

Effect of a whey protein and rapeseed oil gel feed supplement on milk fatty acid composition of Holstein cows

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1 **Interpretive summary: Effect of a whey protein and rapeseed oil gel feed supplement on**
2 **milk fatty acid composition of Holstein cows**

3 *Kliem*

4 Supplementing dairy cow diets with rapeseed oil decreases saturated and increases
5 monounsaturated fatty acids in milk fat. To maximise this effect (and to minimise *trans* fatty
6 acids), the oil needs to be protected from the cow rumen. This study investigated the use of
7 supplemental lipid-protein gels of rapeseed oil, to improve milk fatty acid profile. The
8 supplements were effective at achieving this.

9

10 WHEY PROTEIN GEL OF RAPESEED OIL

11

12 **Effect of a whey protein and rapeseed oil gel feed supplement on milk fatty acid**
13 **composition of Holstein cows**

14

15

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36 ABSTRACT

37 Isoenergetic replacement of dietary saturated fatty acids (**SFA**) with *cis*-monounsaturated fatty
38 acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) can reduce cardiovascular disease
39 (**CVD**) risk. Supplementing dairy cow diets with plant oils lowers milk fat SFA concentrations.
40 However, this feeding strategy can also increase milk fat *trans* FA (**TFA**), and negatively
41 impact rumen fermentation. Protection of oil supplements from the rumen environment is
42 therefore needed. In the present study a whey protein gel (**WPG**) of rapeseed oil (**RO**) was
43 produced for feeding to dairy cows, in two experiments. In Experiment 1 four multiparous
44 Holstein-Friesian cows in mid-lactation were used in a change-over experiment, with 8-d
45 treatment periods separated by a 5-day washout period. Total mixed ration diets containing 420
46 g RO or WPG providing 420 g of RO were fed and the effects on milk production, composition
47 and FA concentration were measured. Experiment 2 involved four multiparous mid-lactation
48 Holstein-Friesian cows in a 4 x 4 Latin square design experiment, with 28-d periods, to
49 investigate the effect of incremental dietary inclusion (0, 271, 617 and 814 g/d supplemental
50 oil) of WPG on milk production, composition and FA concentration in the last week of each

51 period. There were minimal effects of WPG on milk FA profile in experiment 1, but *trans*-18:1
52 and total *trans*-MUFA were higher after 8 days of supplementation with RO than with WPG.
53 Incremental diet inclusion of WPG in experiment 2 resulted in linear increases in milk yield,
54 *cis*- and *trans*-MUFA and PUFA, and linear decreases in SFA (from 73 to 58 g/100 g FA), and
55 milk fat concentration. The WPG supplement was effective at decreasing milk SFA
56 concentration by replacement with MUFA and PUFA in experiment 2, but the increase in TFA
57 suggested that protection was incomplete.

58

59

INTRODUCTION

60 There is evidence that cardiovascular disease (CVD) risk can be reduced by the isoenergetic
61 replacement of saturated fatty acids (SFA) with *cis*-monounsaturated fatty acids (MUFA) or
62 polyunsaturated fatty acids (PUFA) in the human diet (Vafeiadou et al., 2015). In the United
63 Kingdom, milk and dairy products contribute about 25 and 28 % of total SFA consumed by
64 men and women, respectively (Bates et al., 2014), with higher contributions in other countries
65 (Hulshof et al., 1999). Altering the fatty acid (FA) composition of milk and dairy products by
66 replacing SFA with MUFA offers an opportunity to lower SFA intake while maintaining the
67 contribution of these foods to the balanced human diet.

68

69 Plant oil supplements are an effective dietary strategy to decrease milk fat SFA concentrations,
70 by replacement with *cis*-MUFA (Kliem and Shingfield, 2016). However, *cis*-MUFA-rich plant
71 oils (such as rapeseed) and oilseed preparations can undergo biohydrogenation/isomerisation
72 (both of *cis*-9 18:1, and also 18:2 n-6 and 18:3 n-3) in the rumen leading to the formation of
73 *trans*-double bond containing intermediates (Harfoot and Hazlewood, 1997, McKain et al.,
74 2010). Other studies using rapeseed supplements to replace milk fat SFA with *cis*-MUFA, also
75 reported concomitant increases in *trans*-fatty acids (TFA; Givens et al., 2009, Kliem et al.,

76 2011). These comprise of mainly *trans*-18:1 isomers, and smaller quantities of *trans*-containing
77 non-conjugated 18:2 isomers. At current human intake levels ruminant-derived TFA (**rTFA**)
78 are thought not to have negative effects on human health (Mozaffarian, 2006), although further
79 research is required to inform on the isomer-specific effects of rTFA (Gebauer et al., 2011).
80 Prospective studies have proved inconsistent, with some associating rTFA intake with
81 increased CVD risk in women (Laake et al., 2012), and others reporting no such association
82 (Bendsen et al., 2011). A meta-analysis of thirteen clinical studies reported that there was no
83 effect of rTFA on certain risk factors of CVD with intakes up to 4.19 % energy intake (Gayet-
84 Boyer et al., 2014). However, as rTFA and industrially-derived TFA are 40% similar in terms
85 of isomer profile (Mensink and Nestel, 2009), and with mandatory food labelling for *trans* fats
86 in the USA, and legislation to minimise *trans* fats in food (Denmark, Austria, Hungary, Latvia),
87 efforts should be sought to protect any supplemental unsaturated oils from rumen
88 biohydrogenation.

89
90 Rumen inertness technologies for dietary supplements vary widely in their effectiveness
91 (especially for highly unsaturated oils), but lipid composite gels may offer a practical solution
92 (Gadeyne et al., 2016). These gels comprise of an aqueous protein-lipid emulsion which is
93 heat-treated to induce gelatinisation (Rosenberg and DePeters, 2010), and have been used to
94 successfully increase milk PUFA content in dairy cows (Carroll et al., 2006; Heguy et al., 2006)
95 and goats (Weinstein et al., 2016). Comparison with calcium salts has demonstrated that whey
96 protein gel was more effective at enhancing milk PUFA concentration (Heguy et al., 2006).

97
98 The objectives of this study were to assess the effect of feeding a whey protein composite gel
99 of rapeseed oil on feed intake, milk yield and composition, and milk FA profile in lactating
100 Holstein cows. These objectives were addressed in two experiments; experiment 1 was a pilot

101 study to compare the effects of whey protein gel of rapeseed oil with those of unprotected
102 rapeseed oil, with the hypothesis was that the whey protein gel supplement would minimise
103 rumen exposure of the unsaturated FA in rapeseed oil, and therefore result in lower milk TFA
104 concentration than an unprotected rapeseed oil supplement. Experiment 2 was conducted to
105 determine effects of feeding increasing amounts of the whey protein gel of rapeseed oil
106 supplement on milk production and composition.

107

108

MATERIALS AND METHODS

109 *Experiment 1*

110 *Experimental design, animals and management.* All experimental procedures used
111 were licensed, regulated and inspected by the UK Home Office under the Animals (Scientific
112 Procedures) Act, 1996. Four multiparous Holstein cows of mean (\pm standard error) parity of
113 3.5 (\pm 0.25), milk yield of 39.2 (\pm 1.55) kg/d, and 86.2 (\pm 19.88) DIM were used in a
114 changeover study lasting 21 days in total, with 8-d treatment periods. Animals were randomly
115 allocated to one of two treatments (2 cows each) for 8 days (period 1). This was followed by a
116 5 day “wash-out” period when animals reverted to the pre-trial diet, then animals switched
117 dietary treatments for another 8 days (period 2). Cows were housed in a cubicle yard with
118 rubber chip filled mattresses and wood shavings as required as additional bedding. In the
119 cubicle yard individual feeding was achieved using an electronic identification system and
120 pneumatic feed barrier (Insentec, Marknesse, the Netherlands). Clean water was constantly
121 available via a water trough. Cows were milked through a conventional herringbone parlour
122 twice daily at 06:00 and 16:00h.

123

124 *Experimental diets.* Diets were offered as TMR (forage:concentrate ratio 53:47 on a
125 DM basis) with the forage consisting of maize and grass silage, hay and straw (267, 209, 36

126 and 18 g/kg DM respectively; Table 1). Treatments consisted of additional rapeseed oil (**RO**;
127 TMR plus the addition of 420 g food grade RO/d) or whey protein gel of rapeseed oil (**WPG**;
128 TMR plus the addition of 1400 g WPG/d). Supplements were included incrementally so that
129 140, 280, and 420 g of RO or 467, 933 and 1400 g of WPG was fed on days 1, 2 and 3-8,
130 respectively. The RO diet also included added whey protein isolate (UltraWhey 80, Volac
131 International Ltd., Royston, UK; 55, 111 and 167 g for days 1, 2 and 3-8, respectively) to
132 balance the WPG protein content. RO, WPG and whey protein isolate were added to the TMR
133 so that the concentrations of other TMR ingredients were diluted. The WPG supplement was
134 prepared using the methods of (Carroll et al., 2006); whey protein isolate (UltraWhey 80, Volac
135 International Ltd., Royston, UK; composition: DM 955 g/kg fresh weight, protein, fat, lactose
136 and ash 820, 70, 40 and 35 g/kg DM, respectively) was dissolved in tap water at 40°C to create
137 a 17% (w/v) whey protein solution. This was mixed (Silverson LART High Shear Mixer,
138 Silverson Machines Inc., MA, USA) with food grade RO for 5 minutes to yield a product
139 containing approximately 30% oil (fresh weight basis). This mixture was homogenised twice
140 using a single stage high-pressure homogeniser (Rannie, No 2786/54, 100 bar), and the
141 resulting whey protein/rapeseed oil emulsion decanted into 400 g food cans, sealed under
142 vacuum, and heat-treated at 120°C for 138 min (Fraser static steam retort, John Fraser & Sons
143 Ltd., Newcastle-upon-Tyne, UK). This created a gel which contained (on a DM basis) 720 g/kg
144 oil and 280 g/kg whey protein. Following heat treatment, the cans were allowed to cool and
145 stored sealed at 2°C until further use, which was within one month. For feeding, the gel was
146 removed from the can, roughly chopped and mixed with the concentrate portion of the diet
147 using a hand-held rotary paint mixer immediately before TMR preparation. The RO and whey
148 protein isolate were added in a similar manner. During the wash-out period, a basal TMR was
149 fed containing no supplemental fat (Table 1). Cows were offered diets as equal meals at 0830
150 and 1600 h.

151

152 **Experimental sampling.** Prior to the addition of WPG, RO or whey protein isolate, a
153 bulked sample of the basal TMR was taken for nutrient composition and fatty acid (FA)
154 analysis, and stored at -20°C. A sample of the WPG and RO were retained for subsequent FA
155 analysis. Refused feed was removed and weighed daily; fresh weights were recorded and on
156 day 8 of each period a composite of the refused feed was dried at 60°C for 48 h to determine
157 individual daily DM intakes.

158

159 Milk yield was recorded daily throughout the study. Samples of milk for the determination of
160 composition by mid-infrared spectral (MIRS) analysis were collected at each milking
161 throughout the experiment and preserved with potassium dichromate (1 mg/ml; Lactabs;
162 Thompson and Capper, Runcorn, UK). Additional samples of unpreserved milk were collected
163 (am + pm) at the beginning and end of each period (days 1 and 8 from periods 1 and 2), stored
164 at -20°C, composited according the milk yield, and used for FA analysis.

165

166 **Chemical analysis.** The fatty acid profile of RO was analysed using a modified version of the
167 one step transesterification method of Sukhija & Palmquist, (1988). Briefly, 50 mg oil was
168 incubated with an internal standard (1 mg methyl heneicosanoate, Sigma Aldrich Company
169 Ltd., Dorset, UK) at 60°C in the presence of 0.4 M sulphuric acid in methanol and toluene as
170 an extraction solvent, for 2 h (oil) or 3 h (TMR). Following neutralisation, the resulting fatty
171 acid methyl esters (FAME) in toluene were allowed to stand over sodium sulphate for 30 min
172 to remove methanol residues before being quantified by gas chromatography (GC; Bruker 350,
173 Bruker, Germany). The GC was equipped with a flame ionisation detector and 100 m fused
174 silica capillary column (CP-SIL 88, Agilent Technologies, Cheshire, UK), and GC conditions

175 were as published previously (Kliem *et al.*, 2013). FA were quantified using internal standard
176 peak area, and the results were expressed as g/100 g FA.

177

178 Milk fat, crude protein, lactose, urea and casein were determined by MIRS (Foss Electric Ltd.,
179 York, UK) as described previously (Kliem *et al.*, 2008). Milk samples taken at the beginning
180 and end of each period were analysed for FA profile according to the method of Kliem *et al.*
181 (2013a). Briefly, lipid in 1 ml thawed, warmed (to 40°C) milk was extracted in duplicate using
182 a mixture of diethyl ether and hexane (IDF 1: 2010 [E], International Dairy Federation, 2010,
183 Brussels, Belgium) and extracts were transesterified to FAME according to previously
184 described procedures (Kliem *et al.*, 2013a). GC conditions and FAME identification were as
185 described above. Carbon deficiency in the flame ionization detector response for FAME
186 containing 4- to 10-carbon atoms was accounted for using a combined correction factor which
187 also converted FAME to FA (Ulberth *et al.*, 1999). All milk FA results were expressed as g
188 /100 g total FA.

189

190 ***Data analysis.*** Milk yield, DMI, and milk FA concentrations on day 8 of each period
191 and the difference between days 1 and 8 for each period were analysed (n=8) using the mixed
192 procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). The model tested fixed effects
193 of treatment and period, and random effect of cow, with period as a repeated effect within cow.
194 Compound symmetry, heterogeneous compound symmetry, first-order autoregressive,
195 heterogeneous first-order regressive or an unstructured covariance structure was used for
196 repeated measures analysis, based on goodness of fit criteria for each variable analysed. Least
197 squares mean results for day 8 and differences between days 1 and 8 are reported. Differences
198 were deemed significant when $P < 0.05$.

199

200 ***Experiment 2***

201

202 ***Experimental design, animals and management.*** Four multiparous Holstein-Friesian
203 cows of mean (\pm standard error) parity 2.5 (\pm 0.25), milk yield 42.6 (\pm 0.90) litres/day and 116
204 (\pm 1.2) days in lactation were used in a balanced 4 x 4 Latin square design experiment with 14
205 day periods. Cows were housed as in Experiment 1. Cows were milked through a conventional
206 herringbone parlour twice daily at 06:00h and 16:00h.

207

208 ***Experimental diets.*** Diets were offered as a TMR (Forage:concentrate ratio 50:50 on a
209 DM basis) with the forage consisting of maize silage and grass silage (750 and 250 g/kg of
210 forage dry matter respectively; Table 2). Treatments consisted of a control diet containing no
211 supplemental lipid (control) or the same basal diet with whey protein gel fed at incremental
212 inclusion rates calculated to supply 300, 600 and 900 g oil/d (**WPG300, WPG600, WPG900,**
213 respectively) at a predicted DMI of 25 kg/d. The whey protein gel replaced cracked wheat in
214 the TMR. This whey protein gel supplement was manufactured slightly differently to before,
215 in that larger (2.9 kg capacity) food cans were used for the heat treatment, and a higher capacity
216 homogenizer (Rannie 12-16.50 Lab Homogeniser) was used to facilitate preparation of a larger
217 batch. WPG was added to the concentrate portion of the diet as before, prior to TMR mixing.
218 Varying amounts of whey protein were added such that the total amount of whey protein
219 (regardless of treatment) was the same for each diet on a DM basis (Table 1). Diets were
220 formulated to be isonitrogenous. Cows were offered diets as equal meals at 0900 and 1600 h.
221 Uneaten feed was removed and weighed prior to the morning feed.

222

223 ***Experimental sampling.*** Individual forage components of experimental diets and the
224 TMR were sampled daily over the last four days of each period. Oven DM contents were

225 determined daily by drying at 100°C for 23 h to allow for adjustments of fresh weight inclusion
226 rates and to ensure that the DM composition of experimental diets was maintained. Straw and
227 concentrate components were sampled over the same time, and composited. Refused feed was
228 removed and weighed daily; fresh weights were recorded and during measurement weeks
229 (week two of each period) a weekly composite of the refused feed was dried at 60°C for 48 h
230 to determine individual daily DM intakes. Samples of rapeseed oil and whey protein gel were
231 retained at -20°C for subsequent chemical analysis.

232

233 Milk yield was recorded daily and samples of milk for composition and FA analysis were taken
234 as outlined in Experiment 1.

235

236 ***Chemical analysis.*** Samples of each forage, concentrate, whey protein gel and rapeseed
237 oil were analysed for OM, CP, NDF, ADF, starch and water soluble carbohydrate content
238 according to reference procedures outlined previously (Kliem et al., 2008). The TMR ME
239 values were predicted using equations derived from neutral cellulase plus gamanase
240 digestibility (MAFF, 1993). Milk fat, crude protein, lactose, urea and casein were determined
241 by MIRS analysis as described previously. Lipids in 1 ml of milk and appropriate weights of
242 forage, concentrate, whey protein gel and rapeseed oil samples were analysed as in Experiment
243 1.

244

245 ***Data analysis.*** Averages of DMI, FA intake, milk production, milk composition and
246 milk FA concentrations for each cow and treatment (n=16) were analysed using the mixed
247 procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC) and a model testing fixed effects
248 of treatment and period, and random effects of cow, with period as a repeated effect within
249 cow. Data analysed were averaged for the last 4 days of each period (with the exception of milk

250 FA concentration). Compound symmetry, heterogeneous compound symmetry, first-order
251 autoregressive, heterogeneous first-order regressive or an unstructured covariance structure
252 was used for repeated measures analysis, based on goodness of fit criteria for each variable.
253 Orthogonal contrasts were used to test for linear, quadratic and cubic effects of whey protein
254 gel inclusion. Differences were deemed significant when $P < 0.05$.

255

256

RESULTS

257 *Experiment 1*

258 The composition of the basal TMR used in Experiment 1 is presented in Table 1. The rapeseed
259 oil comprised of 60 g/100 g *cis*-9 18:1, 19 g/100 g 18:2 n-6, 8 g/100 g 18:3 n-3, 5 g/100 g 16:0
260 and 2 g/100 g 18:0 (*cis*-13 22:1 content of 0.01 g/100 g). Inclusion of either RO or WPG in the
261 diet had no effect ($P > 0.05$) on DMI or milk, fat or protein yield (Table 2). There was no
262 obvious observed selection against the WPG supplement. Supplementing cow diets with either
263 RO or WPG had very few effects on individual milk FA (Table 3; Supplementary Table 1).
264 Due to the lack of resolution of chromatogram peaks only total CLA was reported, although
265 this will have been predominantly *cis*-9, *trans*-11 CLA. Milk fat 16:0 concentration was lower
266 ($P = 0.036$) after 8 days of WPG supplementation compared with RO, but the difference
267 between days 0 and 8 for WPG was not different ($P = 0.474$). This may have influenced the
268 total SFA concentration, which was numerically ($P = 0.088$) lower after WPG compared with
269 RO supplementation. Supplementation had an effect on TFA concentration in milk fat; RO
270 supplementation increased ($P = 0.015$) *trans*-9 16:1 concentration to a greater extent than WPG
271 (Table 3), after 8 days. In addition, WPG milk fat concentrations of *trans*-6-8 16:1 and several
272 *trans*-18:1 isomers (*trans*-11 18:1, *trans*-12 18:1; Table 3, Supplementary Table 1) were
273 numerically lower compared with RO. This contributed to an overall lower ($P = 0.002$) total
274 *trans* MUFA content after 8 days of supplementation with WPG compared with RO (Table 3).

275 There was no difference ($P = 0.140$) in total TFA between the two treatments after 8 days, but
276 the increase observed in total TFA for both treatments was greater ($P = 0.031$) for the RO
277 treatment (Table 3). Supplementation had no effect on most PUFA, apart from total CLA
278 (Table 3) and *trans*-11, *cis*-15 18:2 (Supplementary Table 1) which was numerically higher
279 following RO supplementation.

280

281 ***Experiment 2***

282 Diets were formulated so that the WPG supplement replaced cracked wheat in the TMR (Table
283 1), thus predicted ME concentration increased with increasing WPG inclusion (Table 2). The
284 rapeseed oil comprised of 61 g/100 g *cis*-9 18:1, 18 g/100 g 18:2 n-6, 9 g/100 g 18:3 n-3, 5
285 g/100 g 16:0 and 2 g/100 g 18:0.

286

287 Incremental inclusion of WPG resulted in a cubic effect ($P = 0.008$) on DMI, which was
288 decreased by 0.5 kg/d at the highest inclusion level (WPG900; Table 4). As with Experiment
289 1, there was no observed selection against the WPG supplement. WPG caused a linear increase
290 ($P < 0.01$) in 16:0, 18:0, *cis*-9 18:1, 18:2 n-6, 18:3 n-3 and total FA intake (Table 4).
291 Incremental inclusion of WPG linearly increased ($P = 0.002$) milk yield (Table 4). There was
292 also a linear ($P = 0.007$) decrease in milk fat concentration when WPG was included
293 incrementally (Table 4).

294

295 WPG supplementation linearly decreased ($P < 0.01$) most short- and medium-chain SFA, apart
296 from 4:0 and 6:0 (Table 5). Milk fat concentration of 16:0 was decreased ($P < 0.001$) by 38 %
297 when comparing control vs WPG900 (Table 5). Linear effects were also observed for 16:1 FA,
298 with the WPG diets linearly increasing ($P < 0.05$) *trans*-6-8, *trans*-9 and *trans*-11-13 16:1, and
299 decreasing ($P < 0.01$) *cis*-9, *cis*-11 and *cis*-13 16:1 (Table 5). There was a tendency for WPG

300 supplementation to linearly increase ($P = 0.087$) milk fat 18:0 concentration (Table 5).
301 Incremental inclusion of WPG linearly increased ($P < 0.05$) total CLA, 18:3 n-3, 20:0, *cis*-8
302 20:1, *cis*-11 20:1 and 20:3 n-3, and linearly decreased ($P < 0.05$) 20:3 n-6, 20:4 n-6 and 20:5
303 n-3 (Table 5).

304

305 In terms of MUFA, increasing WPG supplementation resulted in linear increases ($P < 0.05$) in
306 most *trans*- and *cis*-18:1 isomers identified (Table 6). In milk from control-fed cows, the
307 predominant *trans*-18:1 isomer was *trans*-11 18:1. However, with increasing WPG inclusion,
308 *trans*-10 18:1 became the predominant isomer (Table 6). This isomer showed a tendency ($P =$
309 0.057) to linearly increase with increasing WPG inclusion (Table 6). Milk fat concentration of
310 *cis*-9 18:1 linearly increased ($P < 0.001$) by almost 60 % when comparing control with
311 WPG900 (Table 6). Linear increases in *cis*-11 and *cis*-13 18:1 were also observed. Increasing
312 WPG inclusion linearly increased ($P < 0.05$) *cis*-9, *trans*-13, *cis*-9, *trans*-12, *trans*-9, *cis*-12,
313 *trans*-11, *cis*-15 and *cis*-9, *cis*-12 18:2 isomers (Table 7).

314

315

DISCUSSION

316 Feeding *cis*-MUFA rich plant oil sources to dairy cows is effective at replacing milk fat SFA
317 with *cis*-MUFA (Kliem & Shingfield, 2016). However this dietary strategy also causes an
318 increase in milk fat concentration of TFA. Research continues into protection technologies to
319 minimise rumen exposure of dietary unsaturated fatty acids, but many are not cost effective or
320 are inconsistent in their effectiveness (Jenkins and Bridges, 2007). Formation of whey protein
321 composite gels, where oil is emulsified with an aqueous whey protein phase before being heat
322 treated to form a solid gel matrix, offers one option for rumen protection (Gadeyne et al., 2016).
323 As well as optimising the amount of unsaturated FA available for absorption by the cow, rumen

324 protection of oils also minimises undesirable effects of unsaturated oils on the rumen microbial
325 environment.

326

327 In Experiment 1, supplementing lactating cows with 420 g rapeseed oil as either unprotected
328 oil or WPG had little impact on intake, milk yield and milk composition. Previous studies
329 supplementing cows with similar quantities of unprotected rapeseed oil also reported no effect
330 on milk yield or protein composition (Rego et al., 2009; Halmemies-Beauchet-Filleau et al.,
331 2011), but a decrease in milk fat content was observed when 500 g/d was fed over 28 d (Rego
332 et al., 2009). Experiment 1 was very short-term in comparison, which may in part explain the
333 lack of impact of rapeseed oil or WPG on milk yield and component concentration. Other
334 shorter duration studies supplementing lactating cows with similar quantities of rapeseed oil
335 (DePeters et al., 2001) or WPG of sunflower oil (Carroll et al., 2006) reported similar results
336 in terms of milk yield and composition.

337

338 Experiment 2 involved incremental supplementation of WPG, with treatments providing 271,
339 617 and 814 g supplemental rapeseed oil/d, over longer experimental periods. The slight
340 decrease in DMI with incremental WPG inclusion observed may reflect increased post-ruminal
341 supply of lipid, especially at the higher inclusion level, where DMI was lower than control.
342 Drackley et al. (2007) reported decreased DMI when high oleic acid sunflower oil was infused
343 directly into the abomasum (bypassing the rumen), concluding that free MUFA decrease DMI
344 in a dose-dependent manner. Similarly, Benson and Reynolds (2001) observed a decrease in
345 DMI in cows infused into the abomasum with 400 g rapeseed oil daily, which was associated
346 with an increase in blood concentrations of anorexic gut peptides. It is not known if there was
347 dissociation of lipid from the WPG in the rumen, but at higher intake levels it is possible that
348 some dissociation occurred, which would have a negative impact on the rumen environment

349 (Lock and Shingfield, 2004). Previous research involving whey protein gel complexes of
350 unsaturated oils reported no difference in DMI when compared with control diets containing
351 unprotected oils, but these involved lower supplementation levels (Carroll et al., 2006; Heguy
352 et al., 2006).

353

354 Increasing WPG inclusion in Experiment 2 increased milk yield substantially, despite the small
355 decrease in DMI due to WPG feeding. The WPG incrementally replaced wheat in the diet,
356 increasing the calculated ME content of the treatment diets. A previous study involving
357 incremental supplementation of dairy cow diets with a rumen inert (calcium salt) rapeseed oil
358 reported no effect on milk yield, despite an increase in ME concentration of the diets (Kliem
359 et al., 2013b). This discrepancy may be due to stage of lactation; in the current study cows were
360 at an earlier stage of lactation, when more of their dietary energy is partitioned towards milk
361 energy output (Kirkland and Gordon, 2001).

362

363 Consistent with previous research involving incremental inclusion of unsaturated oils in dairy
364 cow diets, the WPG600 and WPG900 treatment diets resulted in a decrease in milk fat
365 concentration, whilst milk fat concentration was increased by WPG300 (cubic effect). The
366 decrease in milk fat concentration at higher levels of supplementation is in part due to a dilution
367 effect of increased milk yield, as milk fat yield was not affected. In addition, dietary
368 unprotected oils rich in unsaturated fatty acids can decrease milk fat concentration (Glasser et
369 al., 2008), partially due to a negative impact on the rumen environment (Lock and Shingfield,
370 2004), but also due to the inhibition of milk fat synthesis by longer chain unsaturated FA
371 (Barber et al., 1997). The effect on milk fat concentration indicate that at higher
372 supplementation levels there may have been some dissociation of lipid from the whey protein

373 complex, and/or a greater proportion of *cis*-9 18:1, 18:2 n-6 and 18:3 n-3 from the rapeseed oil
374 reaching the mammary gland.

375

376 There were very few differences in milk fat SFA concentration with supplementation of RO
377 compared to WPG in Experiment 1, which is contrary to the results of Carroll et al. (2006) who
378 fed 3 cows a whey protein isolate gel or a whey protein concentrate gel of soybean oil for 8
379 days and observed a significant decrease in 16:0 concentration that accompanied increases in
380 18:2 n-6 and 18:3 n-3 concentrations in milk fat. At the end of the supplementation period,
381 16:0 concentration was lower with WPG than RO, but as there was no difference in change
382 over time this is probably due to a lower day 1 concentration for WPG. Total SFA tended to be
383 lower with WPG, and the WPG supplement appeared to have little effect on change in SFA.
384 This suggests at the inclusion level used for the short supplementation time both RO and WPG
385 had little impact on milk SFA concentration. Other studies involving similar inclusion levels
386 of rapeseed oil supplemented for longer periods. Rego et al. (2009) supplemented grazing cows
387 with 500 g/d rapeseed oil for 28 d, and reported significant decreases in the milk fat
388 concentration of short and medium chain SFA compared with a control diet containing no oil.
389 Jacobs et al. (2011) reported lower concentrations of some SFA in milk fat from cows
390 supplemented with 408 g/d rapeseed oil for 23 d, compared with a control post-trial period
391 lasting 28 d. In Experiment 2, even the lowest level of WPG inclusion (WPG300) resulted in a
392 13% decrease ($P = 0.003$) in milk fat concentration of 16:0, which contributed to a lower total
393 SFA, compared with Control. At the higher intake levels, the effect of WPG inclusion on 8:0,
394 10:0, 12:0, 14:0 and 16:0 was consistent with previous studies involving rapeseed oil. Kliem
395 et al. (2011) supplemented cow diets with 750, 1000 or 1250 kg/d rapeseed oil in the form of
396 milled rapeseed, and reported linear decreases in 6:0, 8:0, 10:0, 12:0, 14:0 and 16:0. In the
397 current study, 16:0 concentration was decreased more than 13 g/100 g FA, which was a similar

398 response to that observed when calcium salts of rapeseed oil were supplemented at similar oil
399 levels (Kliem et al., 2013b). Around half of the 16:0 in milk fat is derived from mammary *de*
400 *novo* synthesis, with the remainder being supplied by the circulation (Hawke and Taylor, 1995).
401 The WPG supplement contained 13 g/100 g FA 16:0, so incremental WPG inclusion increased
402 16:0 intake. Therefore the decrease in milk fat 16:0 is certainly related to inhibition of
403 mammary *de novo* synthesis. In contrast to the study of Kliem et al. (2013b) an increase in milk
404 18:0 concentration was not observed, despite an increased 18:0 intake. Milk fat 18:0 usually
405 increases following supplementation with 18-carbon MUFA and/or PUFA-rich oilseeds
406 (Glasser et al., 2008) following complete biohydrogenation of these FA. The lack of change in
407 milk 18:0 indicates that unsaturated FA in the rapeseed oil present in WPG may have been
408 afforded some protection from rumen biohydrogenation. Carroll et al. (2006) compared
409 different whey protein gel of sunflower oil preparations, and reported no difference in milk fat
410 18:0 concentrations when compared with control diets containing no oil.

411

412 The decreased milk SFA concentration observed when supplementing cow diets with rapeseed
413 oil is generally balanced by an increase in both *cis*- and *trans*-MUFA (Glasser et al., 2008;
414 Kliem and Shingfield, 2016). Milk fat *cis*-9 18:1 is derived from dietary sources, and also from
415 desaturation of 18:0 by mammary Δ^9 desaturase. In Experiment 1, the shorter supplementation
416 time led to no change in milk *cis*-9 18:1 (or total *cis*-MUFA) concentration for both the RO
417 and WPG diets. In Experiment 2, there was a linear increase in *cis*-9 18:1 concentration, which
418 was reflected in total *cis*-MUFA. This *cis*-9 18:1 increase was not as high as some studies have
419 observed for unprotected rapeseed oil, when calculated on a per weight rapeseed oil consumed
420 (1.0 vs 1.7 g/100 g FA increase per 100 g oil from Rego et al., 2009), but was higher than others
421 (Givens et al., 2009; Jacobs et al., 2011) . WPG also linearly increased milk fat concentration

422 of minor *cis*-18:1 isomers, such as *cis*-11, *cis*-13 and *cis*-16 18:1. These isomers have been
423 identified as biohydrogenation intermediates of PUFA such as 18:2 n-6 (Jouany et al., 2007).

424

425 One of the main goals for creating rumen inert supplements of unsaturated oils is to minimise
426 increases in milk fat TFA concentration, which are formed as intermediates of *cis*-MUFA and
427 PUFA biohydrogenation in the rumen. The longer WPG supplementation period of Experiment
428 2 resulted in an increased concentration of most *trans*-18:1 and 18:2 isomers identified, and
429 these concentrations increased linearly with incremental WPG inclusion. *Trans*-11 18:1 is
430 generally the most abundant *trans*-18:1 in milk fat, and is a common intermediate of 18:2 n-6
431 and 18:3 n-3 metabolism in the rumen (Palmquist et al., 2005). In addition to *trans*-11 18:1, *in*
432 *vitro* studies have shown that biohydrogenation of *cis*-9 18:1 by rumen microorganisms can
433 yield a range of *trans*-18:1 isomers, including *trans*-9, *trans*-10 and *trans*-12 18:1, some of
434 which can be further isomerised (Mosley et al., 2002). The highest supplementation level in
435 Experiment 2 resulted in *trans*-10 18:1 being the predominant *trans*-18:1 isomer. This isomer
436 can increase following altered rumen fermentation in response to certain diets, resulting in a
437 shift in 18:2 n-6 biohydrogenation pattern (Bauman et al., 2011). It has been implicated as a
438 possible causative factor in milk fat depression, although abomasal infusion studies report
439 inconsistent results (Shingfield et al., 2009). Intake of 18:2 n-6 in Experiment 2 did increase
440 with incremental inclusion, but not as much as that of *cis*-9 18:1, so it is probably that some of
441 the increase in *trans*-10 18:1 was due to isomerisation of *cis*-9 18:1.

442

443 Recent research has focused on the concentration of *trans*-9 16:1 in milk fat, after studies
444 reported positive associations between circulating concentrations of this FA and human health
445 (Mozaffarian et al., 2010). *Trans*-9 16:1 is thought to arise following rumen metabolism of
446 longer chain PUFA (Shingfield et al., 2012). A human intervention study has reported that

447 some *trans*-9 16:1 may be produced intracellularly following β -oxidation of *trans*-11 18:1
448 (Jaudszus et al., 2014). However it is not known if a similar process occurs in bovine cells. In
449 both experiments, milk concentrations of *trans*-9 16:1 increased in line with *trans*-11 18:1. But
450 as intake of PUFA also increased, it is difficult to say whether milk *trans*-9 16:1 was derived
451 from rumen metabolism, or tissue oxidation of *trans*-11 18:1 from the rumen.

452

453 Results from Experiment 2 demonstrated that increasing supplementation levels of WPG also
454 increase milk fat concentrations of PUFA. The WPG supplement contained 18:2 n-6 and 18:3
455 n-3, and incremental inclusion of WPG resulted in linear increases in the concentration of these
456 FA and their transfer efficiency (18:2 n-6 transfer efficiencies of 0.010, 0.116, 0.118, 0.122, P
457 = 0.002; 18:3 n-3 transfer efficiencies of 0.075, 0.100, 0.105, 0.109, P = 0.001; for control ,
458 WPG300, WPG600 and WPG900, respectively), suggesting that these PUFA were at least
459 partially protected from rumen biohydrogenation. These results improve upon previous data
460 involving unprotected plant oils, which reported transfer efficiencies for 18:2 n-6 and 18:3 n-3
461 of between 0.08 and 0.10, and 0.07 and 0.09, respectively (Halmemies-Beauchet-Filleau et al.,
462 2011). However, increased concentrations of *cis*-9, *trans*-12 18:2 and *trans*-11, *cis*-15 18:2
463 demonstrate that there was some rumen metabolism of 18:2 n-6 (Jouany et al., 2007) and 18:3
464 n-3 (Harfoot and Hazlewood, 1997). Total CLA also increased with incremental WPG
465 inclusion, which reflects increased *trans*-11 18:1 leaving the rumen; most milk *cis*-9, *trans*-11
466 CLA is synthesised by mammary Δ^9 desaturase from *trans*-11 18:1 (Piperova et al., 2002).

467

468 At the highest WPG intake level (814 g rapeseed oil equivalent/d), the increase in total TFA
469 concentration compared with the control diet per 100 g rapeseed oil consumed was 0.52 g/100
470 g total FA. Previous studies from this laboratory have reported varying increases in milk fat
471 TFA, depending on the rapeseed oil preparation (increases of 0.22 and 1.05 g/100 g total FA

472 by Kliem et al. [2011] and Kliem et al., [2013b], respectively). As mentioned previously, the
473 impact of rTFA on human health and disease risk is not entirely clear, and indeed may be
474 isomer-specific. Most studies (prospective cohort and intervention) focus on rTFA as a group,
475 however there is evidence to suggest that consuming butter high in *trans*-11 18:1 or *cis*-9,
476 *trans*-11 CLA may not increase some CVD risk factors (Tholstrup et al., 2006; Tricon et al.,
477 2006). The effect of *trans*-11 18:1 may be metered through its endogenous conversion to *cis*-
478 9, *trans*-11 CLA (Turpeinen et al., 2002). The effect of other *trans* isomers on health is not
479 confirmed, although a butter rich in *trans*-10 18:1 (which was the predominant isomer in milk
480 fat from the high WPG treatments in Experiment 2) has been found to increase CVD risk factors
481 in rabbits when compared with butters rich in *trans*-11 18:1 and a control (Roy et al., 2007).
482 The increases in TFA observed in the current studies would be unlikely to have much of an
483 impact on human health; a previous calculation demonstrated that in order to exceed the
484 maximum recommended level of 2 % EI (SACN, 2007) by changing dairy fatty acid profile
485 alone, would mean producing milk fat containing 17 g/100 g fatty acids (Kliem et al., 2013).
486 However, until isomer-specific effects on human health can be confirmed, increases in rTFA
487 in milk and ruminant meat should be minimised.

488

489 In conclusion, results from the current study suggest that protection of rapeseed oil by the whey
490 protein gel matrix may help to minimise milk TFA concentrations when compared with
491 unprotected rapeseed oil. In addition, WPG is more effective than calcium salts of rapeseed oil
492 FA investigated previously by this group, in terms of minimising milk TFA concentrations.
493 Protection of unsaturated vegetable oils using a lipid composite gel may provide a viable
494 approach to optimising beneficial FA uptake by the cow.

495

496

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