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ARTICLE

# The influence of dietary fat and intestinal pH on calcium bioaccessibility: *an in vitro* study

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*In vivo* studies measuring true fractional calcium (Ca) absorption have shown that dietary fat is a significant predictor of absorption and is influenced by luminal pH levels. However, whether changes in Ca bioaccessibility (CaB) can explain the effects on absorption has not been examined. In the current study, we examined two high fat diets enriched in either monounsaturated fatty acids or saturated fatty acids (SFA), and a low-fat diet (LFD) each with 50 mg Ca, and measured CaB at different intestinal regions during normal acidic or higher (pH=7) gastrointestinal conditions using an *in vitro* gastrointestinal model. During normal pH conditions in the jejunum, there was an interaction between diet and time for CaB ($P < 0.02$), and CaB during the SFA diet was higher than LFD ($P = 0.05$). CaB was reduced by 90 ± 3% during higher compared with normal pH under all dietary conditions ($P < 0.001$). These findings indicate that fat intake, especially SFA enriched, is associated with a greater CaB in the jejunum, and may explain the higher Ca absorption in previous studies. In addition, the marked reduction in CaB under higher pH conditions could have implications in persons taking acid-reducing medications.

Key words: bioaccessibility, calcium, gastrointestinal, high fat diet, monounsaturated fatty acids, saturated fatty acids

## Introduction

Calcium (Ca) absorption occurs throughout the intestinal tract and involves dietary Ca transport across the intestinal lining. There are two pathways for Ca to enter blood circulation from the intestinal lumen: the transcellular and paracellular pathways.[1,2] In the paracellular pathway, dietary Ca moves down the concentration gradient and freely diffuses across gap junctions between enterocytes into the circulation. This passive movement dominates in the jejunum and ileum, and it is independent of hormonal regulations.[2-3] However, other factors such as gastrointestinal pH levels affect paracellular Ca absorption. Because gastric acid is needed for dissolution of dietary Ca into its ionic form ($Ca^{2+}$),[4,5] an increase of gastric pH level due to attenuated HCl secretion can lead to reduced Ca bioaccessibility (CaB) and intestinal Ca absorption.[6] In addition, lowering gastrointestinal acid production is the goal for treatment of ulcers and symptoms of gastritis and heartburn (i.e., proton pump inhibitors, H2 blockers). Achlorhydria characterized as an intestinal pH of >7 is a pathological gastrointestinal condition that commonly leads to intestinal Ca malabsorption.[4, 6, 7]

Fat intake will also affect intestinal Ca absorption. There is evidence that dietary fat interacts with Ca by forming insoluble Ca-fatty acid soap attenuating absorption of fat and cholesterol.[8,9] However, clinical trials show that true fractional Ca absorption is greater in obese individuals than normal weight persons, and that dietary fat is a positive predictor of fractional Ca absorption.[10-12] Evidence in mice also shows that high fat diets (HFD) are associated with a greater true fractional Ca absorption.[13] In this previous mouse study, there was no effect of HFD on gene expression of active Ca transporters whether it was enriched with monounsaturated fatty acids (MUFA) or saturated fatty acids (SFA).[13] Therefore, this study concluded that the increased fractional Ca absorption was due to a greater paracellular transport.[13] Moreover, the type of fatty acids may affect Ca absorption throughout the intestine. For example, studies report that SFA have longer intestinal transit time than other fatty acids.[14,15] Thus, this could increase intestinal Ca bioaccessibility or if the interaction with Ca is prolonged, this may promote Ca-fatty acid soap formation and decrease bioaccessible Ca.[8] In addition, SFA may alter protein structure of tight junctions and negatively affect intestinal fluidity when compared with unsaturated fatty acids.[16-18] Nevertheless, studies of dietary fat on passive Ca absorption are limited, and effects of HFD on CaB remain unknown.

To address these questions, we used a dynamic *in vitro* method[19] to explore the interactions between dietary fat and Ca at different segments of the small intestine and at different time periods during digestion. The primary objective of the current study was to examine effects of HFD (MUFA or SFA) on intestinal CaB *in*

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*vitro* using a gastrointestinal model (TIM-1), with a secondary objective to investigate the intestinal CaB response to changes in gastrointestinal pH levels.

# Materials and methods

***In vitro* gastrointestinal model**

The TIM-1 is an *in vitro* multi-compartmentalized gastrointestinal model (Nutrition and Food Research Institute, Zeist, The Netherlands). This instrument consists of the stomach compartment and three intestinal segments that simulate the intestinal digestion process *in vivo.*[20,21] The utilization of *in vitro* digestion models in research is one way to study the effects of different food matrices on bioavailability and digestion. The TIM-1 system emulates *in vivo* factors, such as meal size and feeding duration, that are sustained with normal gastrointestinal pH, enzymatic activity, intestinal peristaltic movements, nutrient and water absorption, gastric emptying, and approximates normal intestinal transit times.[22,23] To simulate these *in vivo* digestive parameters and conditions, each TIM-1 compartment was filled with pre-specified amounts of pancreatic secretions, bile, and gastric secretions according to the fed state protocol in the TIM-1 system **outlined below**.

**Experimental design**

The current study was performed under the standard fed state **conditions** of the TIM-1, and the following standard TIM solutions were prepared and used: 7% pancreatic solution [Pancrex V powder (α-amylase activity= 25,000 units/g, lipase activity = 25,000 units/g, and protease activity= 1,400 units/g) was obtained from Paines & Byrne, UK], small intestinal electrolyte solution (SIES) (NaCl 5g/L, KCl 0.6g/L, CaCl2 0.3g/L), and gastric electrolyte solution (NaCl 6.2g/L, KCl 2.2 g/L, $CaCl_2$ 0.3 g/L).[21] Prior to feeding, start residues of the duodenum, jejunum, and ileum were infused into each TIM-1 compartment and the temperature was raised to physiological temperature of 37 °C. In addition, the gastric compartment was infused with gastric enzyme solution consisting of 600 U/mL pepsin (P7012, Sigma-Aldrich, MO, USA) and 40 U/mL lipase (F-AP15, Amano Enzyme Inc., Nagoya, Japan) in a gastric electrolyte solution (4.8 g/L NaCl, 2.2 g/L KCl, 0.22 g/L CaCl2, 1.0 g/L NaHCO3). Porcine bile was collected from a slaughterhouse (Farm-to-Pharm, NJ, USA), where multiple collections of bile were pooled, then divided into single-use quantities, and stored at -20 °C. The bile was thawed and filtered using Miracloth (Merck KGaA, Darmstadt, Germany) prior to incorporation into the experimental solutions.

To commence the simulated digestion, each TIM-1 compartment was filled with the corresponding start residues to mimic *in vivo* conditions. The duodenal start residue was consisted of 15 g SIES, 15 g pancreatin solution (7%), and 30 g fresh porcine bile. The jejunal start residue was consisted of 40 g SIES, 40 g pancreatin solution (7%) and 80 g fresh porcine bile. Ileal start residue was consisted of 180 g SIES. After warming the system, the experimental meal was inserted into the gastric compartment, and the 5hr digestion process was initiated. The jejunum and ileum compartments were connected to filtration units (M20S-300-01P, pore size ~50 nm, MiniKros® filter modules, Spectrum Labs, Breda, The Netherlands) to remove the digestive material. During the experiment, samples were obtained from the duodenum and jejunum filtrates as well as the ileum efflux at 1hr intervals. During the digestion period, gastric emptying, intestinal transit time, gastrointestinal pH levels, and secretion fluid amounts were computer-controlled by TIM-1 system. [20,21]

To examine the effects of dietary fat on CaB, 3 types of diets were used and the experiment was repeated in triplicate for each diet. A 10% low fat diet (LFD) and 45% HFD enriched with MUFA or SFA (Research Diets, Inc. New Brunswick, NJ) were used. All diets contained the same amount of dietary Ca and other micronutrients (Table 1) and are identical to those in our *in vivo* Ca absorption study[13]. Pellets of food (10 g; 50 mg Ca) were ground into powder and then hydrated. Each experimental diet was packed into a tea bag to prevent excess digestive residue from clogging the machine (yet not restrict the flow of food) and was inserted into the gastric compartment. Samples (25mL) from the jejunal and ileal compartments were simultaneously collected at the 1, 2, 3, 4, and 5 hr after diet feeding to access CaB. Duodenal solutions are not collected in the TIM-1, however this would not be expected to alter the outcome because the majority of Ca is passively absorbed in the distal jejunum and ileum.[3] Total Ca concentration was measured using a color reagent (o-Cresolphthalein Complexone, CPC) at a

**Table 1 Dietary composition of experimental diets**

| Ingredient | Diet | | |
|---|---|---|---|
| | LFD | MUFA | SFA |
| | g/kg diet | | |
| Casein | 146 | 183 | 200 |
| L-cysteine | 3 | 4 | 0 |
| DL-methionine | | | 3 |
| Corn starch | 540 | 249 | 0 |
| Maltodextrin | 98 | 189 | 0 |
| Sucrose | 98 | 34 | 396 |
| Cellulose | 49 | 61 | 50 |
| Soybean oil | 6 | 36 | 45 |
| Coconut oil | 0 | 0 | 135 |
| Lard | 11 | 64 | 0 |
| Olive oil | 26 | 153 | 0 |
| Mineral mix[2] | 10 | 12 | 35 |
| Vitamin mix V10001 | 10 | 12 | 10 |
| Choline bitartrate | 2 | 2 | 2 |
| Energy, kcal/g diet | 3.9 | 4.8 | 4.6 |
| % Energy[3] | | | |
| Carbohydrate | 75 | 39 | 37 |
| Fat | 10 | 46 | 44 |
| Saturated (%kcal of fat) | 20 | 20 | 41 |
| Monounsaturated (%kcal of fat) | 60 | 60 | 41 |
| Polyunsaturated (%kcal of fat) | 20 | 20 | 18 |

[1]Prepared by Research Diets Inc. New Brunswick. LFD, low fat diet; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.
[2]All diets were maintained to contain 50mg Ca/10g diet
[3]Protein intake ranged from 15-19% in all diets.


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wavelength of 570 nm (Pointe Scientific, CV< 4.6%). Calcium was measured in the food entering the TIM (confirming the reported Ca content in the diets), and in the digestive materials in the samples.

**Gastrointestinal pH conditions**

After diet ingestion, CaB was examined in triplicates under normal acidic conditions (gastric, duodenal, jejunal, and ileal pH are 1.5, 6.5, 6.8, and 7.2, respectively), and under higher neutral gastrointestinal pH conditions (pH of 7 in the stomach and all intestinal sections). For the normal acidic pH condition, the pH levels were 5.5 at the beginning of feeding, then was reduced to pH = 1.5 in the stomach and was maintained at pH levels of 6.5, 6.8, and 7.2 in the duodenal, jejunal, and ileal compartments, respectively. Furthermore, under the higher neutral pH conditions, a pH level of 7 was maintained throughout the gastrointestinal tract including compartments of the stomach, duodenum, jejunum, and ileum using the computer controlled settings on the TIM-1 system.

**Statistical Analysis**

Repeated Measures ANOVA was performed to examine the interaction between diet and time on CaB in the jejunum and ileum over a total 5 hour period. When the interaction for ANOVA results was significant, Tukey's Post-hoc analysis was performed. In addition, repeated measures ANOVA was performed for each intestinal segment using pH as a covariate. The area under the curve (AUC) for each intestinal segment was calculated using the trapezoidal method. The AUC between two points was examined using y = f(x) between x = a and x = b, integrate y = f(x) between the limits of a and b. Two-Factor ANOVA was used to compare between diets and intestinal segments. A P value of <0.05 was considered significant. Values are means ± SD unless otherwise indicated. Statistical analysis was conducted using the SPSS statistical software (IBM, v24.0).

# Results

During normal acidic gastrointestinal pH conditions, Repeated Measures ANOVA indicated that jejunal CaB increased over time ($P < 0.001$) and differed between groups (time x diet) ($P < 0.01$) (Fig. 1A). In addition, CaB increased in the jejunum with the SFA to a greater extent than the LFD at 4 hr ($P = 0.05$). In contrast, jejunal CaB for MUFA and LFD remained relatively stable during the 5 hr digestion period (Figure 1A). In the ileum, there also was an increase over time for CaB ($P < 0.02$); however, the difference between diets was not significant (Fig. 1A). The percent of Ca (from the total input of 50 mg Ca) was 61 ± 9% and 56 ± 7% in the jejunum and ileum, respectively, and did not differ significantly. The AUC for CaB over the 5hr time course showed that it didn't differ due to diet (Fig. 2), but tended to be higher in the jejunum than in the ileum ($P = 0.054$). The average CaB for all diets over the 5 hr period was 29.3 ± 2.9 mg and 25.5 ± 4.7 mg in the jejunum and ileum, respectively.

During the abnormal higher pH condition, CaB was comparable among the three different diet groups at any time point in the jejunum and ileum (Fig. 1B). When both normal and higher pH were analyzed together (using pH as a covariate), there was an interaction between diet and time ($P = 0.007$). While the percent of Ca available during higher pH conditions was similar at the jejunum (6 ± 1%) and ileum (5 ± 1%). CaB AUC showed a trend to be greater in the jejunum than in the ileum ($P = 0.086$). The AUC was also markedly lower under all diet conditions during the higher (pH = 7) compared with the normal acidic gastrointestinal pH conditions ($P < 0.001$) (Fig. 2). Moreover, when comparing higher pH with normal pH conditions, it was found that intestinal CaB was reduced by 90 ± 3% ($P < 0.001$). The CaB for all diets over the 5 hr period averaged only 2.7 ± 0.6 mg and 2.2 ± 0.9 mg in the jejunum and ileum, respectively at higher pH conditions.

An examination of CaB during HFD (MUFA combined with SFA) compared with the LFD, indicated no interactions (diet x time) at normal pH at either intestinal site. At higher pH, Repeated Measures ANOVA also indicated no diet effect on the very low CaB values in the jejunum, and an interaction in the ileum ($P < 0.05$).

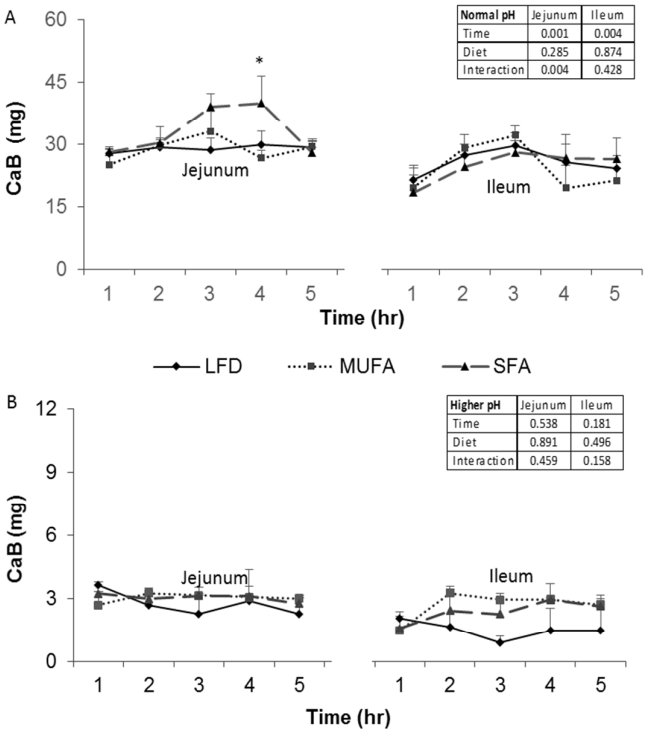


**Fig. 1** Effects of a low fat diet and high fat diets (HFD) enriched with MUFA or SFA on CaB during a 5hr digestion period under (A) normal (acidic) and (B) higher (neutral) gastrointestinal pH conditions in the jejunum and ileum. *Differs from LFD and MUFA using repeated measures ANOVA (Diet x Time), $P < 0.05$. CaB, calcium bioaccessibility; LFD, low fat diet; MUFA, monounsaturated fatty acid HFD; SFA, saturated fatty acids HFD.

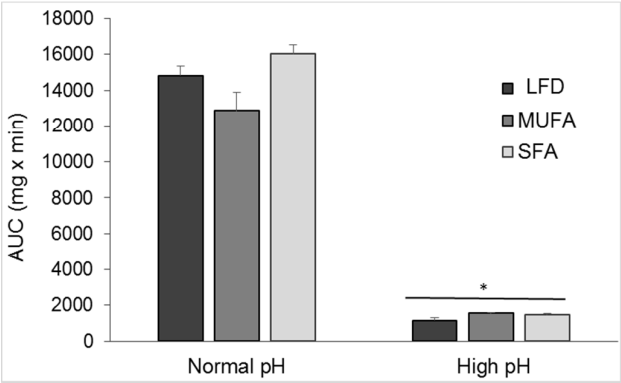


**Fig. 2** AUC of CaB (jejunum combined with ileum) for low fat diet and high fat diets (HFD) enriched with MUFA or SFA under normal (acidic) and higher (neutral) pH conditions. *Differs from the normal gastrointestinal condition, P = 0.005 by two-factor ANOVA (diet x pH). AUC, area under the curve; LFD, low fat diet; MUFA, monounsaturated fatty acid enriched HFD; SFA, saturated fatty acid enriched HFD.

# Discussion

Dietary fat can stimulate or inhibit intestinal Ca absorption in humans and rodents depending on the amount and type of fat intake.[8,10,24] However, an understanding of how the chemical properties of fat influences CaB or absorption has not been addressed, nor has there been an examination of the interaction with different types of fatty acids. In this current study, we examined the role of high dietary fat intake (enriched in MUFA or SFA) on the availability of ingested Ca without consideration of chemical and physiological factors that could potentiate a positive or negative influence using a TIM-1 model.[20,21] Additionally, we investigated these differences under both normal acidic and higher neutral gastrointestinal pH conditions. We found fat intake with excess dietary SFA increases CaB. Also, abnormally elevated gastrointestinal pH markedly impairs CaB during all dietary conditions.

The current *in vitro* study was designed to determine whether CaB could partially explain our *in vivo* findings in mice showing increased Ca absorption with the HFD enriched with SFA or MUFA.[13] Previous findings indicate that compared with the LFD controls, both high fat feedings are associated with a higher Ca absorption.[13] One study in young mice reported a negative effect of fat on Ca absorption, but because they were fed a chronic HFD shortly after weaning, intestinal development may have been impaired or the negative effects could have been due to excess adiposity.[25] We found that CaB was highest with the SFA diet in the jejunum when compared with a low fat diet. These findings may also be applicable in humans, since clinical trials have also shown a positive effect of dietary fat on Ca absorption, [10-12] and SFA is generally high in the western diet.[26] However, the addition of lipids to infant formula does not consistently raise Ca bioavailability or absorption and may be due to the type of fatty acid.[27] Furthermore, the acute effects of fat from a test meal (~100 kcal) from either ice cream (5.6 g fat) or low fat milk (3.3 g fat) was examined in a well-designed study, and indicated no differential effect on Ca absorption.[28] The absence of a difference could be explained by the small amount of fat in either diet or small differences in total fat between the ice cream and milk test meals. Also, it is possible that chronic intake of a HFD is a more important predictor of Ca absorption because over time, it affects cell morphology and causes an increase in intestinal permeability.

The differences between MUFA and SFA on CaB, and Ca-fatty acid soap formation may be explained by the lipid source and type.[24] In our study, the SFA source was largely from coconut oil that is mostly composed of lauric acid (C-12) [fatty acids composition]. In comparison, our MUFA source consisted largely of olive oil that is high in oleic acid (C-18:1) [fatty acids composition]. Lauric acid in triacylglycerol occupies the sn-2 position[29] and remains in the monoacylglycerol form until absorption, decreasing its susceptibility to form Ca-fatty acid soaps.[30] This may explain the unexpected higher CaB (possibly due to fewer Ca-soaps) in the current study with the SFA diet. Moreover, the variation of chain length between fatty acids could play a role. It is possible that longer chain fatty acids in the MUFA (C18:1) than in the SFA (C-12) diet contributed to the lower CaB after digestion. Studies show that as fatty acid chain length increases, absorption rates decrease due to Ca-soap formation.[23-24] This occurs because long chain fatty acids have a melting point higher than body temperature and a higher tendency to form insoluble soaps with Ca.[29] Also, the coconut oil (C-12) used in this study for our SFA enriched diet may explain why we didn't find reduced CaB compared with the study using beef tallow (C-16 and C-18 fatty acids) in the study by Denke et al.[14] On the other hand, in contrast to the jejunum, we found no interaction between dietary fat and CaB in the ileum. This is consistent with the evidence that fatty acids bind with Ca making both less available for absorption in the latter portion of the gastrointestinal tract. We also reported that CaB ranged from 8-12% in the jejunum and only 4-9% in the ileum. These varied ranges in both intestinal segments are supportive of the classic findings by Bronner et al., who reported *in vivo* findings that most Ca absorption occurs in the jejunum.[31] Taken together, the current findings and those of others indicate that SFA increases jejunal CaB, but did not significantly contribute to higher CaB in the ileum or the average AUC for both jejunum and ileum. Because we only examined the direct effect of dietary fat on CaB, other potential influences from biological factors *in vivo* (enzymes and hormones) will play a role in overall intestinal absorption.[1,13] Therefore, it is not possible to make conclusions about HFD on Ca beyond its bioaccessibility in this study. Future studies examining Ca digestion might consider using other gastrointestinal models that can also address the microbial aspects of digestion since probiotics are known to affect Ca bioavailability and bone health.[32]

In addition to nutrient interactions influencing Ca absorption in humans, altered luminal factors such as intestinal pH also affect absorption.[4,6] Achlorhydric patients, who have an absence of HCl secretion and higher intestinal pH, have impaired Ca absorption.[5-7] Also, over the counter antacid medications, such as proton pump inhibitors and H2-blockers lower gastric HCl production and raise pH levels in the gastrointestinal tract. When this abnormal higher gastrointestinal pH was replicated in the TIM-1 system, we found that CaB was about 90% lower compared with normal acidic pH condition. This would have implications for the numerous patients on medications that raise gastrointestinal pH.

The strengths of the current study include applying an *in vitro* gastrointestinal model that can determine nutrient interactions on CaB with high predictive values, and there has been no previous study examining the interaction between dietary fat and Ca. A


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potential limitation was that besides different lipid sources to address the primary hypothesis in this study, the amount of carbohydrate and the sources used in the diets varied. For example, the LFD and MUFA diets contained significant amounts of both cornstarch and maltodextrin while the SFA contained none. Sucrose, on the other hand, was higher in the SFA diet. Studies suggest that carbohydrates, such as lactose, may enhance intestinal Ca absorption.[33-35] While other carbohydrate sources (i.e., corn starch, maltodextrin, and sucrose) will reduce the Ca bioavailability or absorption in rodent overfeeding studies, this has not been shown in human trials.[36] Also, not all rodent studies use controlled purified diets, making the findings difficult to interpret.[37,38] While high intake of phytates consistently show a reduced CaB [39], small differences, if any, in the purified diets used in the current study would be expected, or to affect the findings. In addition, the meal size may influence the bioaccessibility of minerals, as well as the source of calcium [20, 40 ,41] and could be addressed in future studies.

# Conclusion

To conclude, high fat feeding, particularly SFA enriched diet, increases the CaB compared with a low fat feeding in the jejunum. Since the elevated CaB differed between MUFA and SFA, we speculate that there are unique interactions between the SFA and MUFA meal matrix and CaB. A better understanding of how fatty acids affect CaB is needed, and whether this is a factor contributing to the *in vivo* findings of higher Ca absorption associated with high fat intake. Importantly, a clinical trial using a randomized controlled design is needed since all previous human studies have only been observational. In addition, under higher gastrointestinal pH conditions, it was found that CaB is markedly attenuated under all dietary conditions. The very low CaB in the absence of acidic conditions would have implications in a large population of persons taking antacids that raise pH, and would be expected to reduce Ca bioavailability and absorption, and increase the risk for osteoporosis.

# Conflicts of interest

There are no conflicts of interest to declare.

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