n-3 FA that were recorded in the present study with the biodiverse
385 treatment. These differences in intramuscular FA concentrations suggest an increased
386 availability of both 18:2 n-6 and 18:3 n-3 for tissue incorporation. This may reflect a
387 reduction in rumen biohydrogenation of these dietary FA for the lambs grazing
388 biodiverse pastures. The lower *trans*-11 18:1 concentration further illustrates this point,
389 as this is a key intermediate of the biohydrogenation of both PUFA. There are several
390 possible explanations for this. Inhibition of initial lipolysis of plant lipids prior to rumen
391 biohydrogenation (this may have been the mechanism of action observed during *in*
392 *vitro* biohydrogenation of knapweed and selfheal) may have contributed to this effect.
393 In addition, inhibition of biohydrogenation prior to the hydrogenation step that
394 synthesises *trans*-11 18:1 and/or increased rate of passage for animals consuming
395 biodiverse pasture may have resulted in greater amounts of PUFA escaping rumen
396 biohydrogenation. However, the mechanism(s) underlying the finding of the present
397 study are unclear and should be an area of further investigation.
398
399 Total FA concentration was greatest in the subcutaneous fat. Subcutaneous fat total
400 FA content was lower than that observed by Enser *et al*. (1996; 70,572 mg/100 g

401 tissue) and Lourenço *et al.* (2007; 60,900 – 66,900 mg/100 g tissue). This might reflect
402 the lighter carcass weight and therefore level of finish, but may also reflect slight
403 difficulty in separating the subcutaneous fat from muscle. Subcutaneous fat was more
404 susceptible to dietary change, due to the higher total FA concentration when compared
405 with muscle. *Trans*-10 18:1 and *trans*-12 18:1 tend to arise following biohydrogenation
406 of 18:2 n-6 (Jouany et al., 2007). The reason for greater amounts of these FA in
407 subcutaneous fat of lambs grazing the biodiverse pasture is unclear, especially as we
408 hypothesise that biohydrogenation of dietary PUFA may have been lower with
409 biodiverse pastures. It may reflect FA differences for deposition into subcutaneous
410 tissue. Subcutaneous fat from biodiverse treatment lambs contained higher amounts
411 of both 18:2 n-6 and 18:3 n-3 than control lambs, as well as a higher very long chain
412 n-3 FA. There is evidence to suggest that in ruminant animals, 18:2 n-6 is preferentially
413 deposited in phospholipids compared to 18:3 n-3 (De Smet *et al*., 2004), which would
414 suggest a lower n-6:n-3 ratio in subcutaneous fat than the lean muscle tissues.
415 However in the present study the ratio of n-6:n-3 were similar for all the tissues studied.
416
417 Increasing human consumption of 18:3 n-3 has been suggested as a means of
418 increasing synthesis of very long chain (VLC) n-3 FA through tissue elongation and
419 desaturation. Burdge and Calder (2005) concluded that due to poor efficiency of
420 conversion, 18:3 n-3 appears to be a limited source of VLC n-3 FA in humans, and
421 consumption of preformed VLC n-3 FA is a more efficient means of attaining
422 recommended intake levels. The efficiency of conversion of 18:3 n-3 to VLC n-3 FA in
423 ruminant meat has not been measured, but increasing 18:3 n-3 consumption by
424 ruminants has lead to increased amounts of VLC n-3 FA in lean tissues (Scollan *et al*.,
425 2001; Wachira *et al*., 2002). In the present study, the only tissue to display an increase

in VLC n-3 FA when lambs grazed biodiverse pasture was subcutaneous fat, which is likely to be consumed in variable amounts, according to consumer preference, alongside muscle tissue.

In conclusion, the results of the present study suggest that it is possible to manipulate the FA concentration and profile of muscle and subcutaneous fat in lamb by grazing biodiverse pastures. Grazing lambs on the biodiverse pastures established within our project increased overall PUFA content (~30 mg/100 g tissue) of lamb muscle. The three tissues analysed had varying responses to diet reflecting the presence of different lipid classes in each of the tissues. Differences reported from the *in vivo* study may have been more pronounced if the biodiverse species had established at the expected density, especially as the more promising species from the *in vitro* study were not present within the *in vivo* study pastures.

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Table 1 Selected fatty acid content of six plants selected for in vitro biohydrogenation incubations (mixture of four different sampling times; mg/g dry matter)

	Plant[1]					
Fatty acid	BT	K	RP	RC	S	Y
16:0	5.27	4.83	4.20	4.13	5.16	4.25
18:0	0.62	0.59	0.58	0.77	1.01	0.45
18:1 *cis*-9	1.34	1.75	2.28	1.47	5.69	2.44
18:2 *cis*-9, *cis*-12	4.85	6.84	5.25	4.78	5.64	8.95
18:3 n-3	8.94	6.28	5.70	4.59	11.9	4.94
Total 18:2 *cis*-9, *cis*-12 + 18:3 n-3	13.8	13.1	11.0	9.4	17.5	11.4
Total fatty acids	26.6	24.8	21.7	19.8	32.9	24.7

[1] Where BT – Birdsfoot trefoil; K – Knapweed; RP – Ribwort plantain; RC – Red clover; S – Selfheal; Y – Yarrow.

569 **Table 2** Flask content (mg) of selected fatty acids over a 48 h *in vitro* incubation.

Fatty acid	Time (h)	Plant[1]						s.e.m.	*P*[2] (plant)
		BT	K	RP	RC	S	Y		
18:0	0	22.4	21.4	21.9	21.3	22.4	20.6	0.52	0.124
	3	23.0^{b}	22.7^{b}	23.4^{b}	23.9^{ab}	25.3^{a}	24.0^{ab}		0.013
	6	26.9^{bc}	24.6^{d}	25.4^{cd}	25.5^{cd}	29.0^{a}	27.1^{b}		<0.001
	9	29.6^{b}	26.2^{d}	27.4^{cd}	27.6^{cd}	32.1^{a}	28.0^{c}		<0.001
	12	29.8^{b}	26.3^{d}	28.3^{c}	28.0^{c}	33.4^{a}	29.3^{bc}		<0.001
	24	32.2^{b}	30.3^{cd}	29.5^{cd}	29.3^{d}	36.3^{a}	30.5^{c}		<0.001
	48	35.3^{b}	30.7^{d}	31.8^{cd}	31.5^{cd}	37.4^{a}	32.3^{c}		<0.001
18:1 *cis*-9	0	3.04^{c}	3.05^{c}	3.70^{b}	2.91^{c}	6.96^{a}	3.73^{b}	0.068	<0.001
	3	2.46^{c}	2.59^{c}	3.08^{b}	2.61^{c}	5.63^{a}	3.19^{b}		<0.001
	6	2.39^{c}	2.39^{c}	2.89^{b}	2.31^{c}	4.67^{a}	2.82^{b}		<0.001
	9	2.20^{d}	2.23^{d}	2.80^{b}	2.24^{d}	4.14^{a}	2.53^{c}		<0.001
	12	1.88^{d}	1.87^{d}	2.56^{b}	1.98^{cd}	3.31^{a}	2.14^{c}		<0.001
	24	1.33^{d}	1.32^{d}	1.81^{b}	1.39^{cd}	2.26^{a}	1.55^{c}		<0.001
	48	0.91^{d}	1.03^{cd}	1.23^{bc}	1.05^{cd}	1.73^{a}	1.16^{bc}		<0.001
18:1 *trans*-11	0	1.62	1.70	1.67	1.72	1.79	1.79	0.074	0.542
	3	2.47^{b}	3.03^{a}	2.47^{b}	2.51^{b}	2.52^{b}	3.02^{a}		<0.001
	6	3.69^{a}	3.06^{c}	2.63^{d}	3.05^{c}	2.95^{c}	3.29^{b}		<0.001
	9	3.91^{a}	3.28^{bc}	2.61^{d}	3.15^{c}	3.44^{b}	3.33^{bc}		<0.001
	12	3.78^{a}	3.04^{c}	2.53^{d}	2.95^{c}	3.40^{b}	3.14^{c}		<0.001
	24	3.00^{b}	2.62^{d}	2.47^{d}	2.59^{d}	3.41^{a}	2.89^{c}		<0.001
	48	2.91^{ab}	2.45^{cd}	2.36^{d}	2.57^{c}	3.11^{a}	2.87^{b}		<0.001
18:2 *cis*-9, *cis*-12	0	5.57^{c}	6.03^{b}	4.16^{d}	4.99^{d}	5.75^{c}	7.94^{a}	0.078	<0.001
	3	3.86^{d}	4.47^{b}	3.54^{e}	3.57^{e}	4.24^{c}	5.57^{a}		<0.001
	6	3.13^{bc}	3.89^{b}	2.94^{cd}	2.82^{d}	3.22^{b}	4.47^{a}		<0.001
	9	2.51^{b}	3.53^{a}	2.63^{b}	2.47^{b}	2.60^{b}	3.51^{a}		<0.001
	12	1.86^{c}	2.90^{a}	2.17^{b}	2.01^{bc}	1.89^{c}	2.81^{a}		<0.001
	24	1.26^{bc}	2.05^{a}	1.34^{bc}	1.39^{b}	1.13^{c}	1.87^{a}		0.002
	48	0.87^{BC}	1.54^{A}	0.96^{BC}	1.08^{B}	0.85^{C}	1.34^{A}		0.055
18:3 n-3	0	7.60^{b}	3.36^{d}	3.18^{d}	3.75^{c}	9.28^{a}	3.33^{d}	0.075	<0.001
	3	4.18^{b}	1.73^{d}	1.43^{e}	1.98^{c}	6.27^{a}	1.45^{e}		<0.001

	6	2.67^{b}	1.39^{cd}	1.19^{de}	1.41^{c}	4.18^{a}	1.12^{e}	<0.001
	9	1.97^{b}	1.24^{c}	1.05^{cd}	1.12^{c}	3.00^{a}	0.89^{d}	<0.001
	12	1.32^{b}	1.00^{c}	0.89^{cd}	0.87^{cd}	1.83^{a}	0.73^{d}	<0.001
	24	0.84^{a}	0.79^{ab}	0.60^{bc}	0.61^{bc}	0.95^{a}	0.56^{c}	<0.001
	48	0.60^{ab}	0.63^{ab}	0.47^{b}	0.52^{b}	0.76^{a}	0.48^{b}	<0.001

[1] Where BT – Birdsfoot trefoil; K – Knapweed; RP – Ribwort plantain; RC – Red clover; S – Selfheal; Y – Yarrow.

[2] Significance of the effect of plant within sampling time. There were effects (P<0.001) of plant, time and plant by time interaction for all fatty acids presented.

Values within rows with different superscripts are significantly different (P<0.050)

Table 3. Curve fit parameters for the disappearance of 18:2 n-6 and 18:3 n-3 over time.

	Plant[1]					
	BT	K	RP	RC	S	Y
18:2 *cis*-9, *cis*-12						
x^2	0.94	1.58	1.04	1.16	0.84	1.45
y^2	4.55	4.29	3.95	3.75	4.91	6.39
z^2	0.13	0.09	0.11	0.13	0.12	0.13
Curve fit[3]	0.992	0.982	0.976	0.991	0.998	0.995
ED[4]	3.67	3.22	3.12	3.03	3.93	5.18
BP[5]	1.81	2.64	1.86	1.87	1.81	2.66
18:3 n-3						
x	0.75	0.81	0.68	0.62	0.70	0.61
y	6.78	2.49	2.45	3.08	8.64	2.68
z	0.21	0.26	0.30	0.23	0.15	0.33
Curve fit	0.996	0.961	0.944	0.987	0.998	0.975
ED	5.94	2.23	2.22	2.73	7.22	2.46
BP	1.60	1.06	0.90	0.97	2.12	0.84

[1] Where BT – Birdsfoot trefoil; K – Knapweed; RP – Ribwort plantain; RC – Red clover; S – Selfheal; Y – Yarrow.

[2] using the equation $P_t = x + ye^{-zt}$, where P_t is the amount (mg) of 18:2 n-6 or 18:3 n-3 present in the flasks at time *t*, *x* is the non-hydrogenatable fraction (mg), *y* is the hydrogenatable fraction (mg), *z* is the rate of disappearance of fraction *y* (/h), and *t* is incubation time (h; Ørskov & McDonald, 1979)

[3] R-squared value for the curve fit

[4] ED - Effective disappearance (mg/g DM) of 18:2 n-6 or 18:3 n-3 using a ruminal rate of passage (k) of 0.03 (Alcaide *et al*., 2000)

[5] BP – Potential ruminal bypass (mg/g DM) of 18:2 n-6 or 18:3 n-3.

600 **Table 4** Mean fatty acid contents (mg/g dry matter) of the control and biodiverse pastures prior

601 to the commencement of the lamb grazing study.

Fatty acid	Pasture type		s.e.m.	*P*[1]
	Control	Biodiverse		
16:0	4.68	4.11	0.295	0.216
18:0	0.33	0.29	0.022	0.260
18:1 total	0.80	0.63	0.065	0.099
18:2 *cis*-9, *cis*-12	4.02	3.95	0.198	0.793
18:3 n-3	17.8	15.3	0.90	0.094
Total lipid	30.1	26.4	1.67	0.156

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603 [1] Significance of the effect of pasture type

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621 **Table 5.** Fatty acid composition (mg/100g tissue) of tissues from lambs grazing control and biodiverse pasture.

	Forage type			*P*[1]		
	Conventional	Biodiverse	SEM	Treatment	Block	Treatment x block
M. Longissimus Thoracis						
18:1 *trans*-11	45.7	39.6	5.23	0.421	0.306	0.457
18:2 *cis*-9, *cis*-12	58.0	72.7	5.18	0.060	0.358	0.426
18:3 n-3	21.8	27.3	2.35	0.112	0.470	0.334
Total fatty acids	1573	1648	168.1	0.758	0.600	0.189
Total SFA[2]	748	776	81.9	0.808	0.650	0.197
Total *cis*-MUFA[3]	569	592	65.5	0.799	0.585	0.166
Total *trans*-MUFA[4]	76.2	73.6	8.40	0.835	0.386	0.361
n-3 PUFA[5]	37.7	44.5	3.24	0.155	0.495	0.389
n-6 PUFA[6]	92.5	111	7.10	0.078	0.349	0.399
Total PUFA[7]	130	156	10.2	0.094	0.398	0.389
Total CLA[8]	23.0	21.5	2.86	0.716	0.502	0.502
n-6:n-3	2.5	2.5	0.07	0.511	0.278	0.651
VLC n-3[9]	21.1	22.8	1.37	0.389	0.504	0.682
M. Semimembranosus						
18:1 *trans*-11	75.4	61.5	4.40	0.037	0.187	0.018
18:2 *cis*-9, *cis*-12	70.1	84.9	2.90	0.002	0.201	0.099
18:3 n-3	28.4	35.6	2.01	0.020	0.286	0.250
Total fatty acids	2416	2315	147.5	0.634	0.695	0.058
Total SFA	1171	1119	76.4	0.636	0.798	0.079
Total *cis*-MUFA	865	819	55.3	0.557	0.648	0.048
Total *trans*-MUFA	134	115	9.4	0.170	0.311	0.100
n-3 PUFA	47.2	55.2	2.42	0.032	0.252	0.206
n-6 PUFA	111	128	4.2	0.010	0.220	0.104
Total PUFA	158	183	6.2	0.012	0.302	0.110
Total CLA	39.0	32.9	2.88	0.150	0.375	0.111
n-6:n-3	2.4	2.3	0.08	0.748	0.078	0.954
VLC n-3	24.4	25.6	0.68	0.254	0.204	0.204
Sub-cutaneous fat						
18:1 *trans*-11	1638	1494	81.7	0.228	0.077	0.615

18:2 *cis*-9, *cis*-12	707	865	37.1	0.007	0.059	0.532
18:3 n-3	344	510	30.6	0.002	0.169	0.777
Total fatty acids	47261	46723	1368.6	0.784	0.165	0.036
Total SFA	23260	23378	808.0	0.919	0.894	0.138
Total *cis*-MUFA	17681	16744	866.5	0.454	0.177	0.065
Total *trans*-MUFA	2950	2923	87.7	0.826	0.006	0.367
n-3 PUFA	445	631	32.8	0.001	0.207	0.834
n-6 PUFA	939	1126	51.6	0.018	0.071	0.598
Total PUFA	1384	1757	72.9	0.002	0.096	0.762
Total CLA	969	840	64.0	0.170	0.073	0.103
n-6:n-3	2.1	1.9	0.11	0.086	0.186	0.672
VLC n-3	111	135	6.1	0.011	0.985	0.913

[1] Significance of the effect of; T - treatment; B - block; T*B, treatment*block interaction

[2] SFA - saturated fatty acids. Sum of 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 18:0, 19:0, 20:0, 22:0, 24:0.

[3] MUFA - mono-unsaturated fatty acids. Sum of *cis*-9 12:1, *cis*-9 14:1, *cis*-9 15:1, *cis*-9 16:1, *cis*-10 17:1, *cis*-9 18:1, *cis*-11 20:1, *cis*-13 22:1, *cis*-15 24:1

[4] Sum of *trans*-9 16:1, *trans*-6-8 18:1, *trans*-9 18:1, *trans*-10 18:1, *trans*-11 18:1, *trans*-12 18:1, *trans*-13-14 18:1

[5] PUFA – polyunsaturated fatty acids. Sum of 18:3 n-3, 20:5 n-3, 22:3 n-3, 22:5 n-3, 22:6 n-3

[6] Sum of *trans*-9, *trans*-12 18:2, *cis*-9, *cis*-12 18:2, 20:2 n-6, 20:3 n-6, 22:2 n-6, 22:4 n-6.

[7] Sum of n-3 and n-6 PUFA.

[8] CLA – conjugated linoleic acid

[9] VLC – very long chain

633 **Figure captions**

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635 **Figure 1.** The mean liveweight of lambs grazing either a control or biodiverse pasture over a

636 60 d study period. Mixed model analysis concluded an effect (P<0.001) of time but no effect of

637 treatment (P=0.717) or time by treatment interaction (P=0.773).

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