

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

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

3

4 K. E. Kliem¹, A. L. Thomson¹, L. A. Crompton¹, D. I. Givens²

5

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

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

10

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

13

14 Short title: Fatty acid profiles of biodiverse forage species

15

16 **Abstract**

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

27 based sward. Lambs were grazed (~50 days) on biodiverse or control pastures and
28 the effects on the FA composition of *m. longissimus thoracis* (lean and subcutaneous
29 fat) and *m. semimembranosus* (lean) were determined. Biodiverse pasture increased
30 18:2 n-6 and 18:3 n-3 contents of *m. semimembranosus* (+14.8 and +7.2 mg/100g
31 tissue respectively) and the subcutaneous fat of *m. l. thoracis* (+158 and +166 mg/100g
32 tissue respectively) relative to feeding a perennial ryegrass pasture. However, there
33 was no effect on total concentrations of saturated FA in the tissues studied. It was
34 concluded that enhancing biodiversity had a positive impact on muscle FA profile
35 reflected by increased levels of total PUFA.

36

37 **Keywords:** Biodiversity; multispecies swards; fatty acids; biohydrogenation; lamb
38 muscle

39

40 **Implications**

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

49

50 **Introduction**

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

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

84

85 **Material and methods**

86 *Experiment 1: In vitro biohydrogenation of selected species*

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

96

97 *In vitro biohydrogenation.* To Wheaton flasks (capacity 125 ml), 1.0 g (+/- 0.01 g) of
98 each freeze-dried and milled sample was accurately weighed in triplicate, and 90 ml of
99 a reduced anaerobic buffer (Theodorou *et al.*, 1994) added. Flasks were warmed to
100 39°C prior to inoculation with 10 ml strained bovine rumen fluid collected approximately

101 2 h post-feeding from two lactating dairy cows receiving a total mixed diet comprising
102 50:50 forage:concentrate (DM basis), with the forage portion being predominantly
103 maize silage. Flasks were loosely stoppered and vented via a needle. Flasks were
104 incubated at 39°C with regular mixing by agitation of the bottles. Three flasks per plant
105 species were removed following 0, 3, 6, 9, 12, 24 and 48 h incubation, flask contents
106 were frozen at -20°C and then lyophilised. Lyophilised residue was mixed and stored
107 at -20°C before subsequent FA analysis.

108

109 *Fatty acid analysis.* FA analysis of the whole plant material prior to *in vitro*
110 biohydrogenation was performed on triplicate sub-samples of freeze-dried plant
111 material, using a method based on Sukhija and Palmquist (1988) with toluene for
112 extraction and 2% (v/v) sulphuric acid in methanol for methylation. Resulting FA methyl
113 esters (FAME) were analysed on a Varian 3400 CX Gas Chromatograph equipped
114 with a flame-ionization detector, using a temperature programme (Shingfield *et al.*,
115 2003). Identification of FAME peaks was completed using a known external standard
116 (GLC463, Nu-Check Prep., MN, USA). Individual FA concentrations were normalised
117 according to the total lipid content, determined as ether extract (MAFF, 1986). The
118 contents of individual FA were reported on an oven DM basis following measurement
119 of the residual DM content of the freeze-dried samples (after oven drying at 100°C for
120 18 h).

121

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

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

130

131 *Data analysis.* Flask contents of selected fatty acids were analysed for effects of plant,
132 time and their interaction by means of the Mixed model in SAS (v9.4, SAS Institute,
133 Cary, NC, US), which included within sampling time comparison of plant least squares
134 means (analysed using the PDIFF function). Results were considered significantly
135 different when $P < 0.05$. Curves (constructed using the mean of three flasks over the
136 entire incubation period from 0 to 48 h) describing the rate and extent of *in vitro*
137 biohydrogenation (disappearance of 18:2 n-6 and 18:3 n-3) were fitted to the
138 exponential model of Ørskov and McDonald (1979) using SigmaPlot (Systat Software
139 Inc., London). Hydrogenation of FA was described by the equation $P_t = x + ye^{-zt}$, where
140 P_t is the amount (mg) of FA present at incubation time t , x is the non-hydrogenated FA
141 fraction (mg), y is the hydrogenated fraction (mg) and z is the fractional rate of
142 disappearance of y (/h). Curve parameters were compared as in Boufaïed *et al.*
143 (2003); effective disappearance (*ED*) and rumen bypass (*BP*) of 18:2 n-6 and 18:3 n-
144 3 were calculated using a rumen fractional passage rate (k) of 0.03/h (Alcaide *et al.*,
145 2000). This rate describes the passage of small particulate matter in sheep.

146

147 *Experiment 2: Fatty acid profile of lamb*

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

151 had been used to graze sheep and had received approximately 100 kg fertiliser
152 nitrogen/ha/year. The site was divided into ten plots (5 x 2 arrangement; each 60 m x
153 29 m) allocated in a paired block design to either the biodiverse or a control (no
154 additional species sown) treatment. Blocking was completed to account for potential
155 variation in background conditions. The biodiverse plots were power harrowed prior to
156 under-sowing at ~5 kg seed/ha, twice the recommended seed rate (DEFRA, 2004).
157 The weight of each species within the seed mixture was as follows: birdsfoot trefoil (19
158 %), knapweed (24 %), ribwort plantain (32 %), red clover (13 %), selfheal (10 %) and
159 yarrow (2 %; Emorsgate Seeds, Norfolk, UK). These proportions were used so that the
160 same number of seeds per g was included of each species. Establishment of the six
161 'sown' species was completed using 0.75 and 0.25 of the total seed amount (5 kg) in
162 spring and autumn, respectively. Owing to poor establishment of the biodiverse
163 pastures a further ~5 kg seed/ha was applied in late autumn. After sowing the
164 biodiverse plots were rolled and then left undisturbed for at least six weeks. The control
165 pastures received 100 kg fertiliser nitrogen/ha in the first year but no additional fertiliser
166 was applied to the biodiverse pastures.

167

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

176 plots, frozen (-20°C), freeze-dried and milled, and stored at -20°C 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 \pm SEM 26.8 kg \pm 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 \pm s.e.m. control 64.7 \pm 0.93 days, biodiverse 64.3 \pm 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 Carcasses 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 -20°C 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 cm³ pieces,

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

211

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

221

222 **Results**

223 *Experiment 1*

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

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

240

241 There were differences ($P<0.001$) between plants for both 18:2 n-6 and 18:3 n-3, at 0
242 h incubation. Over time both decreased ($P<0.001$) but at different rates. According to
243 the disappearance curves, knapweed contained the highest amount of non-
244 hydrogenatable 18:2 n-6, and selfheal the lowest (Table 3). The effective
245 disappearance of the hydrogenatable fraction was highest for yarrow, with yarrow and
246 knapweed containing the highest amount of rumen bypass 18:2 n-6. Selfheal
247 contained the highest amount of hydrogenatable 18:3 n-3, but had the lowest rate of
248 18:3 n-3 disappearance of all plants. Due to this the ruminal bypass 18:3 n-3 was
249 highest for selfheal.

250

251 *Experiment 2*

252 *Pasture botanical composition and lamb performance.* The number of different species
253 (both sown and unsown) was higher in the biodiverse than the control pastures when
254 expressed per quadrat ($P<0.016$) and per plot ($P<0.019$). However, birdsfoot trefoil
255 was not recorded in any of the biodiverse pastures. The mean contribution of the sown
256 and un-sown (non-grass species) plant species to the overall biomass was 25.4% in
257 the biodiverse pastures. This contribution was largely comprised of ribwort
258 plantain. The total lipid and FA composition of the conventional and biodiverse pastures
259 immediately prior to start of the lamb grazing study is presented in Table 4. There were
260 no statistically significant ($P>0.05$) differences in the individual FA contents of the two
261 pasture types. The predominant FA was 18:3 n-3 and accounted for approximately
262 50% of the total FA.

263

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

269

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

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

279

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

288

289 Subcutaneous fat contained 47,261 and 46,723 mg total FA/100 g tissue from lambs
290 grazed control and biodiverse pastures respectively (Table 5). The content of *trans*-10
291 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-
292 3 and total n-3, n-6, and very long chain n-3 PUFA were all higher ($P < 0.05$) in
293 subcutaneous fat from lambs grazed on biodiverse pasture compared with control
294 pasture (Supplementary table S3).

295

296 **Discussion**

297 *In vitro* biohydrogenation

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

301 plant species may explain some of the differences in plant FA contents observed. The
302 *in vitro* biohydrogenation characteristics were similar for the six plant species studied.
303 Selfheal and birdsfoot trefoil displayed the greatest accumulation of 18:0 but these
304 plants contained the greatest initial amounts of total PUFA. Rate of disappearance of
305 18:2 n-6 was similar for all plants apart from knapweed (mean of plants excluding
306 knapweed, 0.12 mg/h, knapweed 0.09 mg/h). This may indicate that knapweed exerts
307 some other effect on rumen microbes and/or their enzymes, either by inhibiting the
308 initial lipolysis or biohydrogenation itself. Indeed, knapweed resulted in a lower
309 accumulation of both *trans*-11 18:1 and 18:0 which suggests less biohydrogenation.
310 Kumarasamy *et al.* (2003) found that serotonin conjugates extracted from the seeds of
311 knapweed had antimicrobial activity. These conjugates may also be present in other
312 fractions of knapweed and therefore conferring potential antimicrobial effects.

313

314 For 18:3 n-3, both selfheal and birdsfoot trefoil displayed similar high values for ED
315 compared with the other plants, and yet the accumulation of 18:0 for birdsfoot trefoil
316 did not increase at the same rate as that of selfheal. This may be due to birdsfoot trefoil
317 inhibiting the intermediary pathways of biohydrogenation, through, for example, the
318 presence of condensed tannins. After ingestion some condensed tannins from
319 birdsfoot trefoil remain free and unbound that may inhibit the extracellular enzyme
320 action of certain bacteria (Barry and Manley, 1986). Min *et al.* (2002) found that
321 including birdsfoot trefoil in the diet of sheep decreased the population of the rumen
322 bacteria *Butyrivibrio proteoclasticus*, which is one of the few bacterial species that
323 conducts the final step of rumen biohydrogenation of 18:3 n-3 (converting *trans*-11
324 18:1 to 18:0). However, if condensed tannins affect bacterial biohydrogenation in this
325 way, no reduction in the initial rate of disappearance of both 18:2 n-6 and 18:3 n-3 was

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

332

333 Compared with previous *in vitro* research, red clover did not appear to perform better
334 than other species in terms of effective disappearance and by-pass of 18:2 n-6 and
335 18:3 n-3. Van Ranst *et al.* (2013) reported lower lipolysis and biohydrogenation of 18:3
336 n-3 and 18:2 n-6 with silages containing increasing amounts of red clover, which may
337 have been due to the presence of polyphenol oxidase (PPO) within the red clover. In
338 the present study however, red clover was being compared not with ryegrass but with
339 other species which may have exerted similar biohydrogenation-inhibiting effects. An
340 *in vivo* study reported higher concentrations of 18:3 n-3 in rumen fluid following the
341 feeding of a 50:50 grass:red clover silage to lambs, compared with a 100% grass
342 silage, suggesting PPO as a possible reason (Campidonico *et al.*, 2016). However
343 there was no difference between the grass:red clover silage and grass:sainfoin silage
344 treatments, with sainfoin suggested as having a different mechanism of action for
345 inhibiting biohydrogenation.

346

347 *Using biodiverse pasture to alter the fatty acid profile of tissues*

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

351 increased the number of different 'sown' and unsown species present in the
352 experimental plots as compared with the control pastures. On average the biodiverse
353 pastures were shown to contain an average of 16 different plant species per plot,
354 compared with 9 for control pastures. However, despite the higher species richness,
355 the most abundant species, and concomitant contributor to the overall plant biomass,
356 was ribwort plantain. The abundance of the remaining species was low and therefore
357 made a substantially smaller contribution to the available biomass available for
358 grazing. Other studies carried out with the aim of introducing different species to create
359 biodiverse pastures have been, for example, four years in duration (e.g. Hopkins *et al.*,
360 1999; Pywell *et al.*, 2002). Therefore a longer period of establishment is required to
361 enable some slower growing species to proliferate following initial sowing in order to
362 create a truly biodiverse pasture.

363

364 The *m. semimembranosus* total FA concentrations were lower than those measured
365 by Whittington *et al.* (2006) comparing different biodiverse systems with a control
366 pasture. The *m. l. thoracis* total FA concentration was similar to that observed by
367 Lourenço *et al.* (2007) comparing an intensive ryegrass pasture with an established
368 biodiverse pasture. There were few differences in the FA profile between the pasture
369 treatments for *m. l. thoracis*. Lourenço *et al.* (2007) observed a number of differences
370 in this tissue between animals grazing biodiverse or intensive *lolium perenne*-based
371 pastures, including a higher 18:2 n-6 resulting in an increased n-6:n-3 ratio for the
372 biodiverse treatment. In the study of Lourenço *et al.* (2007) the lambs grazed the
373 biodiverse pastures for a period of 12 weeks (84 days) compared to 50 days in the
374 present study. This shorter grazing period may reflect some of the differences in the
375 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

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

429

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

439

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446

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552

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

Fatty acid	Plant ¹					
	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 <i>cis</i> -9	1.34	1.75	2.28	1.47	5.69	2.44
18:2 <i>cis</i> -9, <i>cis</i> -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 <i>cis</i> -9, <i>cis</i> -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

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

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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 <i>cis</i> -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 <i>trans</i> -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 <i>cis</i> -9, <i>cis</i> -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

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

572 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
573 acids presented.

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

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581 **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 <i>cis</i> -9, <i>cis</i> -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

582 ¹ Where BT – Birdsfoot trefoil; K – Knapweed; RP – Ribwort plantain; RC – Red clover; S –

583 Selfheal; Y – Yarrow.

584 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

585 in the flasks at time t , x is the non-hydrogenatable fraction (mg), y is the hydrogenatable

586 fraction (mg), z is the rate of disappearance of fraction y (/h), and t is incubation time (h;

587 Ørskov & McDonald, 1979)

588 ³ R-squared value for the curve fit

589 ⁴ ED - Effective disappearance (mg/g DM) of 18:2 n-6 or 18:3 n-3 using a ruminal rate of

590 passage (k) of 0.03 (Alcaide *et al.*, 2000)

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

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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.	<i>P</i> 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 <i>cis</i> -9, <i>cis</i> -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		SEM	<i>P</i> ¹		
	Conventional	Biodiverse		Treatment	Block	Treatment x block
<i>M. Longissimus Thoracis</i>						
18:1 <i>trans</i> -11	45.7	39.6	5.23	0.421	0.306	0.457
18:2 <i>cis</i> -9, <i>cis</i> -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 <i>cis</i> -MUFA ³	569	592	65.5	0.799	0.585	0.166
Total <i>trans</i> -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
<i>M. Semimembranosus</i>						
18:1 <i>trans</i> -11	75.4	61.5	4.40	0.037	0.187	0.018
18:2 <i>cis</i> -9, <i>cis</i> -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 <i>cis</i> -MUFA	865	819	55.3	0.557	0.648	0.048
Total <i>trans</i> -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 <i>trans</i> -11	1638	1494	81.7	0.228	0.077	0.615

18:2 <i>cis</i> -9, <i>cis</i> -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 <i>cis</i> -MUFA	17681	16744	866.5	0.454	0.177	0.065
Total <i>trans</i> -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

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

623 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.

624 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*-
625 15 24:1

626 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

627 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

628 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.

629 ⁷ Sum of n-3 and n-6 PUFA.

630 ⁸ CLA – conjugated linoleic acid

631 ⁹ VLC – very long chain

632

633 **Figure captions**

634

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 ($P=0.773$).

638