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

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To link to this article DOI: http://dx.doi.org/10.3168/jds.2018-15247

Publisher: American Dairy Science Association

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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**

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35

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

period. There were minimal effects of WPG on milk FA profile in experiment 1, but *trans*-18:1

and total *trans*-MUFA were higher after 8 days of supplementation with RO than with WPG.

Incremental diet inclusion of WPG in experiment 2 resulted in linear increases in milk yield,

cis- and *trans*-MUFA and PUFA, and linear decreases in SFA (from 73 to 58 g/100 g FA), and

milk fat concentration. The WPG supplement was effective at decreasing milk SFA

concentration by replacement with MUFA and PUFA in experiment 2, but the increase in TFA

suggested that protection was incomplete.

INTRODUCTION

There is evidence that cardiovascular disease (**CVD**) risk can be reduced by the isoenergetic

replacement of saturated fatty acids (**SFA**) with *cis*-monounsaturated fatty acids (**MUFA**) or

polyunsaturated fatty acids (**PUFA**) in the human diet (Vafeiadou et al., 2015). In the United

Kingdom, milk and dairy products contribute about 25 and 28 % of total SFA consumed by

men and women, respectively (Bates et al., 2014), with higher contributions in other countries

(Hulshof et al., 1999). Altering the fatty acid (**FA**) composition of milk and dairy products by

replacing SFA with MUFA offers an opportunity to lower SFA intake while maintaining the

contribution of these foods to the balanced human diet.

Plant oil supplements are an effective dietary strategy to decrease milk fat SFA concentrations,

by replacement with *cis*-MUFA (Kliem and Shingfield, 2016). However, *cis*-MUFA-rich plant

oils (such as rapeseed) and oilseed preparations can undergo biohydrogenation/isomerisation

(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

trans-double bond containing intermediates (Harfoot and Hazlewood, 1997, McKain et al.,

2010). Other studies using rapeseed supplements to replace milk fat SFA with *cis*-MUFA, also

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 (± standard error) parity of

113 3.5 (± 0.25), milk yield of 39.2 (± 1.55) kg/d, and 86.2 (± 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

5

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 L4RT 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 -20ºC, 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

7

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

Experiment 2

201

202   ***Experimental design, animals and management.*** Four multiparous Holstein-Friesian

203   cows of mean (± standard error) parity 2.5 (± 0.25), milk yield 42.6 (± 0.90) litres/day and 116

204   (± 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).

11

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

12

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

14

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

15

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

16

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

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