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

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

WHEY PROTEIN GEL OF RAPESEED OIL

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

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ABSTRACT

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

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

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

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

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

MATERIALS AND METHODS

Experiment 1

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

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

and 18 g/kg DM respectively; Table 1). Treatments consisted of additional rapeseed oil (**RO**; TMR plus the addition of 420 g food grade RO/d) or whey protein gel of rapeseed oil (**WPG**; TMR plus the addition of 1400 g WPG/d). Supplements were included incrementally so that 140, 280, and 420 g of RO or 467, 933 and 1400 g of WPG was fed on days 1, 2 and 3-8, respectively. The RO diet also included added whey protein isolate (UltraWhey 80, Volac International Ltd., Royston, UK; 55, 111 and 167 g for days 1, 2 and 3-8, respectively) to balance the WPG protein content. RO, WPG and whey protein isolate were added to the TMR so that the concentrations of other TMR ingredients were diluted. The WPG supplement was prepared using the methods of (Carroll et al., 2006); whey protein isolate (UltraWhey 80, Volac International Ltd., Royston, UK; composition: DM 955 g/kg fresh weight, protein, fat, lactose and ash 820, 70, 40 and 35 g/kg DM, respectively) was dissolved in tap water at 40°C to create a 17% (w/v) whey protein solution. This was mixed (Silverson L4RT High Shear Mixer, Silverson Machines Inc., MA, USA) with food grade RO for 5 minutes to yield a product containing approximately 30% oil (fresh weight basis). This mixture was homogenised twice using a single stage high-pressure homogeniser (Rannie, No 2786/54, 100 bar), and the resulting whey protein/rapeseed oil emulsion decanted into 400 g food cans, sealed under vacuum, and heat-treated at 120°C for 138 min (Fraser static steam retort, John Fraser & Sons Ltd., Newcastle-upon-Tyne, UK). This created a gel which contained (on a DM basis) 720 g/kg oil and 280 g/kg whey protein. Following heat treatment, the cans were allowed to cool and stored sealed at 2°C until further use, which was within one month. For feeding, the gel was removed from the can, roughly chopped and mixed with the concentrate portion of the diet using a hand-held rotary paint mixer immediately before TMR preparation. The RO and whey protein isolate were added in a similar manner. During the wash-out period, a basal TMR was fed containing no supplemental fat (Table 1). Cows were offered diets as equal meals at 0830 and 1600 h.

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

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

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

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

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

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

Experiment 2

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

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

Experimental sampling. Individual forage components of experimental diets and the 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

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

RESULTS

Experiment 1

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

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

Experiment 2

Diets were formulated so that the WPG supplement replaced cracked wheat in the TMR (Table 1), thus predicted ME concentration increased with increasing WPG inclusion (Table 2). The 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 g/100 g 16:0 and 2 g/100 g 18:0.

Incremental inclusion of WPG resulted in a cubic effect (P = 0.008) on DMI, which was decreased by 0.5 kg/d at the highest inclusion level (WPG900; Table 4). As with Experiment 1, there was no observed selection against the WPG supplement. WPG caused a linear increase (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). Incremental inclusion of WPG linearly increased (P = 0.002) milk yield (Table 4). There was also a linear (P = 0.007) decrease in milk fat concentration when WPG was included incrementally (Table 4).

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

supplementation to linearly increase (P = 0.087) milk fat 18:0 concentration (Table 5). Incremental inclusion of WPG linearly increased (P < 0.05) total CLA, 18:3 n-3, 20:0, *cis*-8 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 n-3 (Table 5).

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

Recent research has focused on the concentration of *trans*-9 16:1 in milk fat, after studies reported positive associations between circulating concentrations of this FA and human health (Mozaffarian et al., 2010). *Trans*-9 16:1 is thought to arise following rumen metabolism of 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

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

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

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