Postprandial lipemia and fecal fat excretion in rats is affected by the calcium content and type of milk fat present in Cheddar-type cheeses

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Abstract

The aim of this study was to better understand the effect of calcium on the bioavailability of milk lipids from a cheese matrix using a rat model. Cheddar-type cheeses were manufactured with one of three types of anhydrous milk fat, control, olein or stearin, and salted with or without $CaCl_2$. The cheeses were fed to rats and postprandial lipemia was monitored. Feces were analyzed to quantify fatty acids excreted as calcium soaps. Higher calcium concentration in cheese caused a higher and faster triacylglycerol peak in blood, except for cheeses containing stearin. Furthermore, calcium soaps were more abundant in feces when the ingested cheese had been enriched with calcium and when the cheese was prepared with stearin. Increased lipid excretion was attributable to the affinity of saturated long-chain fatty acids for calcium. Results showed that lipid bioaccessibility can be regulated by calcium present in Cheddar cheese. This study highlights the nutritional interaction between calcium and lipids present in the dairy matrix and confirms its physiological repercussions on fatty acid bioavailability.

Keywords: Cheese, Calcium, Milk fat, Digestion, Calcium soap, Postprandial lipemia.

1. Introduction

When food enters the body, nutrients must be released from the food matrix and broken down into an absorbable form (bioaccessibility). The food matrix acts as a nutrient-release regulator as it disintegrates during the digestion process, which has an impact on the effectiveness of nutrient absorption (Norton, Gonzalez Espinosa, Watson, Spyropoulos & Norton, 2015). Since processing modifies the structure and composition of foods, it can affect their digestibility and, nutrient bioavailability, which corresponds to the fraction of the nutrient that is absorbed and available for physiological functions (McClements, Decker & Park, 2009; Parada & Aguilera, 2007).

Dairy foods are suitable for designing products with enhanced health advantages such as bioactive molecule transport, regulated nutrient release and overall health maintenance (Fruekilde & Høy, 2004; Jaejoon et al., 2011; Turgeon & Rioux, 2011). Among dairy foods, cheese has received special attention because of its widespread consumption. Cheddar cheese is recognized as a source of calcium, which is intrinsically present in milk and is also added during cheesemaking in the form of $CaCl_2$ (Lawrence et al., 2004; Wolfschoon-Pombo, 1997). Calcium plays a key role in determining cheese microstructure. Adding calcium to cheesemilk was shown to reduce the porosity of the cheese matrix, reduce fat losses and increase cheese firmness (Ong, Dagastine, Kentish, & Gras, 2013; Ong, Soodam, Kentish, Powell, & Gras, 2015, Lucey & Fox, 1993). Calcium also plays a major nutritional role in the digestion of milk fat. In the digestive system, calcium enhances lipolysis (i.e. release of fatty acids [FA] from triacylglycerols [TAG]), but limits the absorption of saturated long-chain fatty acids (LCFA). When lipolysis takes place, LCFA accumulate at the lipid droplet surface, which limits lipase access to its substrates (Favé, Coste & Armand, 2004). At intestinal pH (i.e. close to neutrality), LCFA react with calcium, producing insoluble salts, normally referred to as calcium soaps (CS) (Patton, Hamosh, Borgstroem, Lindstroem & Carey, 1985). CS are readily removed from the interface, which enables lipolysis to continue on the newly exposed triglycerides of the fat droplet (Patton & Carey, 1979). This precipitation drives the increase in lipolysis rates (Armand et al., 1992), which in turn results in reduced absorption of LCFA bound to calcium (Hu, Li, Decker & McClements, 2010; Lorenzen, Jensen & Astrup, 2014). The balance between these effects determines the net impact on lipid bioavailability.

Postprandial lipemia is a complex and highly dynamic process that depends on various physiological, genetic and dietary factors that shape the digestion, absorption and metabolic handling of lipids (Armand, 2008; Berry, 2009). The content, composition and structure of fat in a meal are major determinants of postprandial lipemia (Berry, 2009). A small proportion of CS can co-solubilize with fatty acids within the mixed micelles and be gradually absorbed (Jandacek, 1991).

The slower process, in comparison with free FA, can translate into a less abrupt lipemic response. The reduction of the postprandial lipemic peak may influence the metabolism of fatty acids and have a beneficial effect on hyperlipemia and reduce cardiovascular complications in the long term (Lefevre, Kris-Etherton, Zhao & Tracy, 2004; Michalski, 2008; Raynal-Ljutovac et al., 2011; Su, Nzekwu, Cabezas, Redgrave & Proctor, 2009).

Previous studies on *in vitro* digestion of cheese have shown that different structures lead to different behavior during digestion, demonstrating the potential role of the cheese matrix as a lipid-release modulator (Ayala-Bribiesca, Lussier, Chabot, Turgeon & Britten, 2016; Lamothe, Corbeil, Turgeon & Britten, 2012). Additionally, Cheddar-type cheeses with different levels of calcium and different types of milk fats (i.e. with various amounts of LCFA) showed different lipid bioaccessibility profiles during digestion in a static model system (Ayala-Bribiesca, Turgeon & Britten, 2017). In this study, cheeses prepared with a lower proportion of LCFA and enriched with calcium showed a 30% increase in fatty acid bioaccessibility and final lipolysis rates were lower for cheeses prepared with a higher proportion of LCFA. However, to confirm the physiological repercussions of the interaction between calcium and different fatty acids observed *in vitro*, *in vivo* studies are required.

The aim of this study was to assess the effect of calcium on postprandial lipemia and fat excretion following the consumption of Cheddar-type cheeses prepared with anhydrous milk fat (SMF) with different proportions of LCFA. This work was also intended to evaluate the capacity of a relatively simple *in vitro* digestion system used in previous studies (Ayala-Bribiesca et al., 2017) to predict lipid-related physiological responses.

2. Materials and methods

2.1 Cheddar-type cheeses

The cheeses were the same as those used in a previous *in vitro* study, in which detailed information on their manufacture, composition and microstructure is provided (Ayala-Bribiesca et al., 2017). Briefly, Cheddar-type cheeses with different fatty acid profiles were obtained by standardizing their fat content with three different AMF (control, olein and stearin, with final melting points of 35.43, 34.70 and 42.32 °C, respectively) obtained from Fonterra Ltd. (Auckland, New Zealand). Olein AMF had the highest ratio of short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA), the control AMF had the highest amount of unsaturated fatty acids (UFA), and the stearin AMF was the richest in saturated-LCFA (Fig. 1).

The calcium level of the cheeses was modified by salting with NaCl or with a mixture of NaCl + $CaCl_2$. The latter was calculated to maintain a constant ionic strength (Ayala-Bribiesca et al., 2017; Brickley, Lucey & McSweeney, 2009). The cheeses were stored at 4 °C until required for analysis and the experiments, for a total of 15 to 18 weeks. In all, 6 different cheeses were produced with 3 AMF types, each with a regular calcium content and a high calcium content.

All the Cheddar-type cheeses had a composition within the expected ranges for a regular full-fat Cheddar cheese (Health Canada, 2010; Ministry of Justice of Canada, 2014), except for the high calcium content of cheeses that included $CaCl_2$ in the salting step (Table 1). Composition differences among cheeses were within the expected variations for the pilot equipment used.

2.2 *In vivo* protocol

The *in vivo* protocol (Project No. 2011-091-1, Sirul 93470, approved by the Research Ethics Committee of Université Laval and in accordance with the National Institutes of Health Guidelines [National Academy of Sciences, 2011]) was

carried out using 72 young adult male Wistar rats (10 weeks old, Strain code 003, Charles Rivers Laboratories, Montreal, Canada). Upon arrival, the animals were acclimated for 10 days on an ad libitum diet of commercial chow and free access to water. After the acclimation, rats were randomly assigned to each experimental cheese (n = 12).

After the fasting period (11.5 h), a 10-g cheese meal was presented, with any leftovers removed after 30 min. A second cheese meal was presented 7 h later and when the second cheese meal was removed, a 10-g portion of chow was given to the rats in order to avoid the risk of constipation and to prevent any nutritional deficiencies. The chow was left in the cage and no additional food was given to the rats until the next day. Water was always accessible. Weight progression throughout the protocol was normal for all rats, according to the technical specifications of the supplier with no differences among treatments ($P > 0.4$). The rats were trained to ingest the cheese meal within 15 min. As shown in Table 1, a total of 5 rats were excluded during the study due to refusal to rapidly ingest the cheese meal (2 rats) or incomplete results during the postprandial follow-up (3 rats). On day 9, a sample of blood was taken before feeding the cheese meal, to have a fasting baseline. From day 7, feces were collected directly from the animals before the first meal (after the fasting period) and kept at -20 °C until required for analysis. On day 10, an oral lipid-tolerance test (OLTT) was performed after the fasting period using the experimental cheeses as the fat source. The cheese meal was reduced to 7 g to ensure full ingestion of the amount presented and the cages were searched to recover any residue. Cheese ingestion levels were monitored every day before the OLTT and were reported as the mass fraction (%) of ingested meal relative to the total amount of cheese presented.

During the postprandial phase, blood samples from the saphenous vein were collected in EDTA-preconditioned vials every hour for a total of 6 h. No lubricant was used during saphenous sampling, in order to prevent sample contamination with exogenous lipids. Glycemia was monitored at the same time points as the

OLTT. Analyses performed on blood samples included total TAG, non-esterified fatty acids (NEFA) and total cholesterol, determined spectrophotometrically after preparation with commercially available kits (Randox, Kearneysville, USA). Incremental areas under the curve (iAUC) for the postprandial TAG rise were calculated for each animal by the trapezoidal method using each animal's fasting TAG concentration as the baseline. To compute iAUC, missing values during the postprandial phase (i.e. 5% occurrences, randomly distributed, due to hemolysis or untimely sampling and attributed to the absence of lubricant) were imputed from the group's means for the corresponding time point.

After the OLTT, a second meal of 10 g of cheese was presented, followed by the 10-g portion of chow that was left in the cage. On day 11, a cheese meal of 7 g was presented for 30 min and the necropsies were performed after 1 h by complete blooding at the tail and heart puncture under isoflurane anesthesia. Livers had a healthy aspect, with no visual signs of steatosis, and their weight was similar for all treatments (13.01 g, SEM = 0.14), equivalent to 3.18% (SEM = 0.02) of the body weight. Stomachs were harvested to weigh the gastric contents 1 h after the ingestion of the cheese meal.

2.3 Calcium soap quantification

Feces were analyzed to quantify the amount and profile of fatty acids lost as calcium soaps. All solvents were of HPLC grade and obtained from Fisher Scientific (Toronto, Canada), and all other chemicals and standards were obtained from Sigma Aldrich (Oakville, Canada). Fecal samples corresponding to days 8–11 of the *in vivo* protocol were lyophilized to prevent loss of volatile fatty acids (Tangerman & Nagengast, 1996). Fecal samples were pooled for each rat and ground in a porcelain mortar. Lipid extraction was based on the Folch procedure (Folch, Lees & Sloane Stanley, 1957), and according to conditions reported by Bourlieu et al. (2015). A sample of dried feces (i.e. ≈ 50 mg) was precisely weighed in a methylation tube and mixed with 500 µL of water and 200 µL of 2.0 N HCl.

Once the sample was dispersed, 3000 µL of Folch solvent mixture (i.e. 2:1 chloroform-methanol) was added, followed by 200 µL of internal standard containing C7:0, C13:0 and C19:0 dissolved in chloroform. The sample was vigorously mixed using a vortex, then 125 µL of 125 mM NaCl and 750 µL of Folch solvent mixture were added, and the tube was vortexed again. Samples were allowed to stand for 30 min to permit phase separation. Both phases were recovered. The lower chloroform phase containing the lipids was conditioned using a solid-phase extraction (SPE) cartridge to separate out NEFA for GC analysis, whereas the upper aqueous phase was conditioned for Ca, Mg and P quantification.

Amino-propyl Sep-Pak Plus® NH_2 cartridges (WAT020535, Waters, Taunton, MA, USA) were fitted with funnels containing Na_2S0_4 and conditioned with a 10-mL wash of hexane. The chloroform phase was then eluted twice through the SPE cartridges. The cartridges were then washed with 10 mL of a chloroform/isopropanol 2:1 mixture to remove lipids other than NEFA. The latter were desorbed with 3 mL of a 2% formic acid solution in diethyl-ether. All elutions were done at a 2 mL/min rate. NEFA were quantified by gas chromatography (6890A gas chromatograph equipped with a FID and a 7683 injector and autosampler, Agilent Technologies, Mississauga, ON, Canada). Specific settings of the apparatus were according to conditions described in a previous study (Ayala-Bribiesca et al., 2017). Individual fatty acids were identified and calibrated with their respective commercial standards (Sigma Aldrich). FA concentrations were corrected using C7 for C10 and lower, C13 for C12 and C14 and C19 for C17 and higher, including unsaturated FA. C16 was corrected with both C13 and C19 and results were averaged.

For the aqueous phase analysis, samples of exactly 500 µL were diluted with 10 mL of 0.23 M HNO_3, centrifuged for 15 min at 3000 × *g* and filtered with a 0.45-µm PES syringe filter. Ca, Mg and P were quantified from the filtrate using an ICP-OES (Prism 010-00084-1 spectrometer from Teledyne Leeman Labs, Hudson, NH,

USA, equipped with a Burgener Research Peek Mira Mist Nebulizer, PMM4000). Emission lines used for element quantification of Ca, Mg and P were 317.933, 279.080 and 213.618 nm, respectively. Detection was done in radial mode on the argon-plasma torch. Commercial standards (Fisher Scientific, ON, Canada) were diluted in the same acidic matrix and filtered. Results were presented assuming that soluble minerals were entirely in the aqueous phase. For calculations, the volume of the aqueous phase, obtained from the theoretical partition of the methanol in the ternary mixture with water and chloroform (Folch et al., 1957) was used. That volume (i.e. 1720 µL for the conditions set in this study) was representative of the amounts recovered during collection of the aqueous phases.

2.4 Statistical analysis

All statistical analyses were performed with SAS 9.3 TS Level 1M0 (SAS Institute, Cary, NC, USA). The amount of cheese meal ingested was analyzed by analysis of variance (ANOVA) in a split-plot array with the AMF type and salting condition in the main plot and the time after ingestion of the cheese in the sub-plot. Cheese composition, rat weight before necropsy, stomach contents, iAUC and NEFA and calcium in feces were analyzed using a two-way ANOVA for the AMF and calcium level using the general linear models (GLM) procedure. Postprandial responses were transformed ($\log_{10}$) and analyzed with a repeated-measures ANOVA using the MIXED procedure. Model information used for the MIXED procedure: Covariance Structure: Variance Components; Estimation Method: REML; Residual Variance Method: Parameter; Fixed Effects SE Method: Kenward-Roger; Degrees of Freedom Method: Kenward-Roger. Normality of data was verified by the Shapiro-Wilk test. Multiple comparisons were done using least square difference (LSD) with a significance threshold of $P \leq 0.05$.

3. Results and discussion

3.1 Postprandial responses

The cheese ingestion rates for the OLTT on day 10 were over 98% (SEM 0.89), with no differences observed among the experimental cheeses (P = 0.3064). Overall, the rats ingested the cheese test meals in less than 15 min. Although this feeding strategy lacks the narrow timing achieved by gavage, it avoids the need to blend the food to a liquid state, which would have destroyed the original food matrix. No differences were detected for gastric contents 1 h after cheeses had been ingested (9.85 g, SEM = 0.12).

Glucose levels changed during the postprandial phase ($P < 0.0001$) and evolved in a similar way for all cheeses (Fig. 2a), with no effect detected for calcium level or AMF type. This was expected, since the Cheddar-type cheeses had a similar macronutrient composition and contained very small amounts of sugars. One hour after the cheese meal was consumed, there was a drop of glucose to 4.87 mmol/L, which gradually climbed back to its fasting level after 4 h.

The impact of the different experimental cheeses on postprandial lipemia was monitored through plasma concentrations of total cholesterol, NEFA and TAG. Total cholesterol concentration varied over time ($P < 0.0001$), although it was similar for all experimental cheeses, with no effect detected for calcium level or AMF type.

The rise in cholesterol after a fatty meal is part of the normal lipid digestion and handling process and occurs due to cholesterol synthesis and endogenous turnover (Sethi, Gibney & Williams, 1993). The FA composition of the different AMF did not differ drastically and, when combined with endogenous lipids, resulted in similar postprandial cholesterol behavior (Fig. 2b). Dairy products have been reported to induce an increase in HDL cholesterol, in part related to their SCFA and MCFA contents (German & Dillard, 2006; Parodi, 2016), but the cholesterol analysis that was performed does provide data on specific lipoproteins.

NEFA levels were equivalent for all AMF, but varied significantly according to time ($P < 0.0001$) and according to calcium level ($P = 0.0263$). No statistical interactions were detected. One hour after cheese ingestion, overall circulating NEFA reached a peak (2.9 mEq/L) and decreased thereafter, reaching baseline values after 5 h (Fig. 2c). Normally, NEFA decrease after a meal containing fat and carbohydrates due to the secretion of insulin, which inhibits lipolysis within adipocytes (Lairon, Lopez-Miranda & Williams, 2007). However, the experimental cheese meal did not contain large amounts of carbohydrates. The postprandial rise in circulating NEFA occurs because some of them escape uptake and re-esterification in adipose tissue. This rise is exacerbated immediately after a meal, when chylomicrons are hydrolyzed by lipoprotein lipase (Berry, 2009; Lairon et al., 2007). Rats that consumed the high-calcium cheeses presented overall levels of NEFA (1.67 mEq/L) that were slightly higher ($P = 0.0263$) than those that consumed the regular-calcium cheeses (1.52 mEq/L) (SEM = 0.04). It has been shown that this increase in NEFA includes mainly fatty acids of dietary origin, in this case, the cheese test meal, because lipolysis of endogenous lipids is halted in the postprandial state (Berry, 2009). For this reason, higher calcium levels during the digestion process probably enabled an overall increase in lipolysis and lipid uptake. This would have led to higher chylomicron secretion and metabolism, explaining the rise in circulating NEFA.

Finally, TAG levels (Table 2) varied in time ($P < 0.0001$) and differed in magnitude according to AMF type ($P = 0.0023$) and to calcium level ($P = 0.0054$). No statistical interactions were detected. As expected, TAG concentrations increased rapidly after the ingestion of the cheese meal, for all treatments. Peak values were observed during the two first hours of the OLTT, with a sustained level afterwards that was about two times that of the fasting TAG concentration. The AMF type in cheese resulted in different overall TAG concentrations in plasma, with the stearin AMF causing lower levels (1.43 mmol/L) than olein (1.84 mmol/L) and the control (1.72 mmol/L), which were statistically equivalent (SEM = 0.09). The lower TAG levels induced by the stearin AMF compared to the other AMF types can be

282 explained by the higher content of saturated-LCFA of the former, resulting in a
283 higher melting point (42.32 °C), which is above the rats' body temperature. During
284 digestion, solid fats have slower lipolysis and absorption rates than liquid fats
285 (Berry, 2009; Berry, Miller & Sanders, 2007), which can explain the decreased
286 postprandial response of the stearin AMF. Whether the TAG levels remained
287 above the baseline for longer periods in the stearin AMF treatments compared to
288 the others is unknown because the OLTT lasted only 6 h. For treatments with
289 stearin AMF, the TAG concentration seemed to show a rising trend after the drop
290 that occurred after the first TAG peak (Fig. 3). By comparing the iAUC calculated
291 for the different treatments, it was found that the AMF type in cheeses had a
292 significant effect (P = 0.0384), with the stearin inducing the lowest TAG levels after
293 the 6 hours of the OLTT (Table 2). The effect of calcium on iAUC fell short of the
294 significance threshold (P = 0.0583).

295 Despite the lack of statistical significance of the iAUC, cheeses with high calcium
296 content led to significantly higher circulating TAG levels (1.80 mmol/L) than those
297 observed after ingestion of cheeses with regular-calcium content (1.53 mmol/L) (P=
298 *0.0054*; SEM = 0.07). This difference points to the effect that calcium has in terms
299 of enhancing lipolysis and the repercussions on fat absorption. Looking at the TAG
300 concentrations during the OLTT (Fig. 3), there appears to be a trend towards high
301 calcium levels inducing an exacerbated height of the lipemia peak for cheeses
302 containing olein AMF (with higher SCFA and MCFA ratios). Based on the same
303 logic, the effect of high calcium on stearin AMF, with higher saturated-LCFA
304 content, would lead to a less abrupt rise in TAG, possibly with a delay in the
305 lipemia peak, as suggested by the TAG peaks occurring during the first two hours
306 of the OLTT (Fig. 3). These trends are consistent with the effect of calcium on lipid
307 digestion and its increased affinity for saturated-LCFA under intestinal conditions,
308 leading to higher lipolysis rates (Hu et al., 2010; Zangenberg, Müllertz, Kristensen
309 & Hovgaard, 2001), but coupled with slower absorption of the resulting calcium
310 soaps (Boyd , Crum & & Lyman, 1932; Jandacek, 1991; Lorenzen, Nielsen, Holst,
311 Tetens, Rehfeld, & Astrup,, 2007). Unfortunately, the OLTT does not make it

possible to determine whether the net TAG clearance before the return to baseline TAG conditions is equivalent for the different calcium levels or the different AMF types present in the cheeses.

3.2 Quantification of fecal calcium soaps

At the beginning of the protocol, fecal samples were dark in color, which is typical for the chow diet (Jandacek, Heubi & Tso, 2004). As the protocol advanced, feces became representative of the diet with the cheese meals, as evidenced by the change to a very light brown. From day 8, most pellets collected were pale and were kept for analysis. Total FA excretion was affected by the AMF type ($P < 0.0001$) and the calcium content ($P < 0.0001$) of the cheeses. Feces from rats fed stearin AMF cheeses presented total FA excretion levels that were 29% higher than for rats consuming cheeses prepared with the control or the olein AMF (Table 3). Concurrently, higher calcium levels in cheese led to higher FA excretion rates than regular calcium levels (Table 4), which is reflected in the 31.5% increase in total excreted FA.

When broken down into individual fatty acids, differences in excretion rates are mainly due to differences in excretion of saturated-LCFA. Volatile acids in feces were similar for all treatments (C2 3.7, SEM = 0.44; C3 0.3, SEM = 0.12; C4 0.6 SEM = 0.26, in mg/g of dry feces). These acids were likely produced by the intestinal microbial flora (Diem & Lentner, 1970; Pray, Pillsbury & Tomayko, 2012) because dietary SCFA, such as butyric acid from milk fat, are preferentially hydrolyzed by digestive lipases and rapidly absorbed (German, 2008). No MCFA (C6, C8 and C10) were detected in feces, indicating that their absorption was complete. Excreted levels of saturated-LCFA (C12 to C18:0) were influenced by calcium content and AMF type present in the cheeses (in all cases, $P \leq 0.01$). Rats consuming the cheeses prepared with the stearin AMF excreted higher amounts of saturated-LCFA than those consuming cheeses prepared with the other AMF (Table 3). This agrees with studies showing that fats with different melting points

(i.e. due to different TAG structure and FA composition) result in different digestion and absorption behaviors, and in lower absorption levels for higher melting points (Berry, 2009).

Higher calcium levels in cheese led to higher saturated-LCFA excretion levels compared to cheeses with regular calcium levels (Table 4). Excretion of UFA (C18:1, C18:2, C18:3) was similar among treatments, except for oleic acid ($P < 0.0001$), which was excreted in higher amounts by rats fed the high calcium cheeses (Table 4). These results are in agreement with the selectivity of calcium, which preferentially reduces saturated-LCFA like palmitic and stearic acid, and they agree with the decreasing impact on PUFA, MCFA and SCFA owing to their higher water solubility (Mu & Porsgaard, 2005; Owen, Weisgerber, Carr & Harrison, 1995).

Analysis of the aqueous extract of the feces revealed an increase in total calcium and phosphorous for groups consuming the high-calcium cheeses compared to the regular-calcium cheeses (Table 5). Magnesium levels were equivalent for all groups. A calcium increase in dry feces of 7.3 mg/g, equivalent to 12.5% for the regular calcium group, was observed. This increase was in stoichiometric excess if considered solely as CS with respect to the FA increase of 20.5 mg/g observed for the same treatments (i.e. calcium to FA ratio of 0.35, when 0.07 is the calcium ratio in calcium palmitate). Consequently, calcium lost through feces did not consist solely of CS, but may have included calcium phosphates, as suggested by the simultaneous rise in phosphorous level and the low solubility of calcium phosphate salts at intestinal pH (Lorieau, Le Roux, Gaucheron, Ligneul, Hazart, Dupont, & Floury, J. (2018).

When analyzed together, FA and calcium excretion rates point to the formation of CS in the intestine through LCFA complexation by calcium ions, with palmitate and stearate as the predominant soaps. Moreover, the highest excretion levels by far for a single fatty acid were those obtained for palmitic acid. This fatty acid has

368 already been identified as being responsible for elevated plasma cholesterol levels
369 (Grundy & Denke, 1990). The reasons for the increased excretion of oleic acid are
370 unclear, but this trend relates to calcium and not to the relative amount found in the
371 different AMF types. According to Mattson, 1979, oleic acid is less prone to
372 produce CS than saturated-LCFA. However, it has been shown, using a rat model,
373 that some are excreted (Gacs et al., 1977). When compared to saturated fatty acid
374 (stearic acid), oleic acid soaps excretion is significantly lower, but it is still higher
375 than the corresponding PUFA (linoleic acid). Although it is not possible to affirm
376 that excreted fatty acids consisted exclusively of CS, calcium definitely played a
377 role in reducing the bioavailability of saturated-LCFA and oleic acid.

378 While calcium enrichment of cheeses led to higher overall TAG levels after 6 h
379 during the postprandial phase, it increased the amount of FA excreted as calcium
380 soaps, which points to lower FA bioavailability, which was not observed within the
381 time frame of the OLTT. This result shows that the effect of calcium is not
382 straightforward and that it depends on the transit of chyme within the digestive
383 system. The increased concentration of calcium enabled higher lipolysis rates. The
384 rapid release of fatty acids could explain the increase in the lipemic response
385 during the OLTT, especially considering that calcium has a lower affinity for FA,
386 which have a more unsaturated or shorter carbon chain. This means that calcium
387 could form soaps with stearic or palmitic acid, removing them from the fat droplet
388 surface and enabling lipase to continue its lipolytic effect, permitting the release of
389 other FA. Even if the CS produced have slower absorption rates, UFA or shorter
390 FA could still be absorbed and they would be the FA causing the exacerbated
391 lipemia in the presence of high calcium levels. Then, as digestion continued and
392 chyme advanced towards the distal ileum, where pH conditions approach
393 neutrality, CS would have been less prone to be absorbed, resulting in the higher
394 excretion levels observed in this study.

395 Finally, the results obtained through the *in vivo* model are in agreement with those
396 obtained following the *in vitro* digestion of the same experimental cheeses (Ayala-

Bribiesca et al., 2017). Disintegration of the experimental cheeses during *in vitro* digestion was slower for those containing the stearin AMF, when compared to those with the control or the olein AMF (Ayala-Bribiesca et al., 2017), which would partially explain the decreased lipemic response observed *in vivo* for cheeses with the stearin AMF. Additionally, the *in vitro* lipolysis patterns of the experimental cheeses, analogous to intestinal digestion, depict slower lipolysis rates for the stearin AMF than those for the olein and control AMF. This supports the postprandial lipemia patterns observed during the OLTT, where cheeses containing the control and the olein AMF induced higher lipemia, as indicated by the increase in TAG, than cheeses containing the stearin AMF. Lastly, it is interesting to note that with the high-calcium cheese, the extent of *in vitro* lipolysis for the stearin AMF increased towards the end of digestion and eventually reached the same levels as for the olein and control AMF (Ayala-Bribiesca et al., 2017). While this demonstrates the enhancing effect of calcium on lipolysis, it would seem contradictory given the lower lipemic responses obtained *in vivo* for cheeses with the stearin AMF. This discrepancy can be explained by the lack of an absorption process in the *in vitro* model. In a closed *in vitro* vessel, lipolysis products accumulate and the medium eventually saturates, halting lipolysis. In the presence of higher calcium levels, the excess calcium further drives the extent of lipolysis by reacting with FA to produce CS. *In vivo*, CS also occur and some are excreted because their absorption is less efficient than that of the corresponding FA. This reduced bioaccessibility of CS could also explain the decreased lipemic response of the stearin AMF (rich in LCFA), as observed *in vivo*.

4. Conclusion

The results presented show the potential of solid dairy matrices to act as nutrient-release regulators during the digestive process. The study demonstrated that it is possible to modify postprandial lipemia and to increase or decrease fatty acid bioavailability, which could be of interest for specific health requirements. *In vivo*

426 digestion of Cheddar-type cheeses prepared with different AMF types and calcium
427 levels led to different postprandial responses. Cheese with higher proportion of
428 LCFA induced lower circulating TAG levels and higher fatty acid excretion. The
429 amount of solid fat at body temperature is believed to be a major reason for the
430 observed variations of postprandial lipemia. Cheeses with high calcium content
431 induced higher lipemia levels (higher bioavailability during the early postprandial
432 period), but also led to higher fatty acid excretion (decreased net bioavailability).

433 The results presented in this study agree well with those previously obtained with
434 the same experimental cheeses, using an *in vitro* digestive model system. This
435 work illustrates the complementarity of *in vitro* and *in vivo* studies. While *in vitro*
436 approaches are useful for screening and identifying promising experimental
437 conditions, the relevance of the resulting findings can only be validated through *in*
438 *vivo* trials. Once validated, the reliability of the predictions derived from the *in vitro*
439 model increases.

440 This study shows that FA bioavailability depends on the composition and
441 characteristics of fat and it can be modulated by the presence of calcium. A better
442 understanding of the factors controlling lipid bioavailability would allow the
443 development of foods for specific nutritional requirements.

444

445 5. Acknowledgements

446 The financial support of the National Science and Engineering Research Council
447 (Canada), Novalait, Ministère de l'agriculture et de l'alimentation du Québec
448 (MAPAQ), Fonds de recherche du Québec nature et technologie (FRQNT), and
449 Agriculture and Agri-Food Canada (AAFC) is acknowledged. The authors wish to
450 thank Valérie Dumais, Kim Dénault, Christine Dallaire and Marie-Julie Dubois of
451 Dr. Marette's laboratory, Pier-Olivier Tremblay of the Statistical Consulting Service
452 at Université Laval for his assistance with data analysis, as well as Dr. Claire
453 Bourlieu, of INRA-STLO, for her advice on sample preparation for the FA profiles of

454 calcium soaps. The generous contribution of Fonterra Ltd., which provided the
455 AMF fractions, is recognized.

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Figure Captions

Figure 1. Fatty acid subclass composition (molar %) of different anhydrous milk fats (AMF) used to prepare the Cheddar-type cheeses. Calculated from data presented by Ayala-Bribiesca et al. (2017). Subclasses UFA, LCFA, MCFA and SCFA stand, respectively, for unsaturated fatty acids (C18:1, C18:2 and C18:3), long-chain fatty acids (C18:0 to C14:0), medium-chain fatty acids (C12:0 to C8:0) and short-chain fatty acids (C4:0 and C6:0). SEM < 0.1% for all subclasses.

Figure 2. Postprandial progression of (a) glucose (b) total cholesterol and (c) NEFA concentrations in plasma after the ingestion of a cheese meal. Bars indicate SEM.

Figure 3. Postprandial progression of TAG concentration in plasma after the ingestion of Cheddar-type cheese with regular or high calcium content and prepared with olein, control or stearin AMF. Bars indicate SEM.

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Table 1.

Experimental conditions for cheesemaking, cheese composition and the number of rats (n) for each cheese that completed the in vivo protocol.

AMF in cheese	Salting conditions	Moisture %	Fat %	Ca ppm	n
Olein	NaCl	35.5 ^{ab}	33.1 ^{b}	7517 ^{b}	11
	$NaCl + CaCl_2$	35.8 ^{a}	32.6 ^{b}	9854 ^{a}	11
Control	NaCl	34.9 ^{abc}	33.7 ^{ab}	6922 ^{b}	10
	$NaCl + CaCl_2$	34.1 ^{c}	35.3 ^{ab}	9168 ^{a}	12
Stearin	NaCl	34.9 ^{abc}	33.7 ^{ab}	6970 ^{b}	11
	$NaCl + CaCl_2$	34.4 ^{bc}	35.9 ^{a}	9377 ^{a}	12
	SEM	0.22	0.56	117	

$^{a-c}$ Different superscript letters denote significant differences within means reported for each analysis. SEM were obtained from the statistical models. Adapted from Ayala-Bribiesca et al. (2017).

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Table 2.

Incremental area under the curve (iAUC) for TAG appearance in blood 6 h into the postprandial phase after ingestion of experimental cheeses prepared with different types of anhydrous milk fat (AMF).

AMF in cheese	iAUC (mmol × h/L)
Olein	5.99 [a]
Control	4.85 [ab]
Stearin	4.35 [b]
SEM	0.45

[a,b] Different suprscript letters indicate significant differences within columns. SEM was obtained from the statistical model.

Table 3.

Mass fraction of long-chain fatty acids and total fatty acids (FA) recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses prepared with control, olein or stearin AMF.

AMF type	Fatty acids recovered in dry feces (mg/g)								Total FA
in cheese	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C18:1	C18:2	(mg/g)
Control	0.5 [b]	3.7 [b]	0.9 [b]	30.0 [ab]	0.6 [b]	14.5 [b]	9.3 [a]	5.9 [a]	69.7 [b]
Olein	0.7 [ab]	4.0 [b]	0.9 [b]	26.3 [b]	0.6 [b]	13.9 [b]	10.4 [a]	5.7 [a]	67.5 [b]
Stearin	1.0 [a]	5.7 [a]	1.2 [a]	37.2 [a]	1.0 [a]	22.5 [a]	10.0 [a]	5.2 [a]	88.6 [a]
SEM	0.07	0.43	0.09	2.63	0.06	1.39	0.52	0.22	4.88

[a,b] Different index letters indicate significant differences within columns. SEM was obtained from the statistical model.

Table 4.

Mass fraction of long chain fatty acids and total fatty acids (FA) recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses with regular or high calcium content.

Ca level	Fatty acids recovered in dry feces (mg/g)								Total FA
in cheese	C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C18:1	C18:2	(mg/g)
Regular	0.6	3.6	0.8	26.3	0.6	14.5	8.6	5.6	65.0
High	0.9 ***	5.3 ***	1.2 **	36.0 **	0.8 **	19.4 **	11.2 ***	5.5 ns	85.5 ***
SEM	0.06	0.36	0.07	2.20	0.05	1.17	0.44	0.18	4.08

***, **, ns Symbols denote the significance level of the differences (*P* threshold values of 0.001, 0.01 and not significant, respectively). SEM was obtained from the statistical model.

Table 5.

Mass fraction of Ca, Mg and P recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses with regular or high calcium content.

Ca level in cheese	Ca	Mg	P
		mg/g	
Regular	58.4	15.3	31.8
High	65.7 ***	14.3 n.s.	34.2 **
SEM	1.01	0.41	0.51

***, **, n.s. Symbols denote the significance level of the differences (*P* threshold values of 0.001, 0.01 or not significant, respectively). SEM was obtained from the statistical model.

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Figure 1

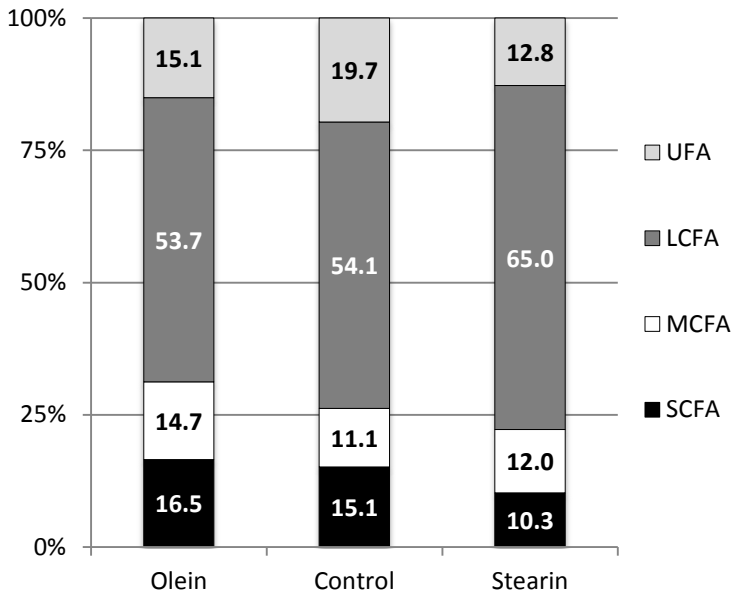


Figure 2

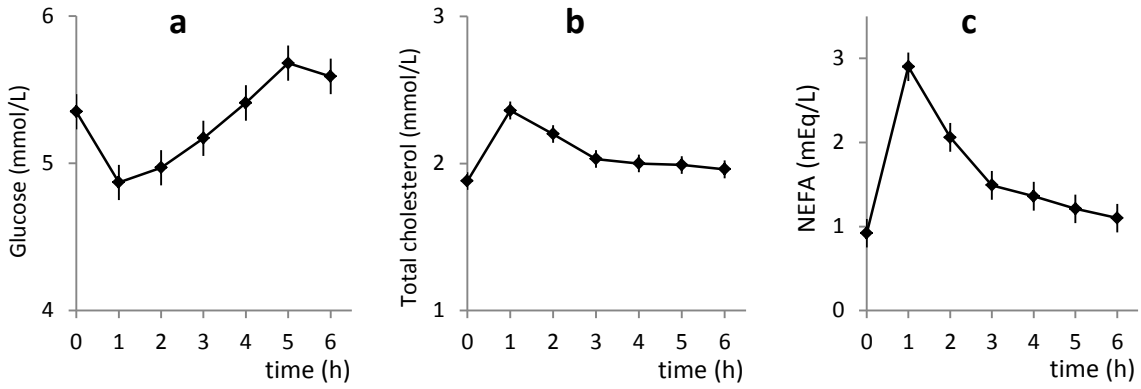
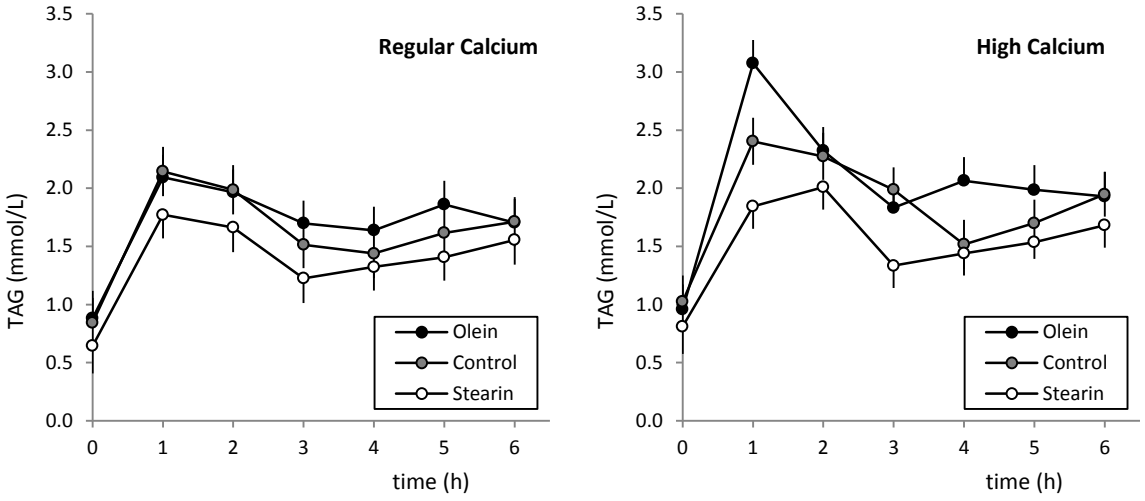

a
Glucose (mmol/L)
6
5
4
0 1 2 3 4 5 6
time (h)
b
Total cholesterol (mmol/L)
3
2
1
0 1 2 3 4 5 6
time (h)
c
NEFA (mEq/L)
3
2
1
0
0 1 2 3 4 5 6
time (h)

Figure 3


Regular Calcium
High Calcium
TAG (mmol/L)
time (h)
Olein
Control
Stearin
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
1
2
3
4
5
6