

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

The Effect of Dietary Calcium and Other Nutritionally Relevant Divalent Cations on Fatty Acid-Soap Formation

A dissertation presented in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy in Nutritional Sciences
at Massey University, Manawatū, New Zealand

Natascha Stroebinger

2016



MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND



Riddet Institute
FOOD | INNOVATION | HEALTH

Abstract

A growing amount of scientific evidence appears to support a relationship between dietary calcium (Ca) and body weight where increased dietary Ca intake leads to weight reduction and the faecal excretion of several fatty acids. One possible mechanism, explaining the effect of dietary Ca on body weight and faecal fatty acid excretion, is the formation of indigestible Ca-fatty acid soaps within the gastrointestinal tract, leading to reduced fat and therefore reduced energy absorption. The objectives of this research were 1) to confirm that dietary Ca reduces fatty acid absorption and that the effect is via the formation of fatty acid soaps, 2) to explore the potential of cations other than Ca to form fatty acid soaps and 3) to investigate where in the gastrointestinal tract Ca-fatty acid soap formation occurs.

In order to investigate the presence of fatty acid-soaps in the gastrointestinal tract, an assay was developed to determine fatty acid-soaps in digesta and faeces. Faecal fatty acid-soap excretion, apparent faecal fatty acid digestibility and apparent faecal Ca digestibility were determined in the growing pig for diets containing different sources of fat (tallow, palmolein oil, olive oil and soya bean oil) and increasing concentrations of Ca (0, 2, 4 and 6 g kg⁻¹ diet). Increasing concentrations of dietary Ca resulted in increased faecal fatty acid excretion ($P < 0.001$), predominantly in the form of fatty acid-soaps (> 80%) for diets containing a fat source rich in saturated fatty acids (tallow and palmolein oil). The fatty acid digestibility of these diets was reduced ($P < 0.001$) by up to 28% when the dietary Ca intake was increased from 0 g Ca kg⁻¹ diet to 6 g Ca kg⁻¹ diet. Moreover, faecal Ca output of the tallow-based diet, for which the fatty acid soap excretion was the greatest, was statistically higher when compared to the oil containing diets.

These results provide evidence that supports the hypothesis that dietary Ca can impair fat absorption via the formation of indigestible Ca-fatty acid soaps but that the effect is largely limited to fat sources rich in saturated fatty acids as evidenced by the reduction in Ca absorption with tallow.

Given that Ca appears to react with fatty acids to form soaps, it was decided to investigate whether other nutritionally relevant divalent cations (magnesium (Mg), zinc (Zn), iron (Fe) and copper (Cu)) were able to form fatty acid soaps. To that end, *in vitro* studies revealed that apart from Ca, other divalent cations such as Zn, Mg, Fe and Cu had the ability to form precipitates in the presence of fatty acids. In general, all the divalent cations examined formed precipitates in the presence of at least some of the fatty acids examined, although the extent to which the divalent cation-fatty acid precipitates (soaps) formed varied depending on the cation and fatty acid present. The precipitation of saturated fatty acids (lauric, myristic, palmitic and stearic acid) when incubated with Zn was comparable with that of Ca. However, the precipitation of unsaturated fatty acids (oleic and linoleic acid) with Zn was greater than that observed for Ca. For Fe and Cu, fatty acid precipitation was less than that observed for Ca.

To investigate where in the gastrointestinal tract fatty acid soaps form, growing pigs were fed diets containing either free fatty acids or an intact triacylglyceride (tallow) and calcium carbonate as the Ca source. The amount of insoluble fatty acid-soap present in the gastrointestinal tract was determined at 10 different locations within the tract. The amount of fatty acid-soaps present increased ($P < 0.05$) at the distal jejunum when the free fatty acid-based diet was fed and at the ileum when pigs received the tallow-based diets, and was correlated with the pH (regardless the diet) of the gastrointestinal tract suggesting that soaps

formed as the pH of the gastrointestinal tract increased. Fatty acid-soap formation in the small intestine of pigs receiving the free fatty acids was almost double than for pigs receiving tallow with their diet. There was little soap formation in the hind gut. With the majority of fatty acid soap formation occurring in the distal small intestine (the major absorption site of fatty acids) fatty acid-soap formation has the potential to reduce fatty acid absorption. Feeding a fat-free diet in addition to the two fat containing diets gave insight into mineral absorption in the absence and presence of dietary fat. The apparent digestibility of Ca, Mg, Zn and Fe was lower ($P < 0.05$) in the presence of dietary fat (free fatty acids or triacylglycerides) suggesting that the formation of divalent cation-fatty acid soaps may have the ability to impair the absorption of divalent cations other than Ca.

In conclusion, high dietary Ca intake leads to increased faecal fatty acid excretion in the form of insoluble fatty acid-soaps. Fatty acid-soap formation can impair the digestibility of Ca and other nutritionally relevant divalent cations such as Zn, Mg and Fe. Moreover, fatty acid-soaps appear to form mainly in the distal small intestine and appear to be associated with gastrointestinal pH. These results contribute to the knowledge of where fatty acid soap formation occurs and provide evidence that fatty acid soap formation can reduce fat absorption and thereby possibly contribute to weight loss.

Acknowledgement

I take this opportunity to present my gratitude to a few people that deserve special mention for their role in the accomplishment of my thesis.

First and foremost, I would like to thank my supervisors Dr. Shane Rutherford, Dr. Sharon Henare and Distinguished Professor Dr. Paul Moughan. Your advice and guidance through this project were invaluable. In particular, Shane your constant encouragement, your immense patience, and you believing in me (more than I ever did) have been appreciated over all these years. With your dedication and critical way of thinking, you always got me back on the right path, but not to forget your quotes from the Matrix or your lectures on how my literature review shall differ from the tedious journey of Frodo described in the book of 'The Lord of the Rings' made our meetings enjoyable. Sharon, I want to thank you for your continuous support, your advice and your sincere interest in my welfare. Not to forget for siding up with me that not every data set needs to be presented as a table and that graphs might be more enjoyable for the reader. Paul, I am very grateful for the opportunity you gave me to be part of the Riddet Institute nutrition team and the learning experience that has come along with the challenges throughout my PhD studies. The group internal Research Days provided a great opportunity to share research results and new experimental ideas and proofed to me that presenting in front of colleagues is not that fearful. I also want to thank you for your valuable scientific inputs, your encouragement and your substantial corrective comments.

I am thankful for the Riddet Scholarship to providing me the financial assistance during the first years of my research. I wish to thank the Riddet Institute for the opportunity of part

time work and the involvement in many interesting projects which allowed broadening of my horizon and earning my daily bread during the final years.

A special thanks goes to Dr. Carlos Montoya for providing statistical guidance to all my projects. Thank you Carlos for all the time you took to explain the statistical models to me and helping me to evaluate my results.

I also want to thank Trent Olson, Stuart Saigeman and Daniel for all their muscle work and help during my animal trials. Guys, the picture of the four of us soaping and shaving pig bums will forever stay with me.

I would like to thank all my colleagues and friends from the Riddet Institute and Massey University. Working amongst you all has been a great pleasure. In particular, thanks to Janiene Gilliland, Nok Sawatdeenaruenat, Leiza Turnbull, Dr. Peter Zhu, and Dr. Sophie Gallier for your assistance with lab equipment, sharing your laboratory skills and providing scientific advices but also for the time spent socialising in the lunch room or the sports hall.

The past years would not have been the same without the support and encouragement of my friends whom I have met throughout my studies. Carlos and Eva, you offered me a home away from home. Movie nights, family-style take away, mid-winter Christmas parties make my time in Palmerston North unforgettable. To my fellow sufferers Filomena, Sonal, Tom, Anne, being able to share experiences, highs and lows, as well as tears but not to forget the unbelievable good times have made these years memorable. The biggest thanks goes to Ajit, for always being there for me, being so kind and patient (enduring all my moods), and still being extremely supportive during the good and difficult stages of PhD life. You pushed me to continue when times were tough and I wanted to throw it all away.

Ein besonderes Dankeschon geht an meine Familie in Österreich. Ich kann euch nicht sagen wie glücklich ich mich schätzen kann, dass ich euch habe. Selbst wenn euch die Entfernung schwer gefallen ist habt ihr mich ständig ermutigt weiterzumachen und nicht aufzugeben. Wenn es finanziell eng wurde habt ihr immer angeboten mich zu unterstützen – das euch meine Ausbildung so wichtig ist bedeutet mir sehr viel und ich hoffe, dass ich euch als erster 'Doktor' in der Familie stolz mache.

Table of Contents

Abstract	I
Acknowledgement	V
List of Tables	XIV
List of Figures	XVII
CHAPTER ONE: Introduction	1
1.1. General Overview of the Topic	1
1.2. Aims and Objectives of the Thesis	2
1.3. Structure of the thesis	3
CHAPTER TWO: Literature Review	5
2.1. Introduction	5
2.2. The Digestion of Fat	7
2.2.1. Characteristics of dietary lipids	7
2.2.2. Gastrointestinal digestion of lipids	9
2.2.2.1. Digestion of dietary triglycerides by gastrointestinal lipases	9
2.2.2.2. Dietary factors influencing fat absorption	10
2.3. The Digestion of Ca	12
2.3.1. Sources of dietary Ca	12
2.3.2. Gastrointestinal digestion and absorption of dietary Ca	12
2.3.2.1. Dietary factors that influence intestinal Ca absorption	15
2.4. A Possible Role of Dietary Ca in the Abatement of Obesity and Its Metabolic Disorders	21
2.4.1. A possible role of dietary Ca on reducing blood lipids	21
2.4.2. A possible role of dietary Ca in weight management	22
2.4.3. Mechanisms by which dietary Ca may effect body weight	29
2.4.3.1. The effect of dietary Ca on fat metabolism	29
2.4.3.2. The effect of dietary Ca on thermogenesis	34
2.4.3.3. The effect of dietary Ca on fatty acid excretion and fat absorption	35

2.5. Interactions between Dietary Ca and Fatty Acids: the Formation of Ca-Fatty Acid Soaps	38
2.5.1. Factors influencing Ca-mediated fatty acid excretion	46
2.5.1.1. The influence of fatty acid chain length and degree of saturation on Ca-mediated fatty acid excretion and Ca-fatty acid soap solubility	46
2.5.1.2. The influence of positional distribution of fatty acids on the triglyceride molecule on Ca-mediated fatty acid excretion	49
2.5.1.3. The influence of bile salts on Ca-fatty acid soap solubility and formation	51
2.5.1.4. Dietary protein intake may influence Ca-mediated faecal fatty acid excretion	53
2.5.2. Fatty acid soap formation with divalent cations other than Ca	54
2.6. Concluding Comments	57
CHAPTER THREE: Development of an Assay to Determine the Amount of Ca-Fatty Acid Soaps in Faeces	59
3.1. Abstract	59
3.2. Introduction	60
3.3. Material & Methods	62
3.3.1. Materials	62
3.3.2. Preparation of synthetic Ca-fatty acid soaps	62
3.3.3. Purity of the synthetic Ca-fatty acid soaps	63
3.3.4. Ca analysis	64
3.3.5. Fatty acid analysis	64
3.3.6. Experiment 1: Testing the efficacy of the Sammons and Wiggs (1960) method for the extraction of Ca-fatty acid soaps from faeces.	65
3.3.7. Experiment 2: Evaluating the solubility of Ca-fatty acid soaps in a range of solvents.	66
3.3.8. Experiment 3: Determination of Ca-fatty acid soaps in faeces after extraction of non-Ca bound fatty acids using a three-step extraction method.	67
3.4. Results & Discussion	71
3.4.1. Purity of synthetic Ca-fatty acid soaps	71
3.4.2. Experiment 1: Testing the efficacy of the method of Sammons and Wiggs (1960) for extracting Ca-fatty acid soaps from pig faeces.	71
3.4.3. Experiment 2: Testing the solubility of Ca-fatty acid soaps in a range of different solvents.	73
3.4.4. Experiment 3: Determination of Ca-fatty acid soaps in faeces by extracting non-Ca bound fatty acid components using a three-step extraction process.	79

3.4.5. Limitations of the soap determination assay	85
3.5. Conclusion	85
CHAPTER FOUR: Effect of Fatty Acids from Different Fat Sources and Dietary Calcium Concentration on Soap Formation in the Growing Pig.	87
4.1. Abstract	87
4.2. Introduction	87
4.3. Material & Methods	90
4.3.1. Materials	90
4.3.2. Diets	90
4.3.3. Molecular distribution of fatty acids in the triglyceride molecule	92
4.3.4. Animal trial	93
4.3.5. Determination of fatty acids in faeces present as soaps	94
4.3.6. Fatty acid analysis	95
4.3.7. Ca and P analysis	96
4.3.8. Trace element analysis	96
4.3.9. TiO ₂ analysis	96
4.3.10. Data analysis	97
4.3.11. Statistical analyses	98
4.4. Results	98
4.4.1. Animal trial	100
4.4.2. Total faecal fatty acids and soap-fatty acids	100
4.4.3. Apparent faecal fatty acid digestibility	107
4.4.4. Faecal Ca and faecal Ca digestibility	110
4.4.5. Faecal P and apparent faecal P digestibility	112
4.4.6. Faecal trace minerals	113
4.5. Discussion	116
4.6. Conclusion	122
CHAPTER FIVE: The Ability of Divalent Cations to Form Fatty Acid-Soaps under Gastrointestinal Conditions.	123
5.1. Abstract	123
5.2. Introduction	124

5.3. Material & Methods	125
5.3.1. Materials	125
5.3.2. Determining divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	125
5.3.3. Investigating the kinetics of divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	128
5.3.4. Investigating the impact of cation concentration on divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	128
5.3.5. Fatty Acid Analysis	129
5.3.6. Divalent Cation Analysis	129
5.3.7. Statistical Analyses	129
5.4. Results	130
5.4.1. Non soap specific precipitation under <i>in vitro</i> simulated gastric and small intestinal conditions	130
5.4.2. Divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	131
5.4.3. The kinetics of divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	139
5.4.4. The impact of cation concentration on divalent cation fatty acid soap formation under <i>in vitro</i> simulated gastric and small intestinal conditions	142
5.5. Discussion	147
5.6. Conclusion	154
CHAPTER SIX: Mapping Fatty Acid Soap Formation Throughout the Gastrointestinal Tract of the Growing Pig.	155
6.1. Abstract	155
6.2. Introduction	156
6.3. Material & Methods	159
6.3.1. Materials	159
6.3.2. Diets	159
6.3.3. Animal trial	162
6.3.4. Chemical analyses	163
6.3.5. Data Analysis	164
6.3.6. Statistical analysis	165
6.4. Results	166
6.4.1. Animal trial	166

6.4.2. Gastrointestinal pH	166
6.4.3. Formation of fatty acid-soaps	167
6.4.4. Ca flow and apparent Ca digestibility along the gastrointestinal tract	172
6.4.5. P flow and apparent P digestibility along the gastrointestinal tract	174
6.4.6. Mg flow and apparent Mg digestibility along the gastrointestinal tract	176
6.4.7. Trace mineral (Fe, Zn and Cu) flow and apparent digestibility along the gastrointestinal tract	178
6.5. Discussion	182
6.6. Conclusion	189
CHAPTER SEVEN: General Discussion	191
7.1. Summary & Discussion	191
7.2. Conclusions & Future Research	202
Bibliography	205



List of Tables

Table 2-1: Major fatty acids in the human diet, their chemical structure, chain length and double bonds.....	8
Table 2-2: Study characteristics of previously performed studies using human subjects investigating the effect of increasing concentrations of dietary Ca on faecal fat/fatty acid excretion.....	42
Table 3-1: Determined molar ratio of Ca to fatty acids in the synthetic Ca-fatty acid soaps ¹ .	71
Table 3-2: Mean recovery (%) of synthetic Ca-fatty acid soaps from spiked fresh pig faeces after extraction using the method of Sammons and Wiggs (1960) with the modifications of Gacs and Barltrop (1977).	72
Table 3-3: Mean ¹ recovery (%) of fatty acids in extraction solvents after incubation of Ca-fatty acid soap with different solvents at 4°C, 24°C and at the solvent's boiling point (b.p.), for 0.5, 2 and 6 h.....	76
Table 3-4: Mean recovery of fatty acids in extraction solvents from faeces spiked with free fatty acids, Na-fatty acid salts, Ca-fatty acids soaps and phospholipids after sequential solvent extraction at different temperatures.....	83
Table 3-5: Mean recovery (\pm SEM) of fatty acids from faeces spiked with free fatty acids, Na-fatty acid salts, Ca-fatty acids soaps and phospholipids (PL) after sequential solvent extraction at different temperatures.	84
Table 4-1: Ingredient composition (g kg ⁻¹ air-dry weight) of the test diets fed to the growing pigs during the experimental period.	91
Table 4-2: Determined fatty acid, calcium and phosphorus contents (g kg ⁻¹ air dry weight) of the 16 experimental diets.....	92
Table 4-3: Fatty acids (FA) and their positional distribution in tallow, palmolein oil, olive oil and soya bean oil.	99
Table 4-4: Mean (n=9) ¹ faecal total palmitic acid (mg kg ⁻¹ DMI) ² and faecal soap-palmitic acid (g kg ⁻¹ DMI) ² for pigs receiving experimental diets differing in fat source and Ca concentration.....	102
Table 4-5: Mean (n=9) ¹ faecal total stearic acid (mg kg ⁻¹ DMI) ² and faecal soap-stearic acid (g kg ⁻¹ DMI) ² for pigs receiving experimental diets differing in fat source and Ca concentration.	103
Table 4-6: Mean (n=9) ¹ faecal total oleic acid (mg kg ⁻¹ DMI) ² and faecal soap-oleic acid (g kg ⁻¹ DMI) ² for pigs receiving experimental diets differing in fat source and Ca concentration. ..	105
Table 4-7: Mean (n=9) ¹ faecal total linoleic acid (mg kg ⁻¹ DMI) ² and faecal soap-linoleic acid (g kg ⁻¹ DMI) ² for pigs receiving experimental diets differing in fat source and Ca concentration.	106
Table 4-8: Mean (n = 9) ¹ apparent faecal palmitic acid digestibility (%) for pigs receiving experimental diets differing in fat source and Ca concentration.....	108

Table 4-9: Mean (n = 9) ¹ apparent faecal stearic acid digestibility (%) for pigs receiving experimental diets differing in fat source and Ca concentration.	109
Table 4-10: Main effects of pooled means across fat source and Ca concentration (n=36) ¹ for faecal Ca (mg kg ⁻¹ DMI), apparent and true faecal Ca digestibility (%) for pigs receiving experimental diets.	111
Table 4-11: Main effects of pooled means across fat source and Ca concentration (n=36) ¹ for faecal P (mg kg ⁻¹ DMI) and apparent faecal P digestibility (%) for pigs receiving experimental diets.	113
Table 4-12: Mean (n=9) ¹ faecal Fe (mg kg ⁻¹ DMI) for pigs receiving experimental diets differing in fat source and Ca concentration.	115
Table 4-13: Mean (n=9) ¹ faecal Zn (mg kg ⁻¹ DMI) for pigs receiving experimental diets differing in fat source and Ca concentration.	115
Table 5-1: Divalent cation ¹ precipitation (μmol) after incubation in the absence of free fatty acids ²	131
Table 5-2: The amounts of fatty acids and divalent cations precipitated under simulated gastric (pH = 2.5) and sequentially simulated intestinal (pH = 4.5-7.5) conditions for several divalent cations and fatty acids.	134
Table 5-3: Kinetics of divalent-cation fatty acid soap formation under <i>in vitro</i> simulated upper gastrointestinal tract conditions ¹ at pH 7.5.	140
Table 5-4: The effect of Ca concentration on Ca-stearic acid soap formation under simulated gastro-intestinal conditions ¹ at pH 7.5.	143
Table 5-5: The effect of Zn concentration on Zn-stearic acid soap formation under simulated gastro-intestinal conditions ¹ at pH 7.5.	144
Table 5-6: The effect of Cu concentration on Cu-stearic acid soap formation under simulated gastro-intestinal conditions ¹ at pH 5.5.	145
Table 5-7: The solubility product for fatty acid soaps and hydroxide salts of different minerals according to the literature.	150
Table 6-1: Ingredient composition (g kg ⁻¹ air dry weight) of the basal and experimental diets.	160
Table 6-2: Determined selected nutrient composition (g kg ⁻¹ air dry weight) of the three experimental diets.	161
Table 6-3: Mean (n = 8) pH throughout the gastrointestinal (GI) tract of the growing pig for the three test diets.	167
Table 6-4: Mean (n = 8) total ¹ fatty acid soap flows for the experimental diets as determined throughout the gastrointestinal (GI) tract.	170
Table 6-5: Main effects of pooled means (n = 8) calculated across gastrointestinal (GI) location or experimental diet for gastrointestinal Ca flow and apparent Ca digestibility.	173
Table 6-6: Mean (n = 8) gastrointestinal P flow (mg kg ⁻¹ DMI) and apparent P digestibility for the experimental diets (A, B and C), determined throughout the gastrointestinal (GI) tract of the growing pig.	175

Table 6-7: Main effects of pooled means (n = 8) across gastrointestinal (GI) location or experimental diet for gastrointestinal Mg flow and apparent Mg digestibility.....	177
Table 6-8: Main effects of pooled means (n = 8) calculated across gastrointestinal (GI) location or experimental diet for gastrointestinal Fe flow and apparent Fe digestibility.....	179
Table 6-9: Main effects of pooled means (n = 8) calculated across gastrointestinal (GI) location or experimental diet for gastrointestinal Zn flow and apparent Zn digestibility.....	180
Table 6-10: Main effects of pooled means (n = 8) calculated across gastrointestinal (GI) location or experimental diet for gastrointestinal Cu flow and apparent Cu digestibility....	181

List of Figures

- Figure 2-1: Stereo specific numbering of fatty acid positions on the triglyceride composed of stearic, oleic and palmitic acid. **A**: Fisher projection of a triglyceride. **B**: Glycerolipid structure of 1-stearoyl-2-oleoyl-3-palmitoyl-triacylglycerol created on LIPID MAPS – Nature Lipidomics Gateway (Fahy *et al.* 2007)..... 9
- Figure 2-2: Intestinal Ca absorption via transcellular and paracellular pathways. 14
- Figure 2-3: Hormone induced lipolysis in adipocytes (**A**) and the influence of intracellular calcium levels on adipocyte fat metabolism (**B**)..... 31
- Figure 3-1: Sequential three-step solvent extraction of non-Ca bound fatty acids from faeces containing free fatty acids, mono-, di-, and triglycerides, phospholipids, Na-soaps and Ca-fatty acid soaps. 68
- Figure 5-1: Amount of lauric acid (◆), myristic acid (■), palmitic acid (▲), stearic acid (×), oleic acid (○) and linoleic acid (●) that precipitated after incubation with Ca (**A**), Mg (**B**), Zn (**C**), Cu (**D**) or Fe (**E**) at increasing pH under *in vitro* simulated gastrointestinal conditions. 136
- Figure 5-2: Kinetics of fatty acid concentration (marker of divalent cation fatty acid soaps) 141
- Figure 5-3: Effect of divalent cation concentration on divalent cation and stearic acid precipitation under simulated gastrointestinal conditions¹ 146
- Figure 6-1: Mean total fatty acid soap flow (n=8) for ■ palmitic (A), ■ stearic (B) and ■ oleic (C) acid determined throughout the gastrointestinal tract of the growing pig receiving experimental diets containing free fatty acids (FFA)  or tallow  171

