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

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2016

MASSEY UNIVERSITY
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Riddet Institute
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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 Rutherfurd, 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 bedeuted mir sehr viel und ich hoffe, dass ich euch als erster 'Doktor' in der Familie stolz mache.

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CHAPTER ONE: Introduction

1.1. General Overview of the Topic

Calcium (Ca) has the ability to form insoluble complexes with free fatty acids and it has been suggested that these complexes, referred to as Ca-fatty acid soaps, can limit the absorption of Ca from the gastrointestinal tract *in vivo*. Ca is a divalent cation and can bind two molecules of free fatty acids. In support of the foregoing hypothesis, increased dietary Ca has been shown to increase total fatty acid excretion in the faeces. This greater faecal fat excretion and therefore reduced fatty acid absorption may be the mechanism for the observation that in some studies, higher dietary calcium intake leads to weight loss in humans. Although it should be pointed out that a number of studies have found no effect of Ca on body weight.

To date, apparent faecal digestibility estimates have been used to assess the effects of dietary Ca on fatty acid digestibility. However, fatty acids and Ca are absorbed mainly in the small intestine with little absorption occurring in the large intestine. Consequently, a more sensible approach would be to assess fatty acid digestibility and calcium absorption to the end of the small intestine (using digesta collected from the terminal ileum) rather than over the entire gastrointestinal tract as is the case when faecal digestibility is determined.

While yet to be investigated, it is also possible that divalent cations other than Ca, such as the nutritionally relevant minerals magnesium (Mg), zinc (Zn), iron (Fe) and copper (Cu), form divalent cation-fatty acid soaps in the same manner that Ca does. If this is the case, then the formation of the latter soaps may reduce the bioavailability of these essential minerals.

The aim of this research is to explore the formation of divalent cation-fatty acid soaps (*in vitro* and *in vivo*) and the effect these soaps have on fatty acid digestibility and mineral absorption. While the studies will generally focus on Ca, other divalent minerals will also be investigated.

1.2. Hypothesis

Dietary calcium and fatty acid interactions in the gastrointestinal tract impair fat digestion via the formation of Ca-fatty acid soaps which evade absorption throughout the intestine. Ca, as a divalent cation has the ability to bind two molecules of free fatty acids, thus forming a rather insoluble complex at intestinal environment. There might be the possibility that other divalent cations form complexes with free fatty acids as well, which might decrease the absorption of these nutritionally relevant divalent cations.

1.3. Aims and Objectives of the Thesis

The aim of this PhD project was to investigate the formation of divalent cation-fatty acid soaps under *in vitro* simulated gastrointestinal conditions and *in vivo* digestion throughout the gastrointestinal tract using the growing pig as an animal model for the adult human. The growing pig, a meal eating omnivore, has been shown to be an appropriate model for studying aspects of digestion in adult humans (Rowan *et al.* 1994). There are no published studies that have focused on divalent cation-soap formation *in vitro* with divalent cations other than Ca, nor on the location within the gastrointestinal tract that fatty acid-soap formation may occur.

The main objectives of this research were:

- To evaluate the published methods for the determination of Ca-fatty acid soaps in faecal material and if necessary develop a new method for determining Ca-fatty acid soaps.
- To investigate the effect of different concentrations of dietary Ca on faecal fatty acid excretion and apparent fatty acid digestibility using a range of different fat sources using the growing pig as an animal model for the adult human.
- To investigate the formation of insoluble soaps derived from fatty acids and a range of nutritionally important divalent cations using *in vitro* models that simulate the conditions present in the stomach and small intestine.
- To investigate Ca-fatty acid soap formation throughout the gastrointestinal tract using the growing pig as an animal model for the adult human in order to determine where in the gastrointestinal tract Ca-fatty acid soaps form.

1.4. Structure of the thesis

The introduction chapter is followed by the literature review that gives an overview covering digestion and absorption of the two components of Ca-fatty acid soaps. Further, a possible effect of dietary Ca on weight management is reviewed and possible mechanisms for the 'Ca weight loss' effect are described. Chapter Three focuses on previously published methods used to determine Ca-fatty acid soaps in faeces and describes the development of a novel method for determining fatty acid soaps which was used for the subsequent experiments in this project. Chapter Four describes the *in vivo* digestion study that investigated the impact of varying dietary Ca concentrations on faecal fatty acid excretion

and apparent faecal fatty acid digestibility for a range of different fat sources. This chapter also includes data on faecal fatty acid soap concentrations and true faecal Ca digestibility. The experimental work investigating the *in vitro* formation of divalent cation-fatty acid soaps with Ca but also other nutritionally relevant minerals such as Mg, Zn, Cu and Fe is described in Chapter Five. The experimental work and the results of the study investigating where Ca-fatty acid soaps are formed in the gastrointestinal tract of the growing pig are presented in Chapter Six. Furthermore, the effect of dietary fat on apparent mineral (Mg, Zn, Fe, Cu) digestibility is also described. The general discussion and conclusions are given in Chapter Seven.

CHAPTER TWO: Literature Review

2.1. Introduction

Obesity is the most common nutritional disorder in the United States and is a serious medical problem globally. The World Health Organisation (WHO) estimated that in 2008 more than one billion adults (≥ 20 years of age) were considered overweight, of which nearly 500 million were obese. Moreover, chronic diseases associated with being overweight or obese, such as type II diabetes, cardiovascular disease, musculoskeletal disorders and certain types of cancer are becoming rapidly more prevalent worldwide. Reducing the intake of fat and highly refined carbohydrates is an obvious approach to reducing body weight but this approach is difficult for many overweight and obese persons. Consequently, considerable research has been undertaken to investigate the efficacy of alternative dietary strategies to weight loss and reducing the prevalence of obesity related diseases. For example, by replacing foods rich in saturated fatty acids with foods rich in polyunsaturated fatty acids (Baum *et al.* 2012), or by increasing the consumption of fibre rich foods (legumes, vegetables, fruits, whole grain) which have been associated with weight loss, reduced blood glucose levels and reduced plasma cholesterol (Ludwig *et al.* 1999; Howarth *et al.* 2001; Pereira and Ludwig 2001). Other compounds in foods have been suggested to contribute to weight management including conjugated linoleic acids, medium-chain triglycerides, capsaicin, and green tea, but the results of studies investigating these types of compounds are conflicting (Kovacs and Mela 2006). Dietary calcium (Ca) has also been purported to play a role in reducing the risk of obesity and its related diseases (Astrup 2011). A growing body of evidence suggests that dietary Ca, either as a dietary supplement or that present naturally in foods, plays a direct role in the prevention and abatement of obesity (Zemel *et al.* 2000) and

obesity-related metabolic disorders, such as hypertension (Sowers *et al.* 1989), hypertriglyceridemia and hypercholesterolemia (Denke *et al.* 1993).

Most observational and population based data, work based on several animal models and a small number of clinical studies in humans support an association between dietary Ca and weight loss and/or body composition (Davies *et al.* 2000; Papakonstantinou *et al.* 2003; Zemel 2003). Furthermore, epidemiological and clinical evidence suggests a role for dietary Ca in the control of serum levels of harmful lipids (Denke *et al.* 1993), and the possibility of a role for Ca in the abatement of obesity and cardiovascular diseases has also been put forward (Astrup 2011). However, the mechanism(s) by which dietary Ca is involved in lowering body fat and serum lipids is not entirely clear. To date, four mechanisms have been proposed in the literature and these are based on 1) a reduction in adipocyte mass, 2) an increase in fat oxidation, 3) an increased thermic effect of food and 4) a decrease in fat absorption and an increase in faecal fat excretion. The last mechanism, an increase in faecal fatty acid output due to increased dietary Ca intake, is hypothesised to occur due to interactions between ionised Ca and free fatty acids, forming complexes referred to as Ca-fatty acid soaps. It is this latter hypothesis that is the focus of this PhD dissertation.

2.2. The Digestion of Fat

2.2.1. Characteristics of dietary lipids

Dietary lipids include triglyceride, phospholipids, sterols, fat soluble vitamins, free fatty acids, and others. Triglycerides are the predominant fat in human diets, constituting approximately 95% of dietary lipids. The structure of triglycerides comprises a glycerol backbone to which three fatty acids are covalently linked via the carboxyl moiety on the fatty acid and a hydroxyl group on the glycerol molecule. In foods, fatty acids are almost exclusively bound to triglycerides and the presence of free fatty acids in foods is rare. Food lipids generally are composed of fatty acids containing between 4 and 24 carbon atoms with long-chain fatty acids containing between 16 and 20 carbon atoms being the most common (see Table 2-1). Fatty acids are divided in to three major classes; saturated fatty acids, monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) which differ in the number of double bonds in the carbon backbone, with saturated fatty acids having no double bonds, monounsaturated fatty acids having one double bond and polyunsaturated fatty acids having up to six double bonds. Unsaturated fatty acids are classified as either n-3 (omega-3), n-6 (omega-6) or n-9 (omega-9) fatty acids depending on the placement of their double bonds. Saturation and chain length play an important role in the solubility of fatty acids in aqueous systems. Fatty acids with less than eight carbons are considered to be short-chained and are relatively soluble in water. With increasing chain length, the solubility of fatty acids in water decreases and saturated fatty acids containing 14 or more carbon atoms are considered to be water insoluble.

Table 2-1: Major fatty acids in the human diet, their chemical structure, chain length and double bonds.

common name	chemical structure	C:D[1]	n-x[2]
Lauric acid	$CH_3(CH_2)_{10}COOH$	12:0	
Myristic acid	$CH_3(CH_2)_{12}COOH$	14:0	
Palmitic acid	$CH_3(CH_2)_{14}COOH$	16:0	
Stearic acid	$CH_3(CH_2)_{16}COOH$	18:0	
Oleic acid	$CH_3(CH_2)_7\mathbf{CH=CH}(CH_2)_7COOH$	18:1	n-9
Linoleic acid	$CH_3(CH_2)_4\mathbf{CH=CH}CH_2\mathbf{CH=CH}(CH_2)_7COOH$	18:2	n-6
ALA[3]	$CH_3CH_2\mathbf{CH=CH}CH_2\mathbf{CH=CH}CH_2\mathbf{CH=CH}(CH_2)_7COOH$	18:3	n-3
AA[4]	$CH_3(CH_2)_4\mathbf{CH=CH}CH_2\mathbf{CH=CH}CH_2\mathbf{CH=CH}CH_2\mathbf{CH=CH}(CH_2)_3COOH$	20:4	n-6
EPA[5]	$CH_3CH_2(\mathbf{CH=CH}CH_2)_4\mathbf{CH=CH}(CH_2)_2COOH$	20:5	n-3
DHA[6]	$CH_3CH_2(\mathbf{CH=CH}CH_2)_5\mathbf{CH=CH}(CH_2)_2COOH$	22:6	n-3

[1]C:D – carbon number:double bonds
[2]n-x omega nomenclature: counting from the methyl group of the fatty acid carbon chain towards the first double bond
[3]ALA – α-linolenic acid
[4]AA – arachidonic acid
[5]EPA – eicosapentaenoic acid
[6]DHA – docosahexaenoic acid

A stereospecific numbering (sn) system is used to denote to which carbon in the glycerol backbone a given fatty acid is attached (Favre and Powell 2013). The primary carboxyl groups are referred to as the sn-1 and sn-3 positions (see Figure 2-1) and can also be referred to as α-positions whereas the secondary carboxyl group is referred to as the sn-2 position or β-position. Most plant oils are low in saturated fatty acids and high in mono- and polyunsaturated fatty acids (with some exceptions such as coconut oil, cocoa butter, palm oil, etc.), whereas animal fat contains considerably more saturated fatty acids. Saturated fatty

acids present in plant oils tend to be present predominantly on the sn-1 and 3 positions of the triglyceride molecules. In contrast, fats of animal origin have a high proportion of saturated fatty acids in the sn-2 position and unsaturated fatty acids tend to be in the sn-1 and sn-3 positions (Christie and Moore 1970; Bracco 1994). Tallow is however, an exception since the tallow molecule has the two saturated fatty acids (stearic and palmitic acids) predominantly in the sn-1 and sn-3 positions with the unsaturated fatty acids being present in the sn-2 position.

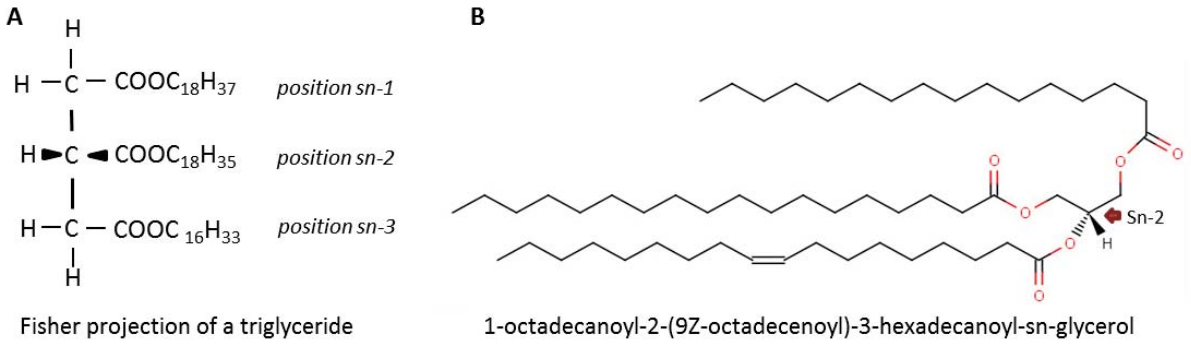


Figure 2-1: Stereo specific numbering of fatty acid positions on the triglyceride composed of stearic, oleic and palmitic acid. **A**: Fisher projection of a triglyceride. **B**: Glycerolipid structure of 1-stearoyl-2-oleoyl-3-palmitoyl-triacylglycerol created on LIPID MAPS – Nature Lipidomics Gateway (Fahy *et al.* 2007).

2.2.2. Gastrointestinal digestion of lipids

2.2.2.1. Digestion of dietary triglycerides by gastrointestinal lipases

Lipid digestion in humans begins in the stomach, where gastric lipase partially digests the triglycerides into diglycerides and free fatty acids. Gastric lipase preferentially cleaves short and medium chain fatty acids from the sn-3 position of a triglyceride releasing between 10 to 30% of the bound fatty acids. Dietary fat enters the duodenum as a mixture of di- and triglycerides and hydrolysed free fatty acids. When the food bolus enters the duodenum, bile (including bile acids, cholesterol, phospholipids and bilirubin; pH 5-6) from the gall bladder

and pancreatic juice and bicarbonates from the pancreas are secreted into the intestinal lumen. Bile salts are involved in the three steps of fat digestion: emulsification, hydrolysis of the triglycerides, and micellar solubilisation of the hydrolysed fatty acids and monoglycerides. In the small intestine, di- and triglycerides are emulsified with bile salts and hydrolysis continues through the action of pancreatic lipases (Fave *et al.* 2004; Bauer *et al.* 2005; McClements *et al.* 2006). Pancreatic lipase cleaves fatty acids from the sn-1 and sn-3 positions of the triglyceride at equal rates resulting in the formation of sn-2 monoglycerides and free fatty acids (Paltauf and Wagner 1976). A rearrangement of fatty acids in the sn-2 position of the monoglycerides into sn-1 or 3 position can also occur, to about 30%, and approximately 75% of the latter transformation can result in complete hydrolysis of the triglyceride molecule into glycerol and free fatty acids (Akesson *et al.* 1978). The resulting free glycerol is absorbed independently and plays no further role in lipid absorption. Monoglycerides can escape hydrolysis by entering the aqueous phase of the intestinal lumen (Hofmann 1963; Hofmann and Borgstrom 1964) since pancreatic lipase acts only at an oil-water interface and cannot hydrolyse water-soluble substrates (Sarda and Desnuelle 1958). The sn-2 monoglycerides are easily incorporated into mixed micelles (Hofmann and Borgstrom 1963) and facilitate micelle formation with other free fatty acids. Lipids incorporated into the mixed micelles are transported to the brush boarder of the enterocyte, where fatty acids and monoglycerides are absorbed.

2.2.2.2. Dietary factors influencing fat absorption

Ingested dietary fat in healthy human subjects is normally nearly completely digested and absorbed. However, some dietary nutrients, such as dietary fibre and Ca, have been reported to affect the digestion and absorption of fat in the gastrointestinal tract.

Dietary fibre

There is compelling evidence in the literature that dietary fibre affects lipid digestion. Studies in human subjects receiving the fibre sources wheat bran, oat bran or bagasse fibre have shown increased faecal fatty acid excretion compared to subjects receiving the control diet devoid of the fibre source (Walters *et al.* 1975; Chen *et al.* 1998). A reduced fat absorption of a fibre rich diet might be the consequence of direct interactions of fibre structures with lipase and/or colipase (Han *et al.* 1999; O'Connor *et al.* 2003) or the formation of a protective multi-layer coating around the lipid droplets (Faldt *et al.* 1993; Ogawa *et al.* 2003; McClements *et al.* 2006), resulting, in both cases, in the reduced hydrolysis of triglycerides. Furthermore, dietary fibre has been shown to bind bile acids *in vitro* (Kern *et al.* 1978), which may prevent emulsification and solubilisation of fat droplets also thereby reducing fat absorption.

Dietary Ca

A number of studies in animals and humans have shown an increase in faecal fat excretion when diets containing high (above the recommended daily intake of 1000 mg Ca per day) concentrations of Ca were consumed (Denke *et al.* 1993; Papakonstantinou *et al.* 2003). Free fatty acids hydrolysed from dietary triglycerides have the ability to complex with ionised dietary Ca within the gastrointestinal environment and form an insoluble complex leading to reduced fat absorption (Gacs and Barltrop 1977). The influence of dietary Ca on ingested fat absorption is discussed in more detail later in the chapter (see section 2.5. Interactions between Dietary Ca and Fatty Acids: the Formation of Ca-Fatty Acid Soaps).

2.3. The Digestion of Ca

2.3.1. Sources of dietary Ca

Ca is present in a wide variety of foods but is also consumed in the form of dietary supplements. In a typical westernized diet more than 70% of dietary Ca intake is derived from milk and milk products, 9-16% from fruits and vegetables particularly green leafy vegetables and dried fruits, 5% from grains, 4% from legumes and 4% from water (Gueguen and Pointillart 2000; Allen and Kerstetter 2005). With the exception of dairy products, most animal derived foods (e.g. red meat, poultry, fish and eggs) contain only small amounts of Ca and account for only a small proportion (approx. 5%) of the daily Ca intake (Fleming and Heimbach 1994).

2.3.2. Gastrointestinal digestion and absorption of dietary Ca

Ca can be consumed either within a food matrix or as an inorganic dietary supplement. When Ca is consumed it is solubilised either via digestion and disintegration of the food material in which the Ca is contained or by direct solubilisation in the acidic milieu in the stomach if the Ca is ingested as an inorganic supplement. Approximately 90% of dietary Ca absorption occurs in the small intestine with the remaining 10% of dietary Ca being absorbed in the colon (Bronner 2009). Ca is transported through the intestinal mucosa into the bloodstream via two mechanisms. The first mechanism involves active transcellular transport and occurs mainly in the duodenum and the upper jejunum of mammals. The second mechanism involves passive diffusion (paracellular transport) and occurs throughout the entire gastrointestinal tract of mammals, although the ileum, and to a lesser degree the large intestine, are the main sites of paracellular transport (Bronner 1987). The relative

contribution of the latter two transport pathways is debatable. It has been traditionally believed that the active vitamin D-dependent transcellular transport pathway is the major Ca absorption mechanism (Ireland and Fordtran 1973). However, more recent studies have suggested that passive diffusion may play a more predominant role (Bronner and Pansu 1999; Bronner *et al.* 2003).

Transcellular Ca transport

The transcellular pathway is an active transport pathway that is saturable due to the limitation in the availability of the Ca binding transport-protein (calbindin) in enterocytes and ATP-dependent active extrusion of Ca into the blood circulation. The transcellular pathway is regulated by the physiological and nutritional availability of vitamin D and involves three steps: 1) entry of ionised Ca across the brush border membranes of epithelial cells at the luminal side; 2) facilitated diffusion across the cytoplasm and 3) active extrusion at the basolateral membrane into the extracellular fluid (Figure 2-2) (Bronner and Spence 1987; Bronner 1992;2001).

The entrance of ionized Ca into the enterocyte cytoplasm occurs via Ca-selective channels. The best understood Ca channel in the intestine is TRPV6 and is thought to be the major Ca-selective channel in transcellular Ca absorption. After Ca enters the enterocyte it must be transported to the basolateral wall of the cell. Therefore, ionised Ca will bind to a cytosolic Ca-binding protein (calbindin-D 9k in humans) which enables the transport of the ion through the cytoplasm (Bronner 1992). The final stage of Ca absorption is the release of Ca from the basolateral membrane into the bloodstream. There are two proteins involved in the process of extrusion, plasma membrane Ca-ATPase and sodium-calcium exchanger. The

Ca-ATPase is an active Ca transporter that requires ATP. The sodium-Ca exchanger, a passive Ca transporter, is only a minor contributor to the efflux of Ca from the enterocyte.

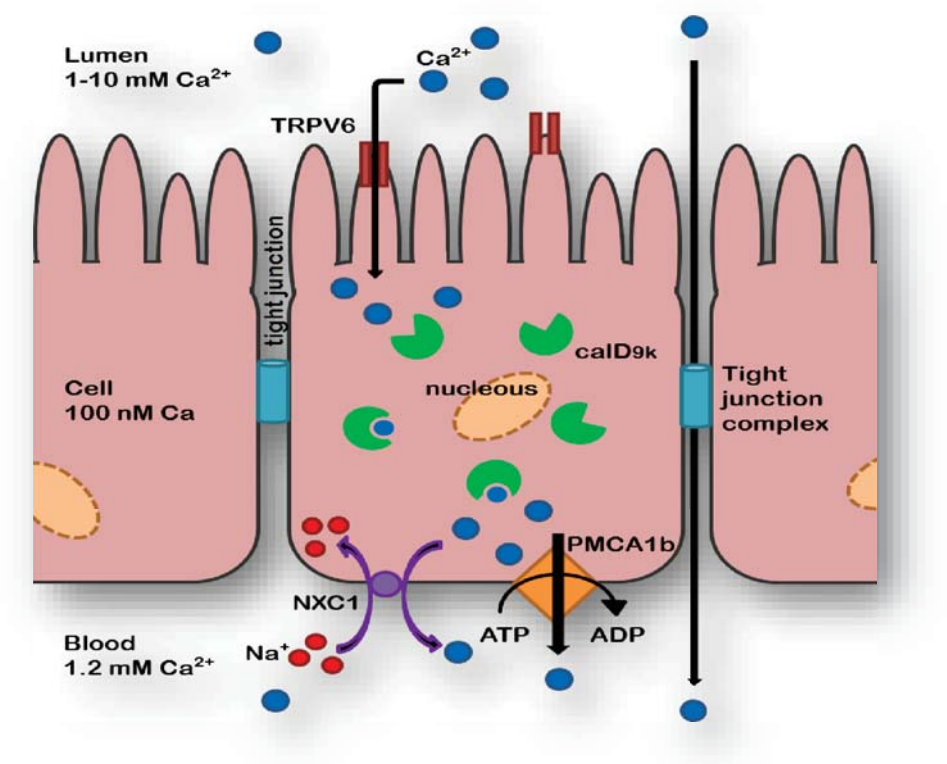


Figure 2-2: Intestinal Ca absorption via transcellular and paracellular pathways. Transcellular Ca transport involves three steps 1) entry of Ca^{2+} into the enterocyte through apical Ca channels (e.g. TRPV6); 2) facilitated diffusion through the enterocyte by Ca^{2+} binding to calbindin ($calD_{9k}$); 3) extrusion of Ca at the basolateral membrane via Ca^{2+}-ATPase (PMCA1b) and a Na^{+}/Ca^{2+} exchanger (NCX1). Paracellular transport takes place across the tight junctions and is driven by a Ca concentration gradient. Figure based on the description and diagram of Slepchenko and Bronner (2001).

Paracellular Ca transport

Apart from the active saturable Ca transport mechanism, Ca can also be absorbed via passive diffusion during its passage through the intestinal tract. Passive paracellular Ca absorption occurs through the tight junctions and is dependent on tight junction permeability and the Ca concentration gradient between the intestinal lumen and the extracellular body fluids (Figure 2-2) (Bronner 2001). Ca ion concentration in the gastrointestinal lumen can be below 1.0 and up to 10.0 mM and is strongly dependent on the dietary Ca intake and most likely

also on endogenous Ca secretion. Ionised Ca within the blood stream is between 1.0 and 1.2 mM, therefore, an intake of dietary Ca resulting in a luminal Ca concentration above 1.2 mM will allow passive diffusion of Ca through the tight junctions. Compared to TRPV6-mediated transcellular transport, which is greatest in the duodenum and decreases significantly in more distal regions of the intestine, paracellular Ca absorption is thought to be relatively constant across the length of the intestine. However, the greatest amount of passive Ca absorption has been reported to occur in the ileum and is most likely influenced by the longer residence time of the digesta in the distal part of the small intestine (Bronner 2001; Hoenderop *et al.* 2005). Since digesta remain for a significantly longer time in the ileum compared to the duodenum, due to the relative length of, and digesta flow rate through, the two intestinal sites, the efficiency of passive transport is much higher in the distal region. Interactions between Ca and other nutrients as a result of the higher pH of the digesta in the distal small intestine is thought to interfere with passive diffusion and may be the limiting step for passive diffusion (Diaz de Barboza *et al.* 2015).

2.3.2.1. Dietary factors that influence intestinal Ca absorption

Foods are a complex materials comprising of many components which can have either a positive or negative influence on Ca absorption. Dietary Ca, once ingested and solubilised from its food matrix in the acidic environment of the stomach, can form insoluble complexes with anti-nutritional factors such as phosphates, phytic acid, oxalic acid, uronic acid, fibres and possibly fatty acids to escape absorption (Allen 1982). On the other hand, dietary components such as proteins (particularly the amino acids lysine and arginine), casein and sugars (such as lactose and resistant sugars), and short chain fatty acids can aid the intestinal absorption of Ca (Allen 1982).

In the following section, nutrients that have either a positive or negative influence on dietary Ca absorption from the gastrointestinal tract are discussed briefly.

Amino acids and casein

A dietary Ca to protein ratio (mg Ca g^{-1} protein) above 20 has been shown to be very favourable in maintaining the Ca balance in the human organism (Klobukowski *et al.* 2014). Dietary proteins are digested to peptides and amino acids in the gastrointestinal tract. The basic amino acids, lysine and arginine, have been shown to enhance gastrointestinal Ca absorption in the rat (Wasserman *et al.* 1957). However, the mechanism for the latter effect is unknown.

Casein, the major protein of bovine milk, has been shown to promote dietary Ca absorption during digestion in the gastrointestinal tract (Scholz-Ahrens and Schrezenmeir 2000). In *in vitro* and *in vivo* experiments, casein has been shown to be digested to phosphopeptides by intestinal proteases which in turn have a high affinity for Ca ions (Reeves and Latour 1958; Naito *et al.* 1972; Sato *et al.* 1991). These casein phosphopeptides are relatively resistant to further breakdown by digestive enzymes and can bind Ca via their serine residues with great affinity (Berrocal *et al.* 1989). It has been suggested that these soluble Ca-phosphopeptides complexes enhance Ca absorption by decreasing the amount of Ca precipitating with phosphorus present in the gastrointestinal tract (Camara-Martos and Amaro-Lopez 2002). In an *ex vivo* absorption experiment using isolated everted sections of rat ileum it has been shown that Ca absorption in the presence of phosphates is reduced to a lesser degree when casein phosphopeptides are present in the synthetic mucosal solution (Erba *et al.* 2001). Moreover Ca solubility in the lower intestinal tract of rats was found to be

double when casein-based diets were fed compared to soya-bean protein- and egg albumin-based diets (Lee *et al.* 1980).

Lactose

Lactose has been shown in several studies to have a positive effect on Ca absorption (reviewed by Kwak *et al.* 2012). *Ex vivo* studies using the rat intestinal tract (Armbrecht and Wasserman 1976; Favus and Angeid-Backman 1984; Armbrecht 1989) and *in vivo* studies using rats (Lengemann *et al.* 1959; Schaafsma and Visser 1980; Greger *et al.* 1989; Buchowski and Miller 1991; Brommage *et al.* 1993) suggest that dietary lactose enhances the absorption of Ca. A number of studies using human infants showed an increase in Ca absorption when lactose was present in the milk formula compared to diets comprising a range of other carbohydrate sources or sugars in place of lactose or its component sugars, glucose and galactose (Kobayashi *et al.* 1975; Ziegler and Fomon 1983; Moya *et al.* 1992; Abrams *et al.* 2002). A positive effect of lactose on Ca absorption in adult men and women appears to be less clear and different study outcomes have raised controversy on this aspect (Tremaine *et al.* 1986; Griessen *et al.* 1989; Schuette *et al.* 1991; Brink *et al.* 1993). How lactose acts to enhance Ca absorption is also unclear but it has been suggested that lactose may bind Ca thereby keeping the Ca in solution and avoiding chelation with phosphate or carbon species.

Short chain fatty acids

Short chain fatty acids, produced during bacterial fermentation of dietary fibre by the microbial flora of the hindgut have been reported to increase colonic Ca absorption. More specifically, a study conducted in human subjects where the short chain fatty acids acetate and propionate were infused rectally into the distal colon showed an increase in Ca

absorption when acetate and propionate were present in the distal colon (Trinidad *et al.* 1999).

Dietary fibre

Dietary fibre includes all plant substances that are not digested by secreted endogenous enzymes in the gastrointestinal tract. Dietary fibre encompasses a wide range of fibres which have different characteristics such as soluble and insoluble fibres that are suspected to decrease gastrointestinal absorption of minerals such as Ca due to mineral binding or physical entrapment of minerals (Torre *et al.* 1991). The ability of dietary fibre to influence mineral bioavailability is related to its cation exchange capacity, its ability to increase the viscosity of the digesta, as well as its water-holding capacity and bulk formation (Frolich 1995). Insoluble (cellulose, hemicellulose, and lignin) as well as soluble fibres (gums and pectin) have mineral binding properties. The dietary fibre matrix contains numerous carboxy, methoxy and hydroxyl groups that can bind soluble cations as the digesta pH increases during gastrointestinal transit. Soluble fibres such as pectin, gums, and β-glucan increase the viscosity of digesta and the unstirred water layer which leads to reduced interactions of minerals with the brush boarder (Torre *et al.* 1991). Fibres (soluble or insoluble) with a high water holding capacity are suspected to reduce paracellular Ca transport by lowering luminal to plasma Ca concentrations. *In vitro* studies have shown that lignin, pectin and gums have a strong ability to hold Ca over the physiological pH range of the intestinal tract (Torre *et al.* 1992; Luccia and Kunkel 2002). On the other hand, *in vivo* studies in rats and humans did not show any decrease in Ca absorption when the above mentioned dietary fibres were consumed (van den Heuvel *et al.* 1998; Greger 1999). These discrepancies between the ability of dietary fibres to effectively bind Ca *in vitro* but not

resulting in significant reduced absorption of Ca *in vivo* may relate to the bacterial fermentation of these fibres in the hind gut releasing Ca from the binding sites and allowing passive diffusion of the mineral in the colon (Trinidad *et al.* 1996). This might suggest that fermentable fibres have little effect on Ca availability overall but rather shift a proportion of the Ca absorption from the small intestine to the hindgut. Furthermore, some soluble fibres such as inulin, oligofructose and fructooligosaccharides have been reported to have enhancing mineral absorption properties (Roberfroid 1993; Ohta *et al.* 1998).

Phytic acid

Cereals, legumes and oil seeds are high in phytic acid. Humans have a limited capacity to hydrolyse phytate molecules in the gastrointestinal tract (Torre *et al.* 1991) but phytic acid in the gastrointestinal tract can interact with multivalent cations and form insoluble complexes which cannot be absorbed from the gastrointestinal tract (Heaney *et al.* 1991; Hurrell *et al.* 1992; Lonnerdal 2000). Of the minerals that are important in mammalian nutrition, Ca availability is the most affected by the presence of phytic acid in the diet.

Oxalic acid

Oxalic acid has been shown to be a strong Ca chelator in both *in vitro* and *in vivo* studies. Green leafy vegetables are rich in oxalic acid and have been reported to significantly reduce Ca absorption from the gastrointestinal tract in humans (Heaney and Weaver 1989). The Ca salt of oxalic acid is known to be soluble in dilute acid, but to what degree plant Ca oxalate solubilises in the stomach after ingestion is uncertain. Ingested Ca bound to oxalic acid of plants may reach the duodenum as Ca oxalate and evade absorption entirely (Weaver and Heaney 1991).

Phosphorus

As is the case with Ca, phosphorus plays an important role in bone mineral balance and soft tissue growth and a dietary Ca to phosphate ratio of 1.3 : 1 has been suggested as optimal to avoid both soft tissue calcification and osteoporosis. Consuming a western diet can result in an increased phosphorus uptake via "hidden" phosphorus sources such as in carbonated beverages and by a high consumption of meat products, which contain 10 - 20 times as much phosphorus compared to Ca, which can in turn interfere with Ca absorption. *In vitro* studies, simulating gastrointestinal pH in a range from 5.5 to 8, have shown that Ca phosphate begins to precipitate at pH 5.6 with maximal precipitation occurring at pH 7 (Van der Meer and De Vries 1985) suggesting that Ca-phosphate precipitation may occur in the gastrointestinal tract which may then interfere with intestinal Ca absorption.

Dietary lipids

Ingested fat has been reported in previous studies to decrease dietary Ca absorption in animals and human infants (Tadayyon and Lutwak 1969a; Nelson *et al.* 1996). Dietary triglycerides are digested to free fatty acids which are suspected to complex with ionised Ca within the gastrointestinal environment and lead to increased faecal Ca excretion (Drenick 1961). The influence of dietary lipids on Ca absorption is discussed in more detail later in this chapter (see section 2.5. Interactions between Dietary Ca and Fatty Acids: the Formation of Ca-Fatty Acid Soaps).

2.4. A Possible Role of Dietary Ca in the Abatement of Obesity and Its Metabolic Disorders

The imbalance between energy intake and energy expenditure is the major contribution risk factor for excess body fat and in extreme cases, obesity. Increased concentrations of serum cholesterol and serum triglycerides, as a consequence of high consumption of saturated long chain fatty acids, along with obesity can lead to the development of cardiovascular diseases. A reduction in fat absorption and deposition is highly desirable from a health standpoint and nutrients that can reduce fat absorption and mobilise stored fat have been the focus of research over the last two decades. A role for Ca in weight management and maintaining a beneficial blood lipid profile has received some support from a number of studies.

2.4.1. A possible role of dietary Ca on reducing blood lipids

Studies focussing on the effect of dietary Ca intake on serum lipid levels have shown a significant decrease in serum triacylglycerol concentrations when feeding high (> 1.2 g Ca 100 g^{-1} diet) versus low (< 0.4 g Ca 100g^{-1} diet) Ca diets to rats (Fleischman *et al.* 1966; Yacowitz *et al.* 1967; Papakonstantinou *et al.* 2003). Furthermore, all studies conducted in rats also reported significant decreases in total serum cholesterol in animals receiving high Ca diets (Fleischman *et al.* 1966; Yacowitz *et al.* 1967; Zhang *et al.* 2012). A number of intervention studies in humans focussing on the effect of dietary Ca on circulating plasma lipid profile have been conducted. Although there appears to be little consensus in terms of the results across the different studies, a decrease in serum triglycerides was observed for several studies when dietary Ca intake was increased for human subjects (Yacowitz *et al.* 1965; Bierenbaum *et al.* 1972; Chai *et al.* 2013). However, the majority of studies did not show a beneficial effect of increased dietary Ca on the serum triglyceride profile as was previously

observed in animal trials. Increasing dietary Ca intake from 900 mg to 2000 mg per day via the use of Ca supplements during intervention periods of 2 to 6 weeks in human subjects led to a 5 - 7% reduction in total serum cholesterol (Denke *et al.* 1993; Ditscheid *et al.* 2005; Chai *et al.* 2013), a 4 - 15% reduction in serum LDL-cholesterol (Bell *et al.* 1992; Denke *et al.* 1993; Shahkhalili *et al.* 2000) and in some studies an increase of 4 to 7% in serum HDL-cholesterol (Bell *et al.* 1992; Reid *et al.* 2002). However, not all studies have demonstrated a hypolipidemic effect for dietary Ca consumption. A few studies did not find any effect of increased dietary Ca intake on plasma lipids (Karanja *et al.* 1994; Bostick *et al.* 2000; Karandish *et al.* 2009; Reid *et al.* 2010). The conflicting results may be related to different study designs and subject numbers across the different intervention studies. Despite conflicting literature reports, evidence does exist to suggest a beneficial effect of dietary Ca on serum lipids in animals and possibly in humans. A possible mechanism how dietary Ca may affect blood lipids is discussed in section 2.4.3.

2.4.2. A possible role of dietary Ca in weight management

The effect of dietary Ca intake and body weight in humans has been examined over the last decade in several observational studies (Davies *et al.* 2000; Zemel *et al.* 2000; Jacqmain *et al.* 2001; Buchowski *et al.* 2002; Huang *et al.* 2011) and a small number of clinical trials (Zemel *et al.* 2004; 2005a; 2005b; 2009; Faghih *et al.* 2011) . The majority of observational studies showed an inverse association between dietary Ca intake and adiposity (Davies *et al.* 2000; Zemel *et al.* 2000). Moreover, Davies *et al.* (2000) re-evaluated four published observational studies that were originally designed to examine the effect of dietary Ca on bone health and also reported an association between Ca intake and body weight. Specifically, Ca intake differing by 1000 mg was associated with an 8.2 kg difference in body weight in young women

(Davies *et al.* 2000). Furthermore, Jacqmain *et al.* (2001) reported that women with a low Ca intake (< 600 mg d^{-1}) were significantly heavier than women with a medium (600-1000 mg d^{-1}) or high (> 1000 mg d^{-1}) Ca intake. Based on the observational studies, it appears that dietary Ca contributes to a lower body mass index, reduced body fat mass or a lower incidence of obesity or a combination of these. One of the disadvantages of observational trials however, is that they do not allow a cause-effect relationship to be determined and therefore are often difficult to interpret. For example, it has been shown that subjects with a low Ca intake also tend to have a poor diet in general (Barger-Lux *et al.* 1992). Therefore, any associations between dietary Ca intake and body weight found in free living subjects may be a result of dietary factors other than Ca. Furthermore, a high Ca intake is often accompanied by a high dairy product intake. Consequently, any effect on reduced body mass index or reduced body weight may be due to nutrients in the dairy products other than Ca, (e.g. conjugated linoleic acid or branched chain amino acids). Therefore, the possible association between dietary Ca and body weight reported by observational studies has to be interpreted with caution and further evidence for an effect of Ca on body composition based on carefully controlled studies is needed.

A considerable number of randomized controlled interventional studies have been conducted in humans to investigate the effect of dietary Ca on body weight or fat mass either as a primary endpoint or as an incidental outcome (for example, studies where the primary focus was the effect of dietary Ca on bone health) (Lanou and Barnard 2008). Compared to observational studies, the human intervention studies have a greater degree of experimental control in the selection of the test subjects and the distribution of the subjects across treatment groups. Nevertheless, the study subjects are still free living and consume a

habitual diet, which may contain nutrients, other than Ca, that have an effect on body weight. Another flaw of these types of studies is the reliability of using body weight or BMI as a measure of changes in body fat content. While it would be expected that a reduction in body fat content will lead to a reduction in body weight loss, a reduction in body weight may not necessarily mean a reduction in body fat content. A change in body weight can be the result of changes in fat mass, protein (muscle) or water content. The water content in the human body can change by up to 2 kg from day to day and is attributed to gastrointestinal contents, bladder content, fullness of glycogen stores (1 g glycogen binds 2.5-3 g water), amount of exercise (influences glycogen and losses via sweat), sodium consumption, low-carb diets and the menstruation cycle for women (Robinson and Watson 1965). Weight measurements performed at baseline and at the end of a study period can reflect such day to day fluctuations in weight and lead to invalid conclusions about the effect of Ca on weight loss.

The results of published interventional studies, designed to evaluate the impact of dietary Ca on body weight either as a primary or secondary endpoint, have not allowed definite conclusions to be drawn. Two studies (Zemel *et al.* 2000; Zemel *et al.* 2005b) showed a reduction in body fat mass, while one study showed lower fat mass accumulation for subjects within the treatment group receiving high amounts of dietary Ca (dietary Ca intake was between 1000 and 1500 mg d^{-1}) in comparison with subjects receiving below 500 mg of Ca per day. Furthermore, another study reporting dietary Ca related weight loss (Recker *et al.* 1996) and another study reporting a smaller weight gain (Caan *et al.* 2007) in the treatment group when Ca intake was increased over several years, support the Ca-weight loss hypothesis. However, other studies either with body weight as the primary endpoint

(Gunther *et al.* 2005a; Haub *et al.* 2005; Wennersberg *et al.* 2009; Yanovski *et al.* 2009; Palacios *et al.* 2011) or as the secondary endpoint (Baran *et al.* 1990; Elders *et al.* 1994; Perez-Jaraiz *et al.* 1996; Cleghorn *et al.* 2001; Chee *et al.* 2003; Wosje and Kalkwarf 2004; Reid *et al.* 2005; Reid *et al.* 2010) did not find an effect of dietary Ca on body weight.

The human intervention studies described above were designed where the dietary caloric intake either met or exceeded the energy requirement and therefore the effect of dietary Ca supplementation tended to focus on maintaining body weight or reducing body weight gain. An alternative approach for investigating the effect of dietary Ca on body weight has used energy restricted diets where the focus has been on weight loss rather than maintaining body weight or reducing body weight gain. One advantage of the intervention studies using energy-restricted diets is that shorter study periods can be used. This in turn improves subject compliance and enables specifically formulated-well controlled diets to be used that will maximise the chance of seeing differences between the control and treatment groups. However, studies that assess the effects of high dietary Ca intake during caloric restricted intervention periods have also provided some inconsistent results. A small number of studies (Zemel *et al.* 2004; 2005a; 2005b; 2009; Faghih *et al.* 2011) documented a significant decrease in body weight, fat mass and/or waist circumference in obese individuals fed a diet high in dietary Ca during energy-restricted diet regimens. For example, Zemel *et al.* (2004; 2005a; 2005b) reported significantly greater weight loss in obese Caucasian and African-American subjects receiving an energy-restricted diet for 12 to 24 weeks when the daily Ca intake was increased from < 500 mg to > 1000 mg by increasing the consumption of dairy products as compared to a control diet. These researchers also examined the effect between dietary Ca supplied in the diet in different forms (dairy vs inorganic Ca carbonate) and

showed a significant effect with both Ca sources on body composition but a more pronounced effect with dairy Ca (Zemel *et al.* 2004). However, in a more recent study, supplementary inorganic Ca had no effect on body weight loss or fat loss, whereas fortifying the energy-restricted diet with Ca from dairy products resulted in significantly greater body weight loss and fat loss compared to the low-Ca diet at the same energy intake (Zemel *et al.* 2009). Other intervention studies involving dietary energy restriction did not show any significant additional decrease in body weight for subjects receiving a diet high in dairy products (1200 – 1400 mg Ca d^{-1}) in comparison to those on a diet low in dairy products (500 or 800 mg Ca d^{-1}) (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). Neither could the results of a 15-week energy-restricted intervention study show any additional body weight loss in subjects supplemented with two tablets of Ca plus vitamin D (1200 mg Ca and 400 IU vitamin D d^{-1}) compared to participants receiving a placebo treatment (Major *et al.* 2007).

Overall, the findings from studies that have examined the effect of dietary Ca on weight loss are not always convincing and the outcomes of the studies are divided, with a smaller number of studies reporting an effect of dietary Ca on changes in body fat and weight loss. A review by Lanou and Barnard (2008) assessing the effect of dairy products or Ca intake on body weight and body fat composition concluded that the majority of current available studies do not support the hypothesis of Ca or dairy products reducing body fat or body weight. A more recent review by Abargouei *et al.* (2012) concluded that the consumption of high-dairy, high-Ca energy-restricted diets may result in a greater weight loss and higher reduction of fat mass compared to conventional energy-restricted diets. Abargouei *et al.* (2012) also performed a meta-analysis that included 10 of the above mentioned randomized controlled trials based on using energy-restricted diets and showed mean weight changes of

-1.29 kg body weight, -1.11 kg fat mass and -2.43 cm waist circumference in subjects consuming a high-dairy diet with a total Ca intake of 1400 mg per day compared to subjects on the control diet receiving 1000 mg of Ca per day.

It needs to be remembered that there are a number of factors that may play a role in weight loss other than dietary Ca that are not taken into account, or at least not well controlled, in these human intervention studies. These factors include diet composition and dietary compliance by the subjects (since the participants were all free living individuals consuming a whole food diet). Dietary Ca appears to show a more pronounced effect on body composition when administered in the form of dairy products rather than inorganic Ca supplements, which may be attributed to other bioactive components such as branched-chain amino acids in the dairy products. Consequently, drawing conclusions on the impact of dietary Ca on weight loss or mechanisms that may be involved must be done with caution.

A disadvantage of human trials will always be the lack of control over free living subjects and the dependency of subject compliance. Dietitian counselling, food records, food weighing protocols and even pre-weight food item supply are no guarantee of total subject compliance. In contrast, hypothesis driven studies can be more easily conducted using animal models and such studies have been performed to test the hypothesis that dietary Ca intake can affect body weight and body fat content. A number of studies using rodents have shown an effect of dietary Ca on body weight when the animals were fed a high-Ca diet compared to a low-Ca diet (Fleischman *et al.* 1967; Stern *et al.* 1984; Metz *et al.* 1988; Zemel *et al.* 2000; Shi *et al.* 2001; Papakonstantinou *et al.* 2003; Sun and Zemel 2004a; Parra *et al.* 2008; Zhang *et al.* 2012). Results obtained from animal trials (rats) indicated a reductive effect of Ca

on body weight gain when the animals were fed high-fat diets (Fleischman *et al.* 1967; Zhang *et al.* 2012). Furthermore, diets high in Ca (10 g kg^{-1} and above) led to a reduced fat mass accumulation in adipose tissue of mice and rats when compared to control groups receiving low dietary Ca concentrations (4 g kg^{-1}) (Zemel *et al.* 2000; Papakonstantinou *et al.* 2003). An elegant study to observe the effect of dietary Ca on body weight loss rather than reduced body weight gain was conducted in agouti mice, a transgenic mouse model prone to dietary induced obesity, receiving energy-restricted diets after having become obese (Shi *et al.* 2001). Energy restriction for 6 weeks on the lowest Ca diet (4 g kg^{-1}) resulted in 5 g body weight reduction, whereas mice receiving 12 g or 24 g Ca per kg diet lost 8.6 g to 13 g body weight compared to a control group fed *ad libitum*. Furthermore, a 42 to 69% reduction in fat pad mass was reported for mice receiving the high-Ca diets. Animal trials testing the effect of dietary Ca sources (supplement vs. milk Ca) concluded that both sources of Ca have an effect on body weight and fat mass, but dairy Ca had the more pronounced effect (Shi *et al.* 2001). Only a small number of studies have reported no effect of dietary Ca on body weight or fat pad mass in rodents (Zhang and Tordoff 2004; Paradis and Cabanac 2005). In summary, a number of studies conducted in animals provide evidence that a high dietary Ca intake has the ability to reduce weight gain induced by increased dietary fat intake. Furthermore, consumption of a diet rich in fat leads to higher weight gain when the dietary Ca concentration is low compared to diets providing a high dietary Ca content.

2.4.3. Mechanisms by which dietary Ca may effect body weight

Most observational and population based data, some of the clinical studies in humans and the majority of the studies using animal models support an association between dietary Ca and body weight loss and/or body composition. Moreover, several mechanisms have been postulated to explain the latter association. These include two metabolic based mechanisms such as 1) reducing the adipocyte mass by shifting the lipolysis/lipogenesis rate towards lipolysis and thereby increasing fat oxidation, and 2) increasing the thermic effect of Ca containing food. A third mechanism has been proposed that is based around dietary Ca reducing the absorption of fatty acids via the formation of indigestible Ca-fatty acid soaps in the gastrointestinal tract. These proposed mechanisms are discussed in more detail below.

2.4.3.1. The effect of dietary Ca on fat metabolism

One hypothesis explaining the effect of dietary Ca on body weight focuses on the role of dietary Ca on the regulation of energy metabolism, including lipolysis and lipogenesis in adipocytes and fatty acid oxidation, via Ca-mediated hormonal regulation. Studies in the agouti mouse showed a reduction in lipogenesis and a stimulation of lipolysis due to a high dietary Ca intake and concomitantly found a reduction in adipose tissue mass and body weight (Zemel *et al.* 2000; Sun and Zemel 2004a). Furthermore, not only animal studies have shown increased lipolysis due to the consumption of a high-Ca diet, but human subjects have also been found to have higher circulating glycerol and free fatty acids concentrations, an indirect indicator for lipolysis, when dietary Ca intake was increased (Zemel *et al.* 2005a; Zemel *et al.* 2005b; Cummings *et al.* 2006; Ping-Delfos and Soares 2011). In addition, adipocyte size tended to be smaller in obese subjects on a dietary weight loss regime when

receiving up to 1300 mg Ca from dairy products for a 12 week period compared to subjects receiving low-Ca diets (< 500 mg Ca d^{-1}) (Van Loan *et al.* 2011).

The calcitrophic hormones, parathyroid hormone and 1,25-dihydroxy-vitamin D_3, play an important role in Ca homeostasis and are upregulated during Ca deficiency. Additional to the classic functions of calcitrophic hormones, such as acting on the intestine, kidneys and bone to increase serum Ca concentrations, more recent research has linked these hormones to a role in regulating body fat levels (see Figure 2-3). The first evidence for such a link was the discovery that obese subjects have elevated circulating 1,25 dihydroxy vitamin D_3 levels (Bell *et al.* 1985; Kerstetter *et al.* 1991) and that serum parathyroid hormone is positively correlated with body fat mass (Parikh *et al.* 2004; Gunther *et al.* 2006). *In vitro* studies using human adipocytes treated with 1,25-dihydroxy vitamin D_3 had a greater influx of Ca into the adipocyte cells, an increased activity of fatty acid synthase, a greater lipolysis inhibition and a greater triglyceride deposition (Zemel *et al.* 2000). It was proposed that low dietary Ca intake leads to an increased concentration of intracellular Ca in the adipocyte which activates phosphodiesterase, which in turn inhibits adipocyte lipases via the degradation of cyclic AMP, leading to decreased levels of cyclic AMP in the adipocyte and further decreasing phosphokinase A activity thereby inhibiting lipolysis (Figure 2-3) (Xue *et al.* 2001).

Supporting evidence for the latter hypothesis which suggests that high dietary Ca intake reduces intracellular Ca influx in adipocytes due to decreased levels of parathyroid hormone and 1,25-dihydroxy vitamin D_3 is provided by studies in both animals and humans. For example, rats on a diet high in Ca (24 g kg^{-1}) which accumulated less fat in the adipose tissue and had lower serum 1,25-dihydroxy vitamin D_3 levels compared to rats in the control group

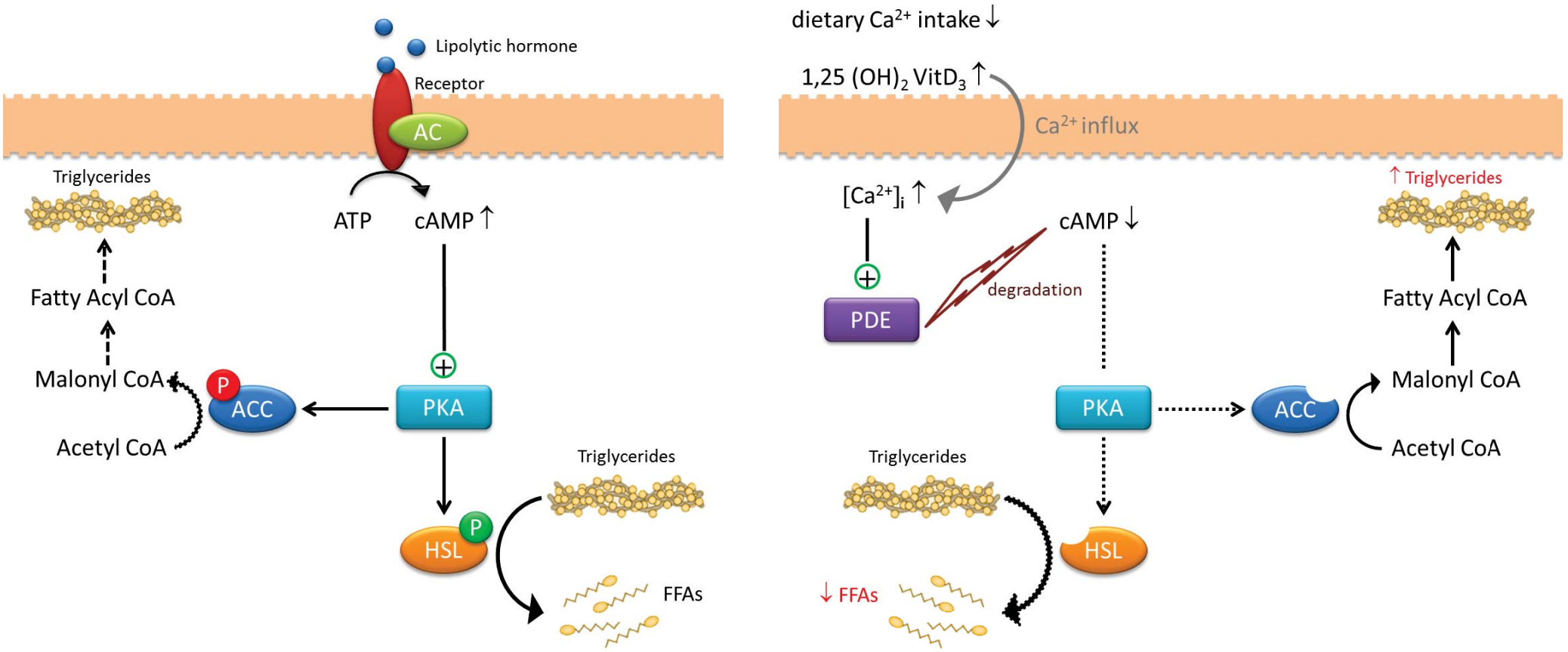


Figure 2-3: Hormone induced lipolysis in adipocytes (**A**) and the influence of intracellular calcium levels on adipocyte fat metabolism (**B**).

A: Lipolysis in adipocytes is mediated by cAMP-dependent activation of PKA and subsequent HSL phosphorylation. Activated HSL releases fatty acids from the triglyceride storage in adipocytes, which will undergo β-oxidation in mitochondria of energy requiring tissue, thereby reducing adipocyte size. PKA additionally phosphorylates ACC, a major enzyme in lipid de-novo genesis. Phosphorylation of ACC inactivates the carboxylase which reduces fatty acid synthase resulting in less triglycerides being stored in the adipocyte.

B: Low dietary Ca intake increases 1,25-dihydroxy Vitamin D which has been shown to increase Ca influx into adipocytes. Increased levels of intracellular Ca activate PDE which in turn degrades cAMP. A reduction in cAMP leads to reduced PKA activity. Consequently, HSL and ACC phosphorylation won't occur, thus leading to a build-up in adipocyte triglycerides. Increasing the dietary Ca intake will reduce the 1,25-dihydroxy vitamin D mediated influx of intracellular Ca; these modifications eventually stimulate lipolysis and inhibit lipogenesis in the adipocyte.

AC – Adenylate cyclase, ATP – adenosine triphosphate, cAMP – cyclic adenosine monophosphate, PKA – cAMP dependent protein kinase A, ACC – AcetylCoA carboxylase, HSL – hormone sensitive lipase, FFAs – free fatty acids, $[Ca^{2+}]i$, PDE – phosphodiesterase

with a lower dietary Ca intake (4 g kg^{-1} diet) (Papakonstantinou *et al.* 2003). It was demonstrated in agouti mice that increased adipocyte intracellular Ca is associated with the development of obesity (Shi *et al.* 2001). Feeding high-fat high-sucrose diets containing 4 g Ca per kg diet to agouti mice resulted in an increase in intracellular Ca and triggered obesity in these mice. Exposing these obese mice to an energy-restricted diet containing 12 or 24 g Ca per kg diet reduced adipocyte intracellular Ca levels, such that the intracellular Ca levels were comparable to that in agouti mice prior to obesity being induced. Furthermore, mice on an energy-restricted diet containing 4 g Ca per kg diet did not experience reduced adipocyte intracellular Ca levels and the observed weight loss was only half that of mice on the high-Ca diets. Additional to a reduction in adipocyte intracellular Ca, an increase in glycerol release from adipocyte cells (indicating increased lipolysis) and reduced fatty acid synthase activity in adipocyte cells (indicating decreased lipogenesis) has been reported for mice receiving an energy-restricted diet containing high Ca concentrations (Shi *et al.* 2001). In addition to the biochemical mechanisms described above, dietary Ca may also affect the lipogenesis/ lipolysis balance at the gene regulation level. Fatty acid synthase m-RNA expression has been shown to be markedly reduced in agouti mice receiving an energy-restricted diet compared to *ad libitum* fed mice. However, fatty acid synthase m-RNA expression was even lower when dietary Ca was increased from 4 to 12 g kg^{-1} in the energy-restricted diet. Boon et al. (2007) reported, in an experiment investigating the effect of varying dietary Ca concentrations on the mRNA expression of fatty acid synthase in the adipose tissue of lean human subjects, that a daily consumption of 2.5 g of Ca for 7 days suppressed mRNA expression, while consuming 1.2 g of Ca per day for 7 days had no effect (Boon *et al.* 2007).

The data obtained using animal models with some support from findings from human studies provide some evidence that diets high in Ca suppress the influx of Ca into adipocytes by lowering 1,25 dihydroxy vitamin D_3 plasma concentrations, thereby shifting adipocyte metabolism from lipogenesis to lipolysis. Free fatty acids released during lipolysis are transported to muscle tissue or the liver where they undergo β-oxidation. Therefore, it would be expected that Ca induced lipolysis would lead to increased fat oxidation. A positive correlation between acute Ca intake (ranging from 477 to 1768 mg d^{-1}) and whole-body fat oxidation has been reported in obese human subjects participating in a 24 hour calorimeter trial (Melanson *et al.* 2003). Furthermore, data from a meta-analysis including eight randomised crossover trials showed that fat oxidation was increased by 11% in subjects with a chronic high Ca intake (~1300 mg d^{-1} for > 7 d) compared to subjects on a low dietary Ca intake (< 800 mg) (Gonzalez *et al.* 2012). It has also been suggested that dietary Ca may be more effective in stimulating fat oxidation in subjects that are dietary energy deficit. Subjects that were in negative energy balance during their stay in the calorimeter (consuming a diet with a 100 kcal deficit and expending 500 kcal extra by doing exercise) had a significantly greater (28%) 24 hour fat oxidation when a high-Ca diet (1400 mg Ca d^{-1} via dairy products), as opposed to a low-Ca diet (500 mg Ca d^{-1}), was consumed the week prior to the examination day and on the examination day (Melanson *et al.* 2005). Moreover, subjects with a habitually low Ca intake had the greatest increase in fat oxidation when a high-Ca diet was administered at least one week prior to the calorimeter visit. Furthermore, fat oxidation appeared to be enhanced to a greater extent in subjects supplemented with Ca salts rather than dairy products during the Ca intervention period (Gonzalez *et al.* 2012). Increased lipolysis and increased fatty acid oxidation, as reported in the above listed studies, would result in decreased adipocyte lipid accumulation, body weight and body fat reduction and

an overall shift of dietary energy from adipose tissue to lean body mass, thereby supporting the proposed hypothesis of dietary Ca having an effect on body weight.

2.4.3.2. The effect of dietary Ca on thermogenesis

While an increased intracellular Ca concentration in adipocytes has been shown to inhibit lipolysis thereby shifting the lipolysis/lipogenesis balance (which is the focus of the mechanism described above), it has also been shown to inhibit the expression of uncoupling protein 2 in adipocytes (Shi *et al.* 2002). Uncoupling protein plays an important role in thermogenesis which has implications for energy metabolism and therefore weight control. Thermogenesis can be induced by exposure to cold or by consuming food, the latter being referred to as dietary-induced thermogenesis and has been shown to be increased after consumption of certain food components, such as piperine, capsaicin, catechines and caffeine (Westerterp-Plantenga *et al.* 2006). Indirect evidence has been published suggesting that dietary Ca can affect thermogenesis. For example, Zemel *et al.* (2000) reported that feeding a high-Ca (12 g kg^{-1}) diet to agouti mice led to an increased core temperature compared to mice fed a low-Ca (4 g kg^{-1}) diet. In addition, evidence has been published suggesting that consuming diets containing high concentrations of Ca (13 g kg^{-1}) can lead to an increased expression of uncoupling proteins in white adipose tissue and skeletal muscle (Sun and Zemel 2004a). The latter findings suggest that dietary Ca stimulates uncoupling protein expression via the suppression of 1,25-dihydroxy vitamin D_3 levels and more energy is released in the form of heat. In contrast, in wild type mice as well as in rats, high dietary Ca did not lead to increased body core temperature (Papakonstantinou *et al.* 2003) or alterations in uncoupling protein expression of white adipocytes (Parra *et al.* 2008) when compared to low-Ca diets. Nonetheless, both studies found a significant effect of dietary Ca on animal

body weight and fat pad mass (Papakonstantinou *et al.* 2003; Parra *et al.* 2008) suggesting that uncoupling proteins may play only a limited role in Ca induced body weight loss. Based on human studies, there is little evidence for an increased dietary-induced thermogenesis following consumption of high-Ca diets (Soares and She-Ping-Delfos 2010). Only one study conducted in humans has shown an effect of dietary Ca on increased dietary-induced thermogenesis (Ping-Delfos and Soares 2011), whereas other studies did not report a difference in dietary-induced thermogenesis following consumption of either a low- or high-Ca meal (Gunther *et al.* 2005b; Jacobsen *et al.* 2005; Melanson *et al.* 2005; Cummings *et al.* 2006; Teegarden *et al.* 2008). Overall, the evidence for the role of dietary Ca induced thermogenesis and body weight is not clear and further research is required.

2.4.3.3. The effect of dietary Ca on fatty acid excretion and fat absorption

While the two mechanisms described above explaining an association between dietary Ca and body fat loss are at the metabolic level, a third mechanism has been proposed that focusses on the interaction of dietary Ca and free fatty acids in the gastrointestinal tract. The latter mechanism proposes the formation of insoluble indigestible Ca-fatty acid soaps in the gastrointestinal tract after ingestion of food containing Ca and fat, thereby increasing the excretion of faecal fatty acids and concomitantly reducing the absorption of fat. Only a few studies in animals and humans have investigated the potential for Ca to reduce fat absorption in conjunction with body weight or fat mass changes. Studies conducted in rats reported a two-fold increase in faecal lipids when the dietary Ca intake of the rats in the Ca intervention group was six-times greater compared to rats in the control group (e.g 2 vs. 12 g kg^{-1} diet or 4 vs 24 g kg^{-1} diet) (Fleischman *et al.* 1967; Papakonstantinou *et al.* 2003). Concomitantly, weight gain in rats on a high-Ca diet was lower compared to rats on a low-

Ca diet. Fleischman *et al.* (1967) calculated that rats receiving the low-Ca diet (2 g kg^{-1}) for 150 days of the experimental period retained 76 g more fat than rats fed the high-Ca diet (12 g kg^{-1}). Furthermore, the rats fed the low-Ca diet gained 115 g more in weight compared to the rats fed the high-Ca diets (Fleischman *et al.* 1967). These data suggest that two thirds of the difference in weight gain between the two different groups of rats could be accounted for by absorption and retention of the ingested fat. Papakonstantinou *et al.* (2003) reported that in another study conducted in rats, the body weight difference between rats receiving low- and high-Ca diets (4 vs 24 g kg^{-1}) was largely due to the inhibition of dietary energy absorption. An increased dietary Ca intake resulted in an energy deficit of 840 kcal over the 85 days of the experimental period. The digestibility of fat (soya bean oil and lard) was reduced by approximately 9% from 93% to 84% in rats fed the high-Ca diet. The reduction in absorbable energy resulted in lower body weight gain and lower fat pad mass in rats receiving the high-Ca diet. Additionally, a reduction in serum triglyceride concentration was observed when the faecal fat excretion was increased (Fleischman *et al.* 1966; Papakonstantinou *et al.* 2003). Dietary supplementation with Ca (an addition of 900 mg d^{-1}) in human subjects receiving a western diet resulted in similar observations to those seen in rats, that being a two-fold increase in faecal fat excretion and a corresponding 13% decrease in the apparent faecal digestibility of dietary fat (Shahkhalili *et al.* 2001a). The study was not designed to determine changes in body weight or fat mass, but blood lipid parameters were determined, and LDL-cholesterol was lower in subjects at the end of the Ca intervention period. Another short term study using overweight human subjects revealed a 2.5-fold increase of faecal fat excretion when study subjects consumed a diet high in Ca (1850 mg d^{-1} via dairy products) compared to a diet low in Ca (500 mg d^{-1}) (Jacobsen *et al.* 2005). The difference of 8.2 g fat excreted daily in the faeces corresponded to an energy deficit of 75 kcal

per day. Jacobsen *et al.* (2005) extrapolated the daily energy deficit to an annual energy deficit of 27,000 kcal per year, which would correspond to a body weight loss of about 3.5 kg per year if the study subjects maintained the same energy intake as observed during the study period (Jacobsen *et al.* 2005). The hypothesis that dietary Ca can increase faecal fat excretion is supported by the literature. However, evidence that the additional excreted fat leads to significant body weight loss is limited and further studies are necessary to estimate the contribution that Ca-fatty acid soap formation may have on body weight loss.

2.5. Interactions between Dietary Ca and Fatty Acids: the Formation of Ca-Fatty Acid Soaps

The interaction between fatty acids and Ca and the impact this interaction may have on the digestion and absorption of Ca or fatty acids was first explored in the early 1900's. Steinitz (1903) observed that in infants, the addition of extra milk-fat to the diet lowered Ca retention. Bosworth *et al.* (1918) was one of the first workers to report an interaction between dietary fatty acids and Ca from the perspective of fat digestion and absorption, rather than that of Ca absorption. The latter workers reported a higher faecal fat output for infants that were bottle-fed with a cow milk-based infant formula when compared to their breast-fed counterparts and hypothesised that the higher concentration of Ca in bovine milk, compared to human breast milk, was responsible for the difference in faecal fat output.

Studies conducted in growing rats and broiler chickens also provide evidence that increasing the concentration of dietary fat can lead to an increase in faecal Ca excretion and a reduced Ca retention. French (1942) investigated the effect of varying dietary fat concentrations of synthetic diets (5, 15, 28 and 45 g of butter fat per 100 g diet) on the absorption of Ca in rats and reported a decrease in the retention of Ca with the increasing dietary fat content where the faecal Ca excretion was 2.6-fold higher in the rats receiving the highest dietary fat content compared to those receiving the lowest dietary fat content. Another study showed that the apparent faecal Ca digestibility determined in weanling rats was 62% when the rats were fed a fat-free diet and remained similar when 5 g 100 g^{-1} of a triglyceride comprising either palmitic, stearic or oleic acid was added to the diet (48%, 54% and 51%, respectively), but reduced to 25% and 46% when 25 g 100 g^{-1} diet of tripalmitin or tristearin was added respectively (Tadayyon and Lutwak 1969b). In contrast, the addition of 25 g 100 g^{-1} diet of

triolein did not alter apparent faecal Ca digestibility compared to the fat-free diet. These latter studies suggest that dietary fat, especially when rich in saturated fatty acids, has the ability to impair Ca absorption but that not all fats display the latter effect.

Insufficient Ca absorption can result in decreased bone mass and lead to osteoporosis. Some published studies have suggested that the decreased absorption of Ca as a result of a high dietary fat intake also resulted in a decrease in bone mineral calcification. For example, Tadayyon and Lutwak (1969b) reported that in rats, not only was apparent faecal Ca digestibility 2-fold lower after consuming a diet containing 25 g 100 g^{-1} tripalmitin compared to a diet containing 5 g 100 g^{-1} tripalmitin but the femur Ca content was also lower (16 mg vs 19 mg Ca in the femur). Similarly, Ca retention was 20% lower in broiler chickens receiving a low-fat diet versus a diet supplemented with 10 g 100 g^{-1} palmitic acid, resulting in a lower bone ash weight and lower bone Ca content in the tibia (Atteh and Leeson 1984). Moreover, in a long term study in rats weaker bones (markedly reduced lumbar vertebrae structural integrity and a reduced femoral neck) were reported over the two year study period when rats received a diet high in lard and sucrose compared to rats receiving a low-fat, complex-carbohydrate diet (Zernicke *et al.* 1995).

It would be expected that if dietary fat can reduce the absorption of Ca from the gastrointestinal tract via the formation of indigestible Ca-fatty acid soaps, then increased dietary Ca would be expected to reduce the absorption of fatty acids. The latter hypothesis has been demonstrated in studies using several different animal species (rats, chickens and dogs) where faecal fat excretion has been shown to increase when dietary Ca was increased. For example, the faecal digestibility of natural and hydrogenated oils and fats was shown to decrease between 12 and 54% in rats when the Ca content of Ca-free diets was increased to

6 g kg^{-1} (Cheng *et al.* 1949). In addition, doubling the dietary Ca concentration of synthetic diets for broiler chickens from 8 to 16 g per kg reduced fat retention in the body by 13 and 44% respectively when either oleic acid or palmitic acid were the sole fat source of the diet (Atteh and Leeson 1984).

While increasing dietary Ca increases faecal fatty acid output, different fatty acids appear to be affected to differing degrees. The order in which fatty acids are affected by an increased intake of dietary Ca is as follows: saturated long-chain fatty acids are the most affected, followed by saturated medium-chain fatty acids, then unsaturated long-chain fatty acids and then saturated short-chain fatty acids. As an example, a study conducted using rats fed diets containing a fat either high in unsaturated fatty acids (corn oil) or high in saturated fatty acids (cocoa butter), showed an increase in faecal fat excretion with increasing dietary Ca concentrations for both fat sources but the effect was greatest for cocoa butter(Yacowitz *et al.* 1967). In another study investigating the effect of dietary Ca on the absorption of fatty acids using trilaurin, trimyristin, tripalmitin and tristearin as fat sources showed that the digestibility of all fatty acids was reduced but the digestibility of the fats containing the longer chain fatty acids (trimyristin, tripalmitin and tristearin) was reduced the most (44 – 54% reduction) compared to the fat containing the shortest chain fatty acid (trilaurin) for which the reduction in digestibility was 28% when 6 g kg^{-1} Ca was added to a Ca-free diet (Cheng *et al.* 1949). In another study, no effect of dietary Ca on fat digestibility was observed when triglycerides comprised solely of unsaturated fatty acids or short chain fatty acids, such as triolein or tributyrin, were fed to rats (Westerlund 1934). The animal studies almost universally demonstrate an effect of dietary Ca on faecal fat excretion for saturated fatty acids of longer chain length.

In addition to animal studies investigating the effect of dietary Ca on faecal fat excretion, several human studies investigating the latter effect have also been carried out. The findings of these studies using human subjects are generally consistent with those reported using animal models (Table 2-2). Across the studies listed in Table 2-2, faecal fat excretion in humans increased by 2-fold, on average, when dietary Ca intake was increased during the intervention period across most of the studies. Furthermore, and as was the case for the animal-based studies, it appears that saturated fatty acids are affected by the presence of dietary Ca to a much greater degree than unsaturated fatty acids. For example, Bhattacharyya *et al.* (1969) reported a 3.5-fold increase in faecal fatty acid excretion in men receiving a Ca-fortified bread in conjunction with a spread rich in saturated fatty acids whereas no increase in faecal fatty acid excretion was observed when the Ca-fortified bread was consumed in conjunction with a spread rich in polyunsaturated fatty acids. Additionally, studies conducted in humans consuming a western diet have shown an increase in saturated long chain fatty acids in the faeces when the dietary Ca intake was increased for the intervention period (Denke *et al.* 1993; Govers *et al.* 1996; Shahkhalili *et al.* 2001a).

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.

Author	Year	Participants	Nr	Intervention	Design	Ca Intake (mg d^{-1})		Faecal fat excretion (g d^{-1})		Duration		Energy	Protein	Fat	sat FA
						Ca (-)	Ca (+)	Ca (-)	Ca (+)	IVP[1]	FCP[2]	(MJ d^{-1})	En%	En%	En%
Bhattacharyya *et al.*	1969	men	11 10	fortified bread ($CaCO_3$)	cross-over	254	2355	1.2 1.2	1.5 4.1*	2 w	7 d	12.6	15	40	4 22
Saunders *et al.*	1988	men & women	8	Ca supplements ($CaCO_3$)	cross-over	na	2400	7.9	16.8*	3 w	7 d	na[9]	15	30	na
Denke *et al.*	1993	men[3,4]	13	Ca fortified juice & muffins + Ca tablets (CCM)[6]	cross-over	410	2200	2.6†	5.4*†	10 d	3 d	11.0	11	34	13
Welberg *et al.*	1994	men & women[3]	24	Ca supplements ($CaCO_3$)	parallel	1450		5.4‡				9.0	15	35	na
							3880		7.0‡	1 w	3 d	9.3	17	39	na
							5490		7.6‡			8.3	17	35	na
Govers *et al.*	1996	men[3]	13	dairy products[7]	cross-over	765	1820	6.7	9.3*	1 w	3 d	13.0	14	35	15
Murata *et al.*	1998	men[5]	9	Ca fortified chocolate (eggshells)	cross-over	504	3939	2.8	7.5*	3 d	3 d	7.7	11	na	na
Shahkhalili *et al.*	2001b	men	12	Ca fortified chocolate ($CaCO_3$)	parallel	na	na	5.9	10.4*	2 w	7 d	na	na	na	na
Shahkhalili *et al.*	2001a	men[3]	9	Ca fortified chocolate (CaCO3)	cross-over	950	1855	4.4	8.4*	2 w	7 d	13.0	14	39	17
Ditscheid *et al.*	2005	men & women[3]	31	Ca fortified bread (CaP)	cross-over	1193	2204	3.9	4.3	4 w	5 d	9.0	16	35	18

Table 2-2 continued

Author	Year	Participants	Nr	Intervention	Design	Ca Intake (mg d^{-1})		Faecal fat excretion (g d^{-1})		Duration		Energy	Protein	Fat	sat FA
						Ca (-)	Ca (+)	Ca (-)	Ca (+)	IVP[1]	FCP[2]	(MJ d^{-1})	En%	En%	En%
Boon *et al.*	2007	men & women[3]	10	dairy products	cross-over	348	1242	4.8	7.2	1 w	3 d	10.0	20	35	na
							2545		7.5						
				Ca supplements ($CaCO_3$)			1242		6.7						
Jacobsen *et al.*	2005	men & women[3]	8	dairy products	cross-over	474		6.0					15		
							1735		14.2*	1 w	3 d	10.0	15	30	na
							1869		5.9				23		
Bendsen *et al.*	2008	men & women[3]	11	dairy products	cross-over	698	2287	5.4	11.5*	1 w	5 d	12.5	15	30	12
Soerensen *et al.*	2014	men	15	dairy products[8]	cross-over	500	1700	3.9	5.2*	2 w	5 d	10.0	15	32	19
							1700		5.7*						
Hjerpsted *et al.*	2016	men & women[3]	23	dairy products (butter vs cheese)	cross-over	451	1220			6 w	2 d	9.6	14 19	34	15

[1]IVP – intervention period
[2]FCP – faecal collection period
[3]Caucasians
[4]participants had moderate hypercholesterolemia
[5]Japanese
[6]CCM – Ca citrate maleate
[7] dairy products (Ca+) vs Ca depleted milk (Ca-)
[8] dairy products cheese and milk (Ca+) vs low dairy diet
[9]na – not available
* significant different from Ca(-) ($P < 0.05$)
[†]only major fatty acids were included (C14:0, C16:0, C18:0, C18:1 and C18:2)
[‡]total fat excretion did not differ between the low Ca and Ca-supplementation periods, but a significant correlation between Ca supplementation and faecal fatty acids ($p = 0.05$) was reported.

As for the mechanism behind the interactions of dietary Ca and fat, it has been suggested that after hydrolysis of the triglyceride molecules in the gastrointestinal tract, free fatty acids and ionised Ca form a complex which has been referred to as a Ca-fatty acid soap. One molecule of Ca has the ability to bind two molecules of free fatty acids and these Ca-fatty acid soaps have been shown to be largely insoluble in water and simulated intestinal fluids (Harrison 1924; Langley *et al.* 1932; Graham and Sackman 1983). Additionally, it has been suggested that these soaps are poorly absorbed as indicated by experiments in rats (Boyd *et al.* 1932; Gacs and Barltrop 1977). Therefore, it is likely that soap formation within the gastrointestinal tract, may be responsible for the observed impact that dietary Ca has on the digestibility of fatty acids and vice versa. Despite this apparently obvious link, little work has been conducted in either animal models or in humans to investigate soap formation and its effect on the availability of fatty acids or Ca.

Direct evidence for the formation of Ca-fatty acid soaps within the gastrointestinal tract is weak. Only a few studies have succeeded in extracting the Ca-fatty acid soap complex from faecal material and recovery values for the methods used to extract the soaps are reported to be poor (Sammons and Wiggs 1960; Owen *et al.* 1995). The first evidence of the formation for the Ca-fatty acid soap complex was provided by Sammons and Wiggs (1960). The latter researchers succeeded in extracting 'white, solvent insoluble solids' from fresh human faeces with diethylether as a solvent. Analysis of the white complexes showed a molar Ca-to-fatty acid ratio of 1:2. However, the recovery of synthetic Ca-palmitic acid soap and Ca-oleic acid soap from fresh faeces with diethylether was only 65%, indicating that either not all soap material could be extracted or that some of the Ca-fatty acid soap dissociated in the solvent into Ca and free fatty acids. Another protocol for the isolation, identification and

quantification of Ca-fatty acid soaps was published by Owen *et al.* (1995). This research group observed that after Soxhlet extraction of faecal material with 72% ethanol, the extracts contained crystalline structures that appeared after overnight cooling at 4°C. The latter workers also performed a recovery study where synthetic Ca-palmitate and Ca-oleate was introduced to fat-free faeces. The research group was able to recover only 25% of the palmitic acid and 23% of the Ca within the reformed crystalline structures when synthetic Ca-palmitic acid soap was added to the faeces. In addition, none of the Ca-oleate precipitate was observed (Owen *et al.* 1995). The latter recovery experiment suggests that Ca-fatty acid soaps composed of different fatty acids have different levels of solubility in organic solvents. Moreover, since the recovery of both palmitic acid and oleic acid soaps were poor this method reported by Owens *et al*. (1995) would not be suitable for the determination of Ca-fatty acid soaps in faecal samples.

Since determining Ca-fatty acid soaps in faeces directly may be difficult since soaps comprising different fatty acids vary in their solubility in solvents, an indirect approach for determining Ca-fatty acid soaps in faeces has been described by a number of different workers (Holt 1919; Toullec 1968; March and MacMillan 1979; Demarne *et al.* 1982). The indirect approaches all aim to extract the fatty acids present in the faeces that are not present in the form of Ca-fatty acids soaps and then assume that the fatty acids remaining in the faeces are present as Ca-fatty acid soaps. The rationale behind the indirect methods is that Ca-fatty acid soaps are relatively insoluble in organic solvents and this has been reported to generally be the case (Harrison 1924). For example, Ca-stearic acid soap and Ca-palmitic acid soap are virtually insoluble in alcohol and petroleum ether and only slightly soluble in chloroform. However, Ca-oleic acid soaps have been reported to be fairly soluble in alcohol,

ether, chloroform and benzene. Therefore, the choice of solvent for extracting fatty acids that are not present in faeces as soaps is critical and should include solvents with the lowest Ca-fatty acid soap solubility across all fatty acids.

2.5.1. Factors influencing Ca-mediated fatty acid excretion

The amount of fatty acids lost in the faeces due to interactions with Ca appears to depend on a number of factors such as the solubility of the dietary Ca source within the gastrointestinal tract, the fat source and its fatty acid composition, the pH of the intestinal environment, as well as the presence of bile (Boyd *et al.* 1932). Fatty acids in food are present in the form of triglycerides and phospholipids (see section 2.2.1) and depend on lipid hydrolysis to be released from the glycerol molecule. Ca in food is usually present in the form of micelles (e.g. casein micelles in milk), complexes (e.g. bound to uronic, oxalic or phytic acid in vegetables and grains) or salts (e.g. carbonate, citrate, phosphate, gluconate, etc. in fortified products or supplements). In order for Ca to form soaps with free fatty acids, the Ca needs to be present in its ionic form thereby necessitating the dissociation of Ca from its 'bound forms'. Once fatty acids and Ca are released and present as free fatty acids and ionized Ca, soap formation must then occur prior to the absorption of the two components from the small intestine.

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

In vivo studies performed in animals and humans have suggested that fatty acid chain length and degree of saturation are a major contributing factor that determines the extent to which Ca-fatty acid soaps form in the gastrointestinal tract. Increasing dietary Ca concentration affects the faecal excretion of saturated long chain fatty acids to a much greater degree than unsaturated fatty acids and shorter-chain fatty acids. A possible explanation for the latter

effect may be 1) that saturated long chain fatty acids take longer to be incorporated into biliary micelles and 2) Ca-fatty acid soaps comprised of saturated long chain fatty acids are relatively insoluble.

Saturated long chain fatty acids might have a greater opportunity to form soaps in the presence of dietary Ca as the absorption/incorporation time into biliar micelles of the latter fatty acids is slower compared to unsaturated long chain fatty acids or short and medium chain fatty acids. For example, Palmitic and stearic acid, which have been found to be excreted to a greater extent in the faeces in the presence of increased dietary Ca concentrations, are absorbed more slowly from the small intestine and therefore are able to travel into more distal regions of the gastrointestinal tract compared to unsaturated fatty acids of similar chain length. This longer residence time in the gastrointestinal tract increases the opportunity for saturated fatty acids to interact with ionized Ca and form insoluble soap complexes. Moreover, dietary Ca has been found to only minimally increase the excretion of oleic acid and linoleic acid, which may in part relate lead to their rapid absorption from the anterior regions of the small intestine, since the more rapid uptake of unsaturated fatty acids into biliary micelles may reduce the contact time of these fatty acids and Ca.

Another factor that will likely influence the presence of Ca-fatty acid soaps in the gastrointestinal tract is the relative solubility of soaps comprising different fatty acids. It has been reported that soaps comprised of saturated long-chain fatty acids are poorly soluble in simulated gastrointestinal conditions and during their passage throughout the gastrointestinal tract (Boyd *et al.* 1932; Gacs and Barltrop 1977; Graham and Sackman 1983). *In vitro* studies testing the solubility of Ca-fatty acid soaps of different chain length (C_8 – C_{18}) in simulated intestinal conditions reported the lowest solubility for Ca-soaps of stearic acid

(Gacs and Barltrop 1977). The solubility for Ca-soaps comprised of medium chain fatty acids (C_{10}-C_{14}) was slightly higher and short chain fatty acid (C_8) soaps were the most soluble. Another observation from *in vitro* studies was that the degree of saturation of the fatty acid comprising the Ca-soap plays an important role in the solubility of the soaps. Ca-fatty acid soaps of oleic and linoleic acid, the latter fatty acids containing one and two double bonds respectively, were highly soluble in simulated intestinal fluids and solubility increased with increasing degree of unsaturation (Gacs and Barltrop 1977). Furthermore, *in vivo* studies using *in vitro* synthesized Ca-fatty acid soaps were performed to investigate the solubility of Ca-fatty acid soaps during their transit through the gastrointestinal tract. Boyd *et al.* (1932) and Gacs and Barltrop (1977) reported that when *in vitro* synthesized Ca-fatty acid soaps were introduced into the rat (either given orally or infused into the duodenum), the absorption of Ca was inversely correlated with fatty acid chain length. Specifically, Ca retention from Ca-butyric acid soap was greater than 50%, but decreased with increasing chain length to 38% for Ca-palmitic acid soap and further to 25% with Ca-stearic acid soap when these soaps were fed to rats in a diet containing 5 g kg^{-1} Ca provided as Ca-fatty acid soaps (Boyd *et al.* 1932). The highest Ca retention value of 90% was reported for soaps composed of the unsaturated fatty acid, oleic acid (Boyd *et al.* 1932). Gacs and Barltrop (1977) reported even lower Ca absorption values from *in vitro* synthesised soaps when infusing the soaps directly into the duodenum of rats to avoid any dissociation of the soaps in the acidic environment of the stomach. The long chain saturated fatty acids, stearic and palmitic acid, were the least soluble during their passage through the small and large intestine and negligible Ca absorption from these Ca-fatty acid soaps was observed. Ca absorption increased with decreasing fatty acid chain length to a maximum of 50% for Ca octanoate. Moreover, a decrease in the degree of saturation of the fatty acids present in the soaps was

correlated with an increase in Ca absorption. Ca-absorption from Ca-fatty acid soaps comprised of the unsaturated fatty acids, oleic and linoleic acid, was 10- and 20-fold higher, respectively, compared to Ca absorption from Ca-stearic acid soaps (Gacs and Barltrop 1977). The difference in absorption of Ca from synthetic Ca-fatty acid soaps across the two studies performed by the different research groups (i.e. 1 and 25% for stearic acid and 10 and 90% for oleic acid reported by Gacs and Barltrop (1977) and Boyd *et al.* (1932) respectively) may be explained by the fact that the stomach was bypassed in the Gacs and Barltrop's (1977) study, while it was not bypassed in the Boyd *et al.* (1932) study. It is assumed that dietary soaps passing through the stomach would partially dissociate, depending on the pH of the gastric environment, as soaps in general have been reported to dissociate readily in acidic environments (Graham and Sackman 1983). However, both studies suggested that Ca-fatty acid soaps comprising saturated long-chain fatty acids were relatively insoluble in the gastrointestinal tract thereby supporting the hypothesis that the greater amount of palmitic and stearic acids in the faeces of animals with a greater dietary Ca intake is most likely due to the formation of insoluble indigestible Ca-fatty acid soaps.

2.5.1.2. The influence of positional distribution of fatty acids on the triglyceride molecule on Ca-mediated fatty acid excretion

Studies in which rats were fed structured triacylglycerol molecules showed a decrease in the absorption of saturated fatty acids in the presence of high dietary Ca concentrations when the fatty acids were present in the sn-1 and 3 positions (Mattson *et al.* 1979; Aoyama *et al.* 1995; Brink *et al.* 1995). For example, rats receiving a diet containing 1-oleoyl-2,3-distearoyl glycerol (OSS) experienced a 14% decrease in the absorption of stearic acid when the dietary Ca concentration was increased from 3 to 9.8 g kg^{-1} diet. In contrast, feeding 2-oleoyl-1,3-

distearoyl glycerol (SOS) to the rats decreased the absorption of stearic acid to a much greater degree (35% decrease) when dietary Ca was increased (Brink *et al.* 1995). Similar observations were reported for structured triglycerides containing palmitic acid where faecal palmitic acid excretion was 2.6-fold higher for the rats receiving 2-oleoyl-1,3-dipalmitoyl glycerol (POP) compared to those receiving 1-oleoyl-2,3-dipalmitoyl glycerol (OPP) at a dietary Ca concentration of 8.1 g kg^{-1} diet (Aoyama *et al.* 1995). It was also of note that the apparent absorption of oleic acid was not influenced by its position on the triglyceride in either of the latter studies (Aoyama *et al.* 1995; Brink *et al.* 1995). In another study, feeding the structured triglycerides triolein, 1,3-dioleoyl-2-stearoyl glycerol (OSO) or 1,2-dioleoyl-3-stearoyl glycerol (OOS) to rats in diets containing different Ca concentrations did not alter oleic acid digestibility for any of the triglycerides given (Mattson *et al.* 1979). The findings from the latter three studies suggest that saturated fatty acids in the sn-1 and sn-3 position are less digestible than those in the sn-2 position. Moreover, it is likely that reduction in digestibility is due to the formation of Ca-fatty acid soaps, since it is likely that sn-1,3 fatty acids are released from the triglyceride during digestion and would be chemically available for Ca complexation, whereas the fatty acids in the sn-2 position remains bound to the glycerol molecule and the resulting monoglyceride incorporated into micelles, thereby rendering the sn-2 fatty acid unavailable for soap formation. In contrast, the digestibility of oleic acid is largely unaffected regardless of the position on the triglyceride molecule.

While most studies have suggested that the positional distribution of unsaturated fatty acids has little effect on their digestibility as a function of dietary Ca concentration (Mattson *et al.* 1979; Renaud *et al.* 1995), one study conducted in humans receiving a western diet high in dairy products appears to contradict the latter conclusions (Bendsen *et al.* 2008). In the study

conducted by Bendsen *et al.* (2008), subject groups consuming different amounts of dairy products, and therefore different amounts of dietary Ca, (up to 2300 mg Ca d^{-1}), experienced a 4.5-fold increase in the faecal excretion of monounsaturated fatty acids compared to subjects on a low-Ca control diet. In contrast, palmitic and stearic acid excretion was only 2-fold higher. Bendsen *et al.* (2008) concluded that the greater increase in faecal excretion of monounsaturated fatty acids compared to that observed for the saturated fatty acids may be due to the fatty acid distribution at the triglyceride molecule in milk fat, with palmitic acid being mainly on the sn-2 position and therefore not available for soap formation, whereas hydrolysed oleic acid from the sn-1 and sn-3 positions was able to complex with Ca in the gastrointestinal tract before its incorporation into biliary micelles. The only other case of increased Ca-mediated oleic acid excretion has been reported in the absence of bile salts in animal models (see section 2.5.1.3). In the absence of bile salts, oleic acid absorption is reduced and therefore oleic acid can travel into more distal regions of the gastrointestinal tract, which increases the opportunity for oleic acid to complex with Ca. Additionally, once formed, oleic acid soaps will be less soluble in the absence of bile salts. The positional distribution of fatty acids on the triglyceride molecule seems to be far more important for Ca-mediated fatty acid excretion of saturated fatty acids compared to unsaturated fatty acids.

2.5.1.3. The influence of bile salts on Ca-fatty acid soap solubility and formation

Another factor that affects the solubility of Ca-fatty acid soaps is the presence of bile salts. An *in vitro* experiment performed with Ca-oleic acid soaps using various solutions of individual bile salts and bile salt mixtures ranging from 5 to 20 mM demonstrated that Ca-oleic acid soap solubility generally increases with increasing bile salt concentration (Graham and Sackman 1982). For example, mixed bile salt solutions resembling human bile (35%

glycocholate, 25% glycochenodeoxycholate, 15% glycodeoxycholate, 13% taurocholate, 8% taurochenodeoxycholate and 5% taurodeoxycholate) in concentrations of 5, 10 and 15 mM together with 1.45 mM phosphatidylcholine led to increased Ca-oleic acid soap solubility from 2 mM to 2.6 mM and 3.2 mM, respectively (Graham and Sackman 1982). *In vivo* oleic acid absorption is relatively unaffected by dietary Ca concentrations which may be a consequence of free oleic acid being rapidly incorporated into biliary micelles, or may be due to the greater solubility of Ca-oleic acid soaps in the presence of bile salts when compared to saturated fatty acids. However, at low concentrations of bile salts or in the absence of bile salts, oleic acid has been reported to be excreted in the faeces to a greater extent. For example, Graham and Sackman (1982) reported a reduced absorption of oleic acid when triolein was fed, together with $CaCO_3$, to bile duct-ligated rats. Bile duct-ligated rats receiving synthetic human bile (as found in pancreatic insufficient patients) absorbed 30% less oleic acid when CaCO3 was present in the diet as compared to diets containing lower amounts of Ca. In addition, the faecal lipid profile shifted from containing mainly triolein in the absence of dietary Ca to containing a greater proportion of free oleic acid, which was suspected to be in the form of Ca-oleic acid soaps (Graham and Sackman 1982). Furthermore, in another study bile duct-ligated rats excreted a greater proportion of insoluble lipid compounds (fatty acids remaining in the faeces after extraction with chloroform:methanol 2:1 v/v) with their faeces compared to control rats (bile duct-intact rats) (Demarne *et al.* 1982). The researchers suggested that the insoluble lipid compounds contained mainly fatty acids precipitated as Ca-soaps. Particularly, the saturated long-chain fatty acids, palmitic and stearic acid were present to a greater extent in the insoluble lipid compounds of faeces of bile duct-ligated rats. The absorption of palmitic and stearic acid was reduced by more than 60%, and also oleic acid absorption was 30% lower in bile duct-ligated rats compared to control

bile duct-intact rats (Demarne *et al.* 1982). Additionally, it has been shown that fatty acid malabsorption can considerably depress the absorption of Ca in bile duct-ligated rats (Kehayoglou *et al.* 1968). Administering a solution of olive oil and $CaCl_2$ to bile duct-ligated rats resulted in a 22% lower absorption of Ca compared to rats with intact bile ducts. The authors concluded that fat absorption was impaired due to the deficiency of bile salts. Moreover they conclude that the lower Ca absorption in the bile duct-ligated rats was due to the formation of insoluble Ca-fatty acid soaps (Kehayoglou *et al.* 1968).

2.5.1.4. Dietary protein intake may influence Ca-mediated faecal fatty acid excretion

Several studies investigating the effect of dietary Ca on faecal fat excretion in humans have been reported where dietary Ca was associated with an increased excretion of faecal fat and fatty acids regardless of the form (either dairy products, Ca-fortified food products or Ca supplements) in which the Ca was consumed (see Table 2-2). However, there are a few studies which did not report an increase in faecal fat excretion with increased dietary Ca (Ditscheid *et al.* 2005; Boon *et al.* 2007; Hjerpsted *et al.* 2011). A common factor in the latter studies where no effect of high dietary Ca intake on faecal fat excretion was reported was that the diets used contained greater amounts of protein than those used in the studies where a "Ca effect" was observed (Boon *et al.* 2007; Hjerpsted *et al.* 2011). For example, Jacobsen *et al.* (2005) reported conflicting effects for high dietary Ca concentrations on faecal fat excretion within the same study when the protein intake differed for the subject groups. The latter research group compared three different diets (see Table 2-2) varying in Ca (500 vs 1800 mg d^{-1}) and in protein content (15 vs 23 E% protein) and observed that when the dietary Ca content was increased and the protein content remained the same (15 E% protein) faecal fat excretion more than doubled (2.4-fold increase). However, no increase in faecal fat

excretion was observed when the increased dietary Ca intake was accompanied by an increase in the protein content of the diet (23 E% protein). Similarly, a Ca-intervention study in subjects with a daily protein intake of 20% of energy showed no differences in faecal fat excretion for varying concentrations of dietary Ca (400 vs 1200 vs 2500 mg Ca d^{-1}) (Boon *et al.* 2007). High protein diets have been reported to improve Ca absorption (Kerstetter *et al.* 1998), thereby likely leaving less Ca available for fatty acid complexation. In another study, Jacobsen *et al.* (2005) reported a 66% higher urinary Ca excretion for the high-Ca/high-protein diet compared to the high-Ca/normal-protein diet which suggested greater absorption of Ca from the intestinal tract for the high protein diet, which would have resulted in less Ca being available for Ca-fatty acid soap formation. A diet high in protein has been suggested to improve Ca absorption (Kerstetter *et al.* 1998) by keeping Ca in solution and preventing precipitation with interfering components such as phosphates (Scholz-Ahrens and Schrezenmeir 2000; Camara-Martos and Amaro-Lopez 2002). As mentioned in section 2.3.2.1, phosphopeptides derived from casein bind ionized Ca to the phosphate group of serine residues, this would render Ca less available for the formation of Ca-fatty acid soaps and explain the lower excretion of fatty acids in the presence of dairy protein.

2.5.2. Fatty acid soap formation with divalent cations other than Ca

Ca is a divalent cation, and therefore when ionised has the ability to bind two free fatty acid molecules forming a Ca-fatty acid complex that is suspected to be insoluble at the pH of intestinal fluids and therefore passes through the gut unabsorbed (Gacs and Barltrop 1977). However, Ca is not the only divalent cation present in the gastrointestinal tract. Nutritionally relevant minerals such as Mg, Zn, Fe and Cu are also present in the gastrointestinal tract and based on their similar charge it is conceivable that these cations could also form soaps with

fatty acids. Moreover, considering that dietary fat can impair Ca absorption most likely via soap formation, the absorption of other divalent minerals may be similarly affected. Despite the obvious similarity between Ca and other divalent minerals little work has been conducted examining the ability of divalent cations other than Ca to form soaps. A few studies investigating the effect of dietary fat on Ca retention have included observations about faecal Mg excretion and digestibility. Of the limited information available, Kaup *et al.* (1990) reported that, feeding rats diets containing 20 g 100 g^{-1} of butter fat resulted in a 20% lower apparent faecal magnesium digestibility and greater faecal Mg excretion when compared to rats receiving a diet containing 5 g 100 g^{-1} of butter fat suggesting that Mg-fatty acid soaps may be the mechanism by which Mg absorption was altered. Moreover, and as is the case for Ca, Mg absorption appears to be influenced to a greater degree by the presence of saturated fatty acids as opposed to unsaturated fatty acids. The latter is evidenced by a study where rats receiving a diet containing 25 g 100 g^{-1} triolein absorbed up to 55% of the ingested Mg (apparent Mg digestibility was comparable to the fat-free control diet) whereas for diets containing 25 g 100 g^{-1} diet of tripalmitin or tristearin only, 30% of the ingested Mg was absorbed (Tadayyon and Lutwak 1969b). However, not all published studies have reported an effect of dietary fat consumption on increased faecal Mg excretion or reduced Mg absorption. For example, comparing fat-free diets to diets supplemented with oleic or palmitic acid and with varying Ca concentrations did not show decreased Mg retention in chicks when fat was present (Atteh and Leeson 1984). Moreover, a study in rats investigating the effect of the positional distribution of stearic and oleic acid in the triacylglycerol molecule did not show an influence of the structured triglycerides on faecal excretion and the apparent digestibility of Mg (Brink *et al.* 1995). In general, the results from some of the latter studies suggest that Mg absorption can be negatively influenced by a diet high in saturated fatty

acids and therefore that Mg-fatty acid soap formation is possible. However, Mg-fatty acid soap formation does not seem to be as profound as Ca-fatty acid soap formation, as studies showing an increase in faecal Ca excretion did not always show an effect on Mg excretion (Atteh and Leeson 1984). Studies investigating the effect of dietary fat on divalent trace mineral absorption (eg Zn, Fe, Cu) are inconclusive (Lukaski *et al.* 1986; Johnson *et al.* 1992; Wapnir and Sia 1996). It has been suggested that an increase in dietary fat increases Fe absorption in rats, regardless of the fat source (Johnson *et al.* 1987). However, some studies suggest a decrease in the absorption of Zn and Fe in the presence of polyunsaturated fatty acids (Lukaski *et al.* 1986; Wapnir and Lee 1990). For example, a study performed with endurance athletes showed increased faecal Zn and Fe excretion in the presence of a diet rich in polyunsaturated fatty acids compared to a diet high in carbohydrates or saturated medium chain fatty acids (Lukaski *et al.* 1986). Moreover, in an *in vivo* study using intestinal perfused rats, arachidonic acid (C20:4) inhibited zinc removal from the intestinal lumen. However, the saturated long chain fatty acid, palmitic acid, had the opposite effect and stimulated zinc absorption. Another study in rats showed improved Fe absorption in the presence of saturated fatty acids (Johnson *et al.* 1992). However, not all studies have showed such an effect of saturated fatty acids on trace element (Fe and Cu) absorption (Johnson *et al.* 1987; Wapnir and Sia 1996). For example, Cu absorption had been reported to decrease significantly in the presence of free palmitic acid and stearic acid when investigated in rats using jejunal perfusion (Wapnir and Sia 1996). The inconclusive results and the limited research conducted regarding divalent cations other than Ca and their ability for fatty acid soap formation requires more research.

2.6. Concluding Comments

An increased dietary Ca intake has been repeatedly reported to increase faecal fat excretion in animals and humans. It has been hypothesised that this Ca-induced increase in faecal fat output leads to a decreased uptake of energy which might further result in a reduction of body weight and body fat. The mechanism by which dietary Ca increases faecal fat output has been suggested to be the formation of Ca-fatty acid complexes, so called Ca-fatty acid soaps, which are insoluble in the intestinal environment and evade absorption in the small intestine.

Although the formation of insoluble Ca-fatty acid soaps is believed to be the cause of the reduced fat absorption, few researchers have attempted to isolate Ca-fatty acid soaps from faecal samples. The currently available methods described in the literature to extract Ca-fatty acid soaps from faecal material appear to poorly recover soaps. However, to determine what impact Ca-fatty acid soap formation has on faecal fat excretion, it is necessary to be able to determine the amount of fat excreted in the form of fatty acid-soaps.

The formation of Ca-fatty acid soaps is believed to not only alter the absorption of fat but has also been shown to reduce the absorption of Ca from the gastrointestinal tract in studies conducted in animals and human infants. If the formation of Ca-fatty acid soaps could potentially impair Ca absorption, other divalent cations may also be able to complex with fatty acids leading to decreased absorption of essential minerals from the intestinal tract.

Further research is necessary to definitively address the hypothesis that Ca-fatty acid soap formation is the mechanism for increased faecal fatty acid excretion in the presence of dietary Ca. This can only be done by determining Ca-fatty acid soaps in faecal material, and to date

this has not been done well. Moreover, understanding where in the gastrointestinal tract soaps are formed would provide important knowledge about the behaviour of dietary Ca and fatty acids in relation to soap formation in the gastrointestinal tract.

CHAPTER THREE: Development of an Assay to Determine the Amount of Ca-Fatty Acid Soaps in Faeces

3.1. Abstract

The quantification of Ca-fatty acid soaps in the faeces is of importance to understand how fatty acids behave in the gastrointestinal tract. However, the previously published method to extract Ca-fatty acid soaps from faeces has been reported to have a rather poor recovery (65%). Consequently, it was the aim of this study to develop a new assay for determining Ca-fatty acid soaps in faeces. Since no suitable solvent has been reported for extracting soaps, this study used an indirect approach whereby all the non-soap specific fatty acid compounds were extracted from the faeces leaving the Ca-fatty acid soaps behind, rather than extracting the soaps themselves. Synthetic Ca-fatty acid soaps of different chain lengths (C_{12}-C_{18}) and degree of saturation ($C_{18:0}$ – $C_{18:2}$) were incubated with a range of solvents (hexane, isopropanol, and water were selected for the extraction of non-polar to polar non-soap fatty acid components based on literature reports for the solubility of the compounds in the solvents) to find solvents for which the non-soap fatty acid compounds were highly soluble but the fatty acid soaps were insoluble. The three solvent's extractions were combined to give a three-step extraction method. When applied to faeces 100% of the free fatty acids, 100% of the phospholipids and almost all of the Na-fatty acid salts (100% for Na-laurate and Na-oleate; 95% for Na-stearate) were removed and 98% of Ca-lauric acid soap, 99% of Ca-stearic acid soap and 93% of oleic acid soap were recovered. The newly developed method appears to be suitable for determining fatty acids in the form of soaps in faeces and thereby permitting the determination of the amount of Ca-fatty acid soaps excreted in the faeces for fat containing foods.

3.2. Introduction

Over the past two decades there has been considerable interest in the study of dietary calcium (Ca) and its influence on body weight. Several observational studies (Davies *et al.* 2000; Zemel *et al.* 2000; Jacqmain *et al.* 2001) and a smaller number of clinical trials (Zemel *et al.* 2004; 2005a; 2005b) have shown an association between dietary Ca intake and body weight, body fat, and weight loss in humans.

Several hypotheses, not mutually exclusive, have been mooted to explain the effect of dietary Ca on weight loss and body composition (Major *et al.* 2008). Zemel *et al.* (2000) proposed a hypothesis based on the metabolic role of Ca in human adipocytes, in which when Ca is consumed in abundance, metabolic pathways are triggered that lead to increased lipolysis and the inhibition of lipogenesis. Another hypothesis is based on observations from several animal and human studies demonstrating an increase in faecal fat excretion after consumption of a Ca-rich diet (Denke *et al.* 1993; Brink *et al.* 1995; Shahkhalili *et al.* 2001a; Papakonstantinou *et al.* 2003; Jacobsen *et al.* 2005; Bendsen *et al.* 2008). The latter hypothesis is based on the proposed formation of indigestible Ca-fatty acid soaps in the intestinal tract resulting in decreased fat absorption. When ionised, Ca can bind two free fatty acids forming a Ca-fatty acid complex that is reported to be insoluble at the pH of intestinal fluids (Graham and Sackman 1983) and is therefore suspected to pass through the gut unabsorbed (Gacs and Barltrop 1977).

In addressing the latter hypothesis, several workers have attempted to detect and quantify Ca-fatty acid soaps in faeces. In the early 1960's Sammons and Wiggs (1960) quantified soaps based on the molar ratio of Ca-to-fatty acids (1:2) in 'white, solvent insoluble solids' that they extracted from fresh human faeces with diethyl ether. However, extraction experiments

using faeces spiked with synthetic Ca-palmitic and Ca-oleic acid soaps suggested that extraction recoveries were only around 65%. Watkins *et al.* (1974) adopted the same method to investigate the presence of Ca-fatty acid soaps in the faeces of neonate infants but did not test the efficacy of the extraction method. Meanwhile, Gacs and Barltrop (1977) using ^{47}Ca, refined the method of Sammons and Wiggs (1960) by adding sucrose to rat faecal homogenates and reported Ca-fatty acid soap recoveries of 75-90% depending on the chain length of the fatty acid. Owen *et al.* (1995) who used a different protocol for the isolation of Ca-fatty acid soaps, based on Soxhlet extraction of faecal material, reported rather poor recoveries of synthetic Ca-palmitic acid soap while synthetic Ca-oleic acid soap was not recovered at all.

Several studies have reported that increasing dietary Ca concentration leads to increased faecal fat excretion and the latter effect appears to be a result of increased soap formation (Denke *et al.* 1993; Welberg *et al.* 1994; Shahkhalili *et al.* 2001a; Jacobsen *et al.* 2005; Bendsen *et al.* 2008). Yet, none of the latter studies made any attempt to actually determine the amount of Ca-fatty acid soaps in the faeces. That studies focussing on the dietary Ca-faecal fat interaction have not always determined soaps in the faeces limits their interpretation, and an accurate quantitative method for determining Ca-fatty acid soaps in faecal material is greatly needed. Consequently, the aim of this study was to evaluate the efficacy of a soap extraction method (Sammons and Wiggs (1960) as modified by Gacs and Barltrop (1977), and if necessary develop a new method for determining soaps in faeces. The long term aim was to apply this method to faeces obtained from study subjects to investigate the effect of dietary Ca on faecal fatty acid soap excretion.

3.3. Material & Methods

3.3.1. Materials

Lauric, myristic, palmitic, stearic, oleic, linoleic acids, Na-stearate, methyl-nonadecanoate, and tert-butylhydroquinone (TBHQ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). $CaCl_2$, n-hexane, petroleum ether, diethyl ether, chloroform, isopropanol, ethanol, methanol, HCl and H_2SO_4 were purchased from Merck (Darmstadt, Germany). All chemicals used were analytical grade.

Fresh untreated faecal samples were obtained from pigs receiving a semi-synthetic experimental diet containing (per kg): soya bean oil (130 g); lactic casein (177 g); cellulose (51.4 g); sucrose (105.9 g); NaCl (3 g); NaH_2PO_4 (10 g); vitamin-mineral premix (2 g); TiO_2 (3 g). Diets were made up to 1 kg with purified wheat starch. Ca was not present in the vitamin-mineral premix and the final Ca concentration in the diet was 0.4 g kg^{-1}.

To obtain fat-free faeces, fresh faeces were freeze dried and thereafter acidified with 6 M HCl to liberate any cation-bound fatty acids. The resulting free fatty acids and other lipids were extracted three times with diethyl ether. The defatted faeces were washed with distilled water to remove any liberated minerals after which the pH of the faeces was found to be 7. The defatted faecal samples were dried and an aliquot was analysed for the presence of fatty acids to demonstrate that all fatty acid containing compounds had been removed.

3.3.2. Preparation of synthetic Ca-fatty acid soaps

Ca-fatty acid soaps containing either lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (18:1) or linoleic acid (C18:2) were prepared as described by Graham and Sackman (1983). Briefly, 2 g of each fatty acid was individually

dissolved in 100% ethanol (150 mL). $CaCl_2$ was dissolved in 20 mL distilled water and added to each fatty acid-ethanol solution such that the molar ratio of Ca to each fatty acid was 2:1. The pH of the mixture was adjusted to 7.0 with 1 M NaOH prior to heating under reflux for 1 h. To avoid fatty acid oxidation of unsaturated fatty acids, tert-butylhydroquinone (0.05 mg g^{-1} fatty acid) was added to mixtures containing oleic and linoleic acid. After cooling the mixtures to room temperature the soaps were separated via filtration using a Buchner funnel and filter paper. Unreacted fatty acids were removed from the soaps by washing three times with ethanol at 50°C for saturated fatty acids and at room temperature for unsaturated fatty acids. The unreacted Ca was removed by washing the soap material twice with distilled water. All soap preparations were dried and stored in a desiccator at 4°C.

3.3.3. Purity of the synthetic Ca-fatty acid soaps

To test the purity of the prepared Ca-fatty acid soaps, the soaps were dissociated and the molar ratio of Ca to fatty acids determined. The soaps were disassociated by acidifying the Ca-fatty acid soap (20 mg) with 6 M HCl (2 ml) and then heating the suspension to either 80°C for the saturated fatty acid soaps or 25°C for the unsaturated fatty acid soaps. The free fatty acids were extracted three times with 3 ml hexane with the washings being pooled in a 10 ml volumetric flask. After extraction, the solution was made up to volume and a 1 ml aliquot was transferred into a glass screw cap tube for fatty acid analysis (see section 3.3.5). The aqueous layer was made up to volume with distilled water and Ca was then determined using an atomic absorption spectrometer (see section 3.3.4).

3.3.4. Ca analysis

Test Ca solutions were diluted as required and $SrCl_2$ (1000 μg mL^{-1} in the final solution) was added to bind any phosphates or sulphates present that can interfere with the Ca determination. Samples were then analysed using flame atomic absorption spectrometry (AAS) at a wavelength of 422.7 nm with the current of the Hollow Cathode Lamp being 5 mA. The height of the burner was 8 mm and the air-acetylene ratio was 6:1.

3.3.5. Fatty acid analysis

Fatty acid analysis was conducted as described by Zhu *et al.* (2011) with slight modifications. Briefly, samples (50 mg of freeze dried faeces or 1 mL hexane containing extracted fatty acids) were added to screw-cap glass tubes to which 1 mL of internal standard solution (1 mg mL^{-1} methyl-nonadecanoate in hexane), 0.7 mL 10 M NaOH and 5.3 mL methanol were added. The tubes were incubated for 1.5 h at 65°C with vigorous shaking every 20 min. After cooling the tubes to room temperature in a cold water bath, 580 μL 12 M H_2SO_4 was added and the tubes were remixed on a vortex mixer and incubated for another 1.5 hours at 65°C with vigorous periodic shaking. The tubes were cooled to room temperature and the fatty acid methyl esters (FAMEs) extracted by the addition of 3 mL hexane and vortex mixing for 1 min, followed by centrifugation at 1000 g for 10 min. The hexane layer, which contained the FAMEs, was transferred into glass tubes and evaporated under a stream of nitrogen. FAMEs were then re-dissolved in 1 mL hexane and transferred into gas chromatography (GC) vials and stored at -18°C prior to GC analysis. FAMEs were separated on a Supelcowax® 10 (30 m x 0.20 mm x 0.20 μm) fused silica capillary column installed in an Agilent Technologies 7890A gas chromatograph equipped with a flame ionisation detector and split/splitless injector (Agilent Technologies, Victoria, Australia).

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.

The method described by Sammons and Wiggs (1960) was designed to allow extraction of Ca-fatty acid soaps from faecal samples. Consequently, fresh untreated faecal samples obtained from pigs (as described in section 3.3.1) were used to test the efficacy of the proposed method for Ca-fatty acid soap extraction by determining the soap recovery from samples spiked with synthetic Ca-fatty acid soaps. Fresh pig faeces (250 g) were homogenised with distilled water (approximately 700 mL) in a kitchen blender (Warring Commercial Blender, Model HGB2WTS3) and were either spiked with freshly prepared Ca-fatty acid soaps (suspended in a small amount of ethanol) of either lauric, stearic or oleic acid, or left unspiked. Sucrose was added to the homogenates to give a final concentration of 60 mM, based on the modification of Gacs and Barltrop (1977). Aliquots of 10 mL were taken from the faecal homogenates and freeze dried for total fatty acid determination (see section 3.3.5). Further aliquots (10 mL) of homogenised faeces were transferred into separation funnels, 30 mL of diethyl ether was added and the separation funnel contents were mixed by gently inverting the funnels several times. Once phase separation was complete, the diethyl ether layer (expected to contain the insoluble Ca-fatty acid soaps and liberated fatty acids) and the water layer (expected to contain Ca-salts, Na-soaps and micellar lipids) were collected separately. The aqueous phase was extracted with diethyl ether three more times. The combined diethyl ether fractions were washed twice with distilled water, centrifuged at 3000 g for 10 min and the supernatant was removed. The precipitate (expected to contain the insoluble Ca-fatty acid soaps) was washed twice with diethyl ether and any residual solvent was evaporated under a stream of nitrogen. The precipitate was acidified with 6 M HCl and incubated for 30 min at either 80°C for the faeces spiked with Ca-lauric acid soap and

Ca-stearic acid soap or at room temperature for the faeces spiked with Ca-oleic acid soap. The fatty acids present were then extracted as described in section 3.3.3 and determined as described in section 3.3.5. For the diethyl ether supernatant (expected to contain soluble/dissociated Ca-fatty acid soaps) and the aqueous phase (expected to contain Ca-fatty acid soaps that could not be extracted from the faeces) the fatty acids were determined as described in section 3.3.5 except that fatty acids were extracted from the aqueous layer by acidification and hexane extraction.

Evaluation of the Sammons and Wiggs (1960) method for the extraction of Ca-fatty acid soaps from faeces pointed to the need to develop a different methodological approach. A three-step extraction method, which is presented fully below (see section 3.3.8), was therefore developed and as part of this the solubility of Ca-fatty acid soaps in a range of solvents was determined.

3.3.7. Experiment 2: Evaluating the solubility of Ca-fatty acid soaps in a range of solvents.

The solubility of synthetic Ca-fatty acid soaps was determined using different solvents (hexane, petroleum ether, diethyl ether, chloroform, dichloromethane, isopropanol, ethanol, methanol and water) and different combinations of incubation temperature and extraction time. Each assay was performed in duplicate at three separate times (n=3). Synthetic Ca-fatty acid soaps (20 mg) were weighed into 12 mL screw cap glass tubes and dispersed in 10 mL of each solvent separately. The tubes were mixed on a vortex mixer and kept either at 4°C, at room temperature (24°C) or were incubated at the boiling point of the solvent for 6 h. After 0.5, 2 and 6 h the tubes were centrifuged at 1500 g for 5 min and 1 mL aliquots of the resulting solvent supernatants (hexane, petroleum ether, diethyl ether, isopropanol, ethanol

or methanol) or solvent subnatants (chloroform, dichloromethane or water) were taken for fatty acid analysis as described in section 3.3.5. Any insoluble material remaining after the 6 h incubation step was acidified as described in section 3.3.3 and analysed for the molar ratio of Ca to fatty acids.

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.

The aim was to extract fatty acids that had not been incorporated into Ca-fatty acid soaps, including free fatty acids, Na-fatty acid soaps, neutral fats (eg. mono, di, triglycerides) and phospholipids from faeces, leaving the Ca-fatty acid soaps remaining in the faeces which could then be determined using fatty acid analysis. Solvents with the lowest Ca-fatty acid soap solubility, as determined in section 3.3.3, were chosen for the extraction of non-Ca bound fatty acids. The sequential three-step extraction method was performed as follows (Figure 3–1): Step 1) extraction with hexane (a non-polar solvent) to remove free fatty acids and triglycerides; step 2) extraction with hexane-isopropanol (a more polar solvent mixture) to remove neutral fats (eg. diglycerides, monoglycerides, phospholipids) (the latter solvent mixture was proposed by Hara and Radin (1978) as being suitable for the extraction of polar lipids); step 3) aqueous extraction to extract the Na-fatty acid soaps.

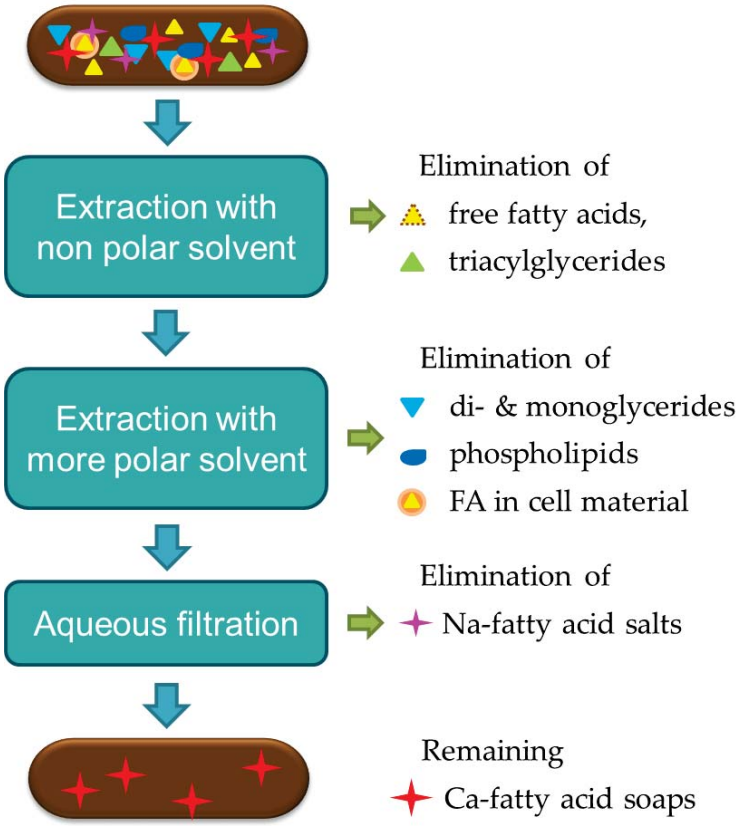


Figure 3-1: Sequential three-step solvent extraction of non-Ca bound fatty acids from faeces containing free fatty acids, mono-, di-, and triglycerides, phospholipids, Na-soaps and Ca-fatty acid soaps.

Step 1: Extraction with hexane to remove free fatty acids from the faecal matrix

Hexane (3 mL) was added to all of the faecal samples and mixed using a vortex mixer to allow extraction of the free fatty acids. Of the four replicate samples, one duplicate set (set A) was kept at room temperature for 10 min while the second duplicate set (set B) was incubated at 60°C for 10 min. After incubation, the tubes were again mixed on a vortex mixer. Each tube was then centrifuged at 1500 g for 5 min and each supernatant transferred into a separate screw cap glass tube. Each of the precipitates was washed twice with 2 mL hexane, with the washings being pooled with the supernatant for each sample separately. The solvent was then removed from each tube by evaporation under a stream of nitrogen. The extracted free fatty acids were then determined as described in section 3.3.5. The precipitated faeces underwent extraction with hexane-isopropanol (Step 2).

Step 2: Extraction with hexane-isopropanol to remove polar lipids from the faecal matrix

A mixture of hexane-isopropanol (3:2) (3 mL) was added to the faecal precipitate from the previous hexane extraction step. Samples were extracted with hexane-isopropanol for 15 min where again one duplicate set (set A) was kept at room temperature and the second duplicate set (set B) was incubated at 37°C. The samples were centrifuged at 1500 g for 5 min and each supernatant transferred into a separate glass tube. The extraction step was repeated and the supernatant pooled with the previous supernatant for each sample separately. The extracted free fatty acids were then determined as described in section 3.3.5. The faecal precipitates were dried under a stream of nitrogen to remove any residual hexane-isopropanol prior to commencing step 3.

Step 3: Aqueous extraction of Na-soaps from the faecal matrix

Distilled water (6 mL) was added to the tubes containing the faecal precipitates that had been previously extracted with hexane and hexane-isopropanol. The tubes were mixed on a vortex mixer and incubated for 30 min. Duplicate set A was kept at room temperature, whereas duplicate set B was incubated at 80°C. After incubation, all the samples were filtered through a Buchner funnel (previously heated to 95°C for the duplicate set B) attached to a vacuum pump. All the tubes were quantitatively washed with distilled water either at room temperature for duplicate set A or water heated to 80°C for duplicate set B. The amounts of Na-fatty acid soaps in the filtrate were then analysed by firstly acidifying the filtrate, extracting with hexane and then determining the extracted fatty acids as described in section 3.3.5. The residue present on each filter disc containing the insoluble Ca-fatty acid soaps, was dried overnight, inserted into screw top glass tubes and prepared for fatty acid analysis as described in section 3.3.5.

Preparation of model faecal samples to test the efficacy of the three-step extraction method for removing non-Ca bound fatty acids

Model faecal samples containing known amounts of free fatty acids, Na-fatty acid salts, neutral fat and Ca-fatty acid soaps comprising medium chain saturated, long chain saturated and long chain unsaturated fatty acids were prepared. Defatted faecal samples (treated as described in section 3.3.1) were spiked with either: 1) Ca-laureate, Na-stearate and free oleic acid; 2) Ca-stearate, Na-oleate, and free lauric acid; 3) Ca-oleate, Na-laureate and free stearic acid. An additional faecal sample was spiked with phospholipids (Sigma-Aldrich) to evaluate the efficacy of the three-step extraction method for removing phospholipids. The defatted freeze dried faeces (50 mg) were weighed in quadruplicate into screw cap glass tubes. Ca-fatty acid soaps, Na-soaps and free fatty acids (approximately 3 mg of each) were added and mixed into the faecal samples. To spike the faeces with phospholipids, 3 mg of the phospholipids were dissolved in distilled water (1 mL) and 50 mg of defatted freeze dried faeces were added, mixed and dried overnight under vacuum at 37°C. In addition, faeces containing the added phospholipids were also spiked with 3 mg of Ca-fatty acid soaps, Na-soaps and free fatty acids as described above to investigate the behaviour of Ca-fatty acid soaps, Na-soaps and free fatty acids in the presence of phospholipids during the three extraction steps.

3.4. Results & Discussion

3.4.1. Purity of synthetic Ca-fatty acid soaps

The purity of the synthetic Ca-fatty acid soaps was assessed based on the determined molar ratio of Ca to fatty acids. The molar ratio of Ca to fatty acids in the Ca-fatty acid soaps synthesised with lauric, myristic, palmitic, stearic, oleic or linoleic acid after purification is shown in Table 3-1. The determined molar ratios were generally close to the expected ratio of 1:2, indicating that soaps had been formed. The poorest ratio was observed for the Ca-linoleic acid soaps, which had a molar ratio of Ca to fatty acids of 1:1.6. The lower ratio may indicate incomplete soap formation or may be due to the oxidation of linoleic acid as it can oxidise quickly when exposed to air.

Table 3-1: Determined molar ratio of Ca to fatty acids in the synthetic Ca-fatty acid soaps[1].

Fatty acid present in the soap	MW[2]	molar Ca:fatty acid ratio
Lauric acid	200.32	1:1.8
Myristic acid	228.38	1:2.1
Palmitic acid	256.42	1:2.0
Stearic acid	284.48	1:1.9
Oleic acid	282.46	1:1.8
Linoleic acid	280.45	1:1.6

[1]The molar ratio of Ca to fatty acids in the synthetic soaps was analysed in duplicates at three different times; mean values (n=3) from fatty acid analysis and Ca-analysis were used to calculate the molar ratio of the synthetic soaps.
[2]molecular weight (MW) of the individual fatty acids

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.

Sammons and Wiggs (1960) used diethyl ether as their extracting solvent and reported a recovery of 65% for both spiked synthetic Ca-palmitic and spiked synthetic Ca-oleic acid soap. In contrast, in the present study the recovery of spiked Ca-oleic acid soap was only 32% (Table 3-2). The recoveries of the saturated fatty acid soaps using diethyl ether extraction were 68% and 54% for Ca-stearic acid and Ca-lauric acid soaps, respectively. A

considerable proportion (41, 30 and 43% of the fatty acids from the Ca-lauric acid, Ca-stearic acid and Ca-oleic acid soaps, respectively) was found to be present in the aqueous phase and not in the diethyl ether phase. Furthermore, Ca-oleic acid soap was relatively soluble in diethyl ether (> 20%) further suggesting that in general, diethyl ether is an inappropriate solvent for the determination of Ca-fatty acid soaps (Table 3-2).

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).

Fatty acid (FA)	FA (mg)[1] added as Ca-FA soap into faeces[2]	FA recovered as Ca-FA soap[3] (%)[1]	FA present in the diethyl ether fraction[4] (%)[1]	Unrecovered FA in faeces[5] (%)[1]
Lauric acid[6]	8.9 ± 0.07	54 ± 9.3	0.9 ± 0.13	41 ± 9.5
Stearic acid[6]	9.2 ± 0.08	68 ± 7.1	0.3 ± 0.06	30 ± 6.8
Oleic acid[6]	7.4 ± 0.10	32 ± 2.6	22 ± 5.8	43 ± 8.7

[1]Mean values ± standard error of the mean (SEM); the analysis was conducted in duplicates at three different times (n=3).
[2]The amount of each fatty acid added to the faeces in the form of synthetic Ca-fatty acid soap.
[3]The insoluble material extracted with diethyl ether and precipitated with centrifugation (3000 g, 10 min)
[4]Fatty acids soluble in the diethyl ether phase.
[5]The remaining faeces (aqueous phase) was acidified and extracted with hexane.
[6]Total recoveries of lauric, stearic and oleic acid were 96%, 98% and 97%, respectively, indicating that most of the fatty acids added to the faecal samples were accounted for.

Ca-fatty acid soaps have been reported by other workers to be relatively insoluble in organic solvents and water with Ca-soaps comprising unsaturated fatty acids being more soluble in several organic solvents as compared to Ca-soaps of saturated long chain fatty acids (Harrison 1924). Specifically, it was found that Ca-oleic acid soap was soluble in chloroform and benzene when incubated at the solvent's boiling point whereas Ca-palmitic and Ca-stearic acid soaps were only slightly soluble. Moreover, Ca-fatty acid soaps were reported to be insoluble in alcohol and petroleum ether, as well as water (Harrison 1924). In contrast, Ca-oleic acid soap was reported to be soluble in alcohol and ether (Beilstein 1920; Martindale and Westcott 1920) which is consistent with the findings of the present study where 22% of the oleic acid from the added Ca-oleic acid soap was soluble in diethyl ether but almost none

of stearic acid from the added Ca-stearic acid soap was soluble in the diethyl ether phase. Overall, the method described by Sammons and Wiggs (1960) was inadequate for the extraction of soaps present in pig faeces and it was concluded that a new approach for determining Ca-fatty acid soaps in faeces was needed. The solubility of certain Ca-fatty acid soaps is low in a number of solvents (Harrison 1924). Therefore, an alternative strategy was proposed whereby the aim was not to extract the soaps directly, but rather to extract all the non-Ca bound fatty acids instead. It was assumed that faeces contain non-Ca bound fatty acids in the form of: 1) free fatty acids (Bohle and Starck 1967); 2) tri, di- and monoglycerides (Bohle and Starck 1967; Matthys *et al.* 1972); 3) phospholipids (Cotton 1972; Davies *et al.* 2014); 4) ionised fatty acid salts (Na/K) (Watkins *et al.* 1974); 5) sterol esters (Matthys *et al.* 1972). Consequently, the first step of the method development was to investigate the solubility of Ca-fatty acid soaps in a range of different solvents to ascertain which solvents would be suitable for extracting non-Ca bound fatty acids at the exclusion of the Ca-fatty acid soaps.

3.4.3. Experiment 2: Testing the solubility of Ca-fatty acid soaps in a range of different solvents.

The aim was to identify solvents known to be capable of dissolving free fatty acids, ester-bound fatty acids and ionised fatty acid salts but that would not solubilise Ca-fatty acid soaps. Free fatty acids and triglycerides are reported to be highly soluble in non-polar solvents such as hexane, toluene, and cyclohexane, moderately polar solvents such as diethyl ether and chloroform and insoluble in polar solvents such as methanol (Zamora and Hidalgo 2004). Sterol esters are reported to be soluble in isooctane, hexane and isopropanol (FAO 2008). Monoglycerides and phospholipids are reported to be only slightly soluble in hexane

but do dissolve readily in more polar solvents such as methanol, ethanol and chloroform (Zamora and Hidalgo 2004). The Na- and K-salts of fatty acids are reported to be soluble in water but rather insoluble in organic solvents (Bartley 1989).

The Ca-fatty acid soap solubility in a range of solvents is presented in Table 3-3. Ca-fatty acid soaps of lauric acid, myristic acid, palmitic acid and stearic acid were insoluble (solubility = 0-3%) in hexane, petroleum ether and diethyl ether at all the incubation times and temperatures tested. The Ca-fatty acid soaps of the latter fatty acids were also insoluble in isopropanol at 4°C and at room temperature at all incubation times (solubility = 0-3%), whereas at the boiling point of the solvent the synthetic Ca-fatty acid soaps were slightly soluble (solubility = 7-22%). Furthermore, Ca-fatty acid soaps containing saturated long chain fatty acids (palmitic and stearic acid) were relatively insoluble in ethanol (solubility = 0-8%) and water (solubility = 0-5%) at all incubation times and temperatures tested. However, Ca-fatty acid soaps containing saturated medium chain fatty acids (lauric and myristic acid) were partially soluble (solubility = 7-33%) in ethanol and water at the boiling point of the solvents. The solubility of all four tested Ca-fatty acid soaps containing saturated fatty acids increased with time and temperature when incubated in chloroform (lauric acid soap solubility was 5% at 4°C for 0.5 h and increased to 58% at the solvent's b.p. for 6 h; stearic acid soap solubility was 1% at 4°C for 0.5 h and increased to 19% at the solvent's b.p. for 6 h), dichloromethane (lauric acid soap solubility was 5% at 4°C for 0.5 h and increased to 60% at the solvents b.p. for 6h; stearic acid soap solubility was 1% at 4°C for 0.5 h and increased to 18% at the solvents b.p. for 6h), and methanol (lauric acid soap solubility was 4% at 4°C for 0.5 h and increased to 77% at the solvent's b.p. for 6h; stearic acid soap solubility was 1% at 4°C for 0.5 h and increased to 10% at the solvent's b.p. for 6 h).

Incubation at the boiling point of the latter solvents (chloroform, dichloromethane and methanol) resulted in more than 50% of Ca-lauric acid and Ca-myristic acid soaps being solubilised. Across the different incubation times and temperatures the Ca- fatty acid soaps containing unsaturated fatty acids (oleic and linoleic acid) were least soluble in isopropanol (average solubility across all incubation times and temperatures was 18 and 22% for Ca-oleic and Ca-linoleic acid soap, respectively), followed by water (average solubility across all incubation times and temperatures was 21 and 25% for Ca-oleic and Ca-linoleic acid soap, respectively), hexane (average solubility across all incubation times and temperatures was 22 and 30% for Ca-oleic and Ca-linoleic acid soap, respectively), diethyl ether (average solubility across all incubation times and temperatures was 25 and 35% for Ca-oleic and Ca-linoleic acid soap, respectively) and ethanol (average solubility across all incubation times and temperatures was 36 and 40% for Ca-oleic and Ca-linoleic acid soap, respectively). Solubility of Ca-oleic and Ca-linoleic acid soap was very similar and generally high for chloroform, dichloromethane and methanol (average solubility across all incubation times and temperatures for the latter three solvents was 57 and 62% for Ca-oleic and Ca-linoleic acid soaps, respectively).

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.

Ca-soaps of:	Soluble fatty acids (%) in solvent								
	4°C			RT (24°C)			b.p. of solvent		
	0.5 h	2 h	6 h	0.5 h	2 h	6 h	0.5 h	2 h	6 h
	HEXANE (b.p. 60°C)								
Lauric acid	1	1	1	1	1	2	1	1	2
Myristic acid	0	0	1	1	1	1	1	1	2
Palmitic acid	0	0	0	0	1	1	1	1	3
Stearic acid	0	0	0	0	1	1	1	1	2
Oleic acid	1	2	4	6	25	38	7	34	84
Linoleic acid	1	2	5	8	32	76	14	49	91
	PETROLEUM ETHER (b.p. 40-60°C)								
Lauric acid	0	1	1	0	1	1	1	1	2
Myristic acid	0	1	1	0	1	1	1	1	2
Palmitic acid	0	0	0	0	0	1	1	1	3
Stearic acid	0	0	0	0	0	1	1	1	2
Oleic acid	1	3	2	2	27	41	22	53	90
Linoleic acid	1	2	4	8	36	74	28	56	89
	DIETHYL ETHER (b.p. 37°C)								
Lauric acid	1	1	1	1	1	2	1	2	2
Myristic acid	0	1	1	0	1	1	1	2	2
Palmitic acid	0	0	1	0	1	1	2	3	3
Stearic acid	0	0	0	0	0	1	2	2	2
Oleic acid	1	6	15	12	36	49	18	39	48
Linoleic acid	1	11	19	18	49	55	31	50	63
	CHLOROFORM (b.p. 61°C)								
Lauric acid	5	13	16	25	39	43	49	54	58
Myristic acid	5	10	14	11	21	37	41	49	52
Palmitic acid	1	2	2	6	15	22	26	32	35
Stearic acid	1	2	2	1	3	6	11	16	19
Oleic acid	18	39	51	48	56	68	76	81	89
Linoleic acid	24	43	64	52	59	72	82	88	93
	DICHLOROMETHANE (b.p. 40°C)								
Lauric acid	4	15	17	24	38	44	50	52	56
Myristic acid	4	10	13	12	24	36	39	45	50
Palmitic acid	1	2	2	6	12	19	25	29	33
Stearic acid	1	2	2	1	3	7	9	15	17
Oleic acid	17	40	49	50	58	71	72	76	81
Linoleic acid	21	42	61	52	62	76	80	84	89

Table 3-3 continued

Ca-soaps of:	Insoluble Ca-fatty acid soaps (%) in solvents								
	4°C			RT (24°C)			b.p. of solvent		
	0.5 h	2 h	6 h	0.5 h	2 h	6 h	0.5 h	2 h	6 h
	ISOPROPANOL (b.p. 85°C)								
Lauric acid	1	1	1	1	1	2	14	14	14
Myristic acid	1	1	1	1	1	2	18	19	22
Palmitic acid	0	0	1	0	1	3	7	9	10
Stearic acid	0	0	1	0	1	3	7	8	8
Oleic acid	2	10	11	4	12	13	31	38	37
Linoleic acid	2	9	12	8	16	16	43	48	46
	ETHANOL (b.p. 80°C)								
Lauric acid	1	2	1	1	3	8	33	37	9
Myristic acid	1	1	1	1	7	10	7	10	11
Palmitic acid	1	0	1	1	1	1	2	8	7
Stearic acid	0	0	1	1	1	1	2	3	4
Oleic acid	5	13	16	15	22	26	58	85	84
Linoleic acid	7	16	17	23	29	31	64	87	88
	METHANOL (b.p. 65°C)								
Lauric acid	4	19	31	23	48	46	78	74	77
Myristic acid	1	8	12	6	24	28	51	64	73
Palmitic acid	1	3	8	3	15	21	34	27	33
Stearic acid	1	2	4	2	9	4	17	11	10
Oleic acid	34	41	46	39	58	69	66	77	77
Linoleic acid	37	39	47	45	61	73	72	81	82
	WATER (b.p. 100°C)								
Lauric acid	1	1	1	1	1	16	15	22	28
Myristic acid	1	1	1	1	1	8	9	13	17
Palmitic acid	0	0	0	0	1	1	1	2	5
Stearic acid	0	0	0	0	1	1	1	2	4
Oleic acid	1	2	8	3	8	14	38	51	65
Linoleic acid	1	3	10	5	10	18	50	57	71

[1]All analyses were conducted in duplicates at three different times (n = 3). The coefficient of variance (CV) between replicates ranged from 2% to 22% with a mean CV of 8%.

The aim of this experiment was to identify solvents and extraction conditions that would solubilise non-Ca bound fatty acid compounds but not solubilise Ca-fatty acid soaps. The use of a similar indirect approach to determine Ca-fatty acid soaps has been reported previously (Holt 1919; Toullec 1968; March and MacMillan 1979; Kaup *et al.* 1990). For example, March and MacMillan (1979) used diethyl ether extraction (Soxhlet method) in an attempt to remove the non-soap fatty acids of chicken excreta and measured the remaining soap fatty acids. However, based on the results of the present study many of the published indirect soap determination assays appear to have limitations. March and MacMillan (1979) used an extended Soxhlet extraction to extract non-soap fatty acids but the results given here suggest that extended extraction times (6 h and above, as commonly used for Soxhlet extraction) may reduce the actual amount of some Ca-fatty acid soaps (e.g. oleic acid soap) present in faeces. Another example of inaccuracies arising from application of the indirect approach of Ca-fatty acid determination by extracting non-Ca bound fatty acid components from the faeces would be the use of the Folch method (chloroform-methanol extraction) (Toullec 1968; Kaup *et al.* 1990). Results from the present study clearly showed that Ca-fatty acid soaps had the highest solubility when incubated with chloroform or methanol and that fat extraction with these solvents would lead to an underestimation of actual Ca-fatty acid complexes present in the faeces.

Identifying solvents with the lowest Ca-fatty acid soap solubility as performed in the presently reported study permits the extraction of non-Ca bound fatty acid material without co-extracting the Ca-fatty acid soaps. Within that context, the solvents hexane, petroleum ether and diethyl ether were deemed to be suitable for the determination of Ca-fatty acid soaps comprised of saturated medium chain fatty acids (lauric and myristic acid) at all

extraction temperatures tested, while the solvents isopropanol, hexane, petroleum ether and water may be suitable for the determination of Ca-fatty acid soaps comprised of unsaturated fatty acids (oleic and linoleic acid) if low incubation temperatures and incubation times shorter than 2 h are used. Finally, hexane, petrol ether, diethyl ether, isopropanol, ethanol and water all appeared to be suitable solvents for the determination of Ca-soaps comprising long chain saturated fatty acids (palmitic and stearic acid). Overall, hexane, a hexane/ isopropanol mixture and water were chosen for determining Ca-fatty acid soaps based on the Ca-fatty acid soap solubility. Further, hexane has been reported to be a suitable solvent for the extraction of free fatty acids and triglycerides (Zamora and Hidalgo 2004), mixtures of isopropanol and non-polar solvents have been reported to be suitable for extracting polar lipids (phospholipids and sphingolipids) (Guckert and White 1988; Markham *et al.* 2006), and water has been reported to solubilise ionised fatty acid salts (Bartley 1989). The latter three solvents therefore formed the basis of the three-step method developed here to determine Ca-fatty acid soaps in faeces, a method which relies on the removal of non-fatty acid compounds by extracting any fatty acid containing material that would interfere with the quantitative determination of fatty acids present as Ca-fatty acid soaps.

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.

In order to determine Ca-fatty acid soaps in faeces, a three-step sequential extraction method was designed whereby non-Ca bound fatty acid compounds were extracted leaving the Ca-fatty acid soaps in the faeces which were then determined using fatty acid analysis. The three consecutive steps included: Step 1 extraction with hexane to remove free fatty acids; Step 2, extraction with hexane-isopropanol (3:2) to remove neutral fats such as phospholipids; Step 3

aqueous extraction to solubilise Na-soaps. In this experiment the efficacy of the latter approach was tested using model faecal samples containing enriched samples of free fatty acids, phospholipids, Na-fatty acid salts and Ca-fatty acid soaps. The tested fatty acids, lauric, stearic and oleic acid, were chosen to represent saturated medium chain fatty acids, saturated long chain fatty acids and unsaturated long chain fatty acids, respectively. The phospholipids used were derived from egg yolk and soyabean, and contained 29% palmitic acid, 8% stearic acid, 16% oleic acid 44% linoleic acid and 3% α-linolenic acid. Table 3-4 and Table 3-5 summarize the recoveries of the fatty acids added to the fat-free, freeze dried faecal samples in the form of free fatty acids, Na-fatty acid salts or Ca-fatty acid soaps using the three sequential extraction steps, in either the individual solvents (Table 3-4) or for the spiked faecal sample overall (Table 3-5). Complete recovery of all three free fatty acids was observed after hexane extraction at 60°C for 10 min, whereas hexane extraction at room temperature led to a lower recovery (27%) of stearic acid. Successive extraction with hexane and then hexane-isopropanol at room temperature recovered 70% of the free stearic acid (Table 3-4) with 30% remaining unrecovered in the faecal sample (Table 3-5). Moreover, free lauric and oleic acids were completely removed from the faecal sample after hexane and then hexane-isopropanol extraction at room temperature (Table 3-4). The recovery of the sodium salts (Na-laureate, Na-stearate and Na-oleate) when extracted with hexane followed by hexane-isopropanol were very low (<5.2%) regardless of the extraction time and temperature used. In contrast, when extracted with water at either room temperature or at 80°C for 30 min the recoveries for Na-fatty acid salts were generally greater than 90%. The exception was Na-stearate for which the extraction recovery was low (8.2%) when extracted with water at room temperature for 30 min (Table 3-4). More than 90% of stearic acid added as Na-stearate was recovered from the spiked faeces (Table 3-5).

The Ca-fatty acid soaps of lauric acid, stearic acid and oleic acid were insoluble and therefore poorly extracted using hexane at either temperature (mean recovery across all Ca-fatty acid soaps and temperatures was 2.6%), hexane followed by hexane-isopropanol at either temperature (mean recovery across all Ca-fatty acid soaps and temperatures was 2.1%) and hexane followed by hexane-isopropanol and then water extraction at low temperature for 30 min (mean recovery across all Ca-fatty acid soaps was 0.4%). However, when extracted with water at 80°C for 30 min the recoveries of lauric and oleic acid from the added Ca-fatty acid soaps were much higher, being 18 and 63%, respectively (Table 3-4). The extraction recoveries observed for the Ca-fatty acid soaps were consistent with the solubility results presented in Table 3-3. Satisfying recoveries of lauric and oleic acid from spiked faeces with Ca-lauric or oleic acid soap were only achieved when extractions were performed at room temperature (97.5 and 92.8% for lauric and oleic acid, respectively). Extractions at elevated temperature lowered the recovery of lauric acid from Ca-lauric acid soap to 77.9% and that of oleic acid from Ca-oleic acid soap to 20.9% (Table 3-5).

Phospholipids were partially extracted in hexane with the remaining phospholipids being completely removed with the hexane-isopropanol extraction (Table 3-4). Since phospholipids may have an emulsifying effect on Ca-fatty acid soaps and therefore increase their solubility, the interactive effects between phospholipids and free fatty acids, Na-fatty acid salts or Ca-fatty acid soaps were investigated using spiked samples containing all of the latter fatty acid components together with the phospholipids (data not shown). Overall, the recovery of the Ca-fatty acid soaps remaining after the non-Ca-bound fatty acids were extracted was similar whether phospholipids were present or not (mean recovery across fatty acids,

extraction time and temperature combination in the presence or absence of phospholipids differed by less than 7%).

It is clear that for the sequential extraction methods tested, the selection of the extraction time and temperature combination is important to remove non-Ca fatty acid compounds while leaving the Ca-fatty acid soaps behind. This is particularly notable for free stearic acid and Na-stearate, for which quantitative extraction required higher extraction temperatures during the organic solvent extractions and the aqueous extraction, respectively. In contrast, when carrying out the aqueous extraction step at 80°C for 30 min appreciable amounts of Ca-oleic and Ca-lauric acid soaps were removed. Furthermore, 15% of Ca-oleic acid soap was lost during the incubation steps with the organic solvents at elevated temperatures. Overall, it appears that different extraction conditions are required depending on the Ca-fatty acid soap present. More specifically, to determine Ca-fatty acid soaps of saturated long-chain fatty acids extraction of non-Ca bound fatty acids was best achieved using hexane extraction for 10 min at 60°C, followed by hexane-isopropanol extraction at 37°C for 30 min and an aqueous extraction for 30 min at 80°C. For determining Ca-fatty acid soaps containing unsaturated fatty acids and saturated medium-chain fatty acids, using hexane extraction for 10 min at room temperature, followed by hexane-isopropanol extraction at room temperature for 30 min and an aqueous extraction for 30 min at room temperature was optimal.

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.

		mg FA[01]	Recovery of FA[0] in extraction solvents % ± SEM[02]			Recovery of FA[0] in extraction solvents % ± SEM[02]		
			Sequential extraction at RT[0] (A)			Sequential extraction at elevated temp. (B)		
	Extraction solvents:		Hexane[3] RT[0]/10'	Hexane-isopropanol[4] RT[0]/2x15'	Water[5] RT[0]/30'	Hexane[3] 60°C/10'	Hexane-isopropanol[4] 37°C/2x15'	Water[5] 80°C/30'
Model sample 1[6]								
Ca	lauric acid	2.96 ± 0.06	2.3 ± 0.67	0.2 ± 0.04	0.1 ± 0.27	3.7 ± 0.64	1.3 ± 0.68	15.7 ± 3.01
Na	stearic acid	2.87 ± 0.03	ND[10]	ND	8.3 ± 1.13	0.6 ± 0.27	1.1 ± 0.43	91.8 ± 1.67
free	oleic acid	3.53 ± 0.15	99.1 ± 0.16	ND	ND	99.0 ± 0.05	ND	ND
Model sample 2[7]								
Ca	stearic acid	2.87 ± 0.05	ND	ND	ND	ND	0.6 ± 0.08	ND
Na	oleic acid	2.96 ± 0.06	1.9 ± 0.15	5.2 ± 0.54	92.0 ± 9.28	5.1 ± 1.19	4.8 ± 0.34	90.4 ± 3.12
free	lauric acid	3.03 ± 0.02	98.8 ± 0.28	0.9 ± 0.11	ND	99.6 ± 0.07	ND	ND
Model sample 3[8]								
Ca	oleic acid	2.84 ± 0.02	1.4 ± 0.20	2.1 ± 0.54	1.2 ± 0.11	8.2 ± 0.48	6.8 ± 0.42	62.6 ± 1.56
Na	lauric acid	3.02 ± 0.05	ND	0.4 ± 0.12	98.8 ± 0.27	ND	2.7 ± 0.57	96.4 ± 4.12
free	stearic acid	2.92 ± 0.04	27.3 ± 1.12	41.6 ± 0.48	ND	99.4 ± 0.04	ND	ND
Model sample 4[9]								
FA of PL[0]		2.28 ± 0.07	16.4 ± 1.28	82.8 ± 3.94	ND	39.8 ± 2.32	58.9 ± 2.89	ND
palmitic acid		0.66 ± 0.09	14.8 ± 1.34	84.7 ± 3.98	ND	38.2 ± 2.84	60.3 ± 3.28	ND
stearic acid		0.18 ± 0.06	13.7 ± 0.22	85.2 ± 1.57	ND	37.7 ± 1.72	61.4 ± 2.17	ND
oleic acid		0.36 ± 0.06	16.9 ± 1.25	82.3 ± 2.85	ND	40.6 ± 2.02	57.9 ± 2.35	ND
linoleic acid		1.01 ± 0.07	18.8 ± 1.37	80.2 ± 4.08	ND	42.0 ± 2.79	56.8 ± 3.15	ND
α-linoleic acid		0.07 ± 0.01	17.9 ± 1.08	81.8 ± 1.62	ND	40.3 ± 1.91	58.4 ± 1.94	ND

[0]FA – fatty acids, SEM – standard error of the mean, RT – room temperature, PL – phospholipids
[1]Calculated amount of fatty acids (FA) present in the faeces as either Ca-FA soap, Na-FA salt or free fatty acid.
[2]Standard error of the mean; the analyses were conducted in duplicates at three different times (n=3).
[3]Hexane extraction was performed with 3 mL hexane at room temperature (A) or at 60°C (B) for 10 min.
[4]Hexane-isopropanol extraction was performed with 3 mL solvent mix of hexane-isopropanol (3:2) at RT (A) or at 37°C (B) twice for 15 min.
[5]Aqueous extraction was performed with 6 mL distilled water for 30 min at either RT (A) or 80°C (B).
[6]Sample 1: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-lauric acid soap, Na-stearate and free oleic acid.
[7]Sample 2: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-stearic acid soap, Na-oleate and fee lauric acid.
[8]Sample 3: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-oleic acid soap, Na-laureate and free stearic acid.
[9]Sample 4: 50 mg fat free, freeze dried faeces were spiked with approximately 3 mg of phospholipids (PL).
[10]ND – not detected

Table 3-5: Mean recovery (± 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.

		mg FA[01] added	Recovery of FA from added material in faeces after extractions at RT[2] (A)	Recovery of FA from added material in faeces after extractions at elevated temp.[2] (B)
Model sample 1[3]				
Ca	lauric acid	2.96 ± 0.06	97.5 ± 0.42	77.9 ± 1.86
Na	stearic acid	2.87 ± 0.03	90.9 ± 0.98	5.8 ± 0.61
free	oleic acid	3.53 ± 0.15	ND[7]	ND
Model sample 2[4]				
Ca	stearic acid	2.87 ± 0.05	99.4 ± 0.29	99.2 ± 0.17
Na	oleic acid	2.96 ± 0.06	ND	ND
free	lauric acid	3.03 ± 0.02	ND	ND
Model sample 3[5]				
Ca	oleic acid	2.84 ± 0.02	92.8 ± 2.17	20.9 ± 2.60
Na	lauric acid	3.02 ± 0.05	0.1 ±± 0.05	ND
free	stearic acid	2.92 ± 0.04	30.8 ± 1.00	ND
Model sample 4[6]				
FA of PL[0]		2.28 ± 0.07	ND	ND
palmitic acid		0.66 ± 0.09	ND	ND
stearic acid		0.18 ± 0.06	ND	ND
oleic acid		0.36 ± 0.06	ND	ND
linoleic acid		1.01 ± 0.07	ND	ND
α-linoleic acid		0.07 ± 0.01	ND	ND

[0]FA – fatty acids, SEM – standard error of the mean, RT – room temperature, PL – phospholipids
[1]Calculated amount of fatty acids (FA) present in the faeces as either Ca-FA soap, Na-FA salt or free fatty acid.
[2]Determined amount of FA that remained in the faeces after the three-step solvent extraction;
desirable recoveries: 90-100% Ca-fatty acid soaps, 0-5% non-soap fatty acid components (e.g. free fatty acids, Na-fatty acid salts,
[3]Sample 1: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-lauric acid soap, Na-stearate and free oleic acid.
[4]Sample 2: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-stearic acid soap, Na-oleate and free lauric acid.
[5]Sample 3: 50 mg fat-free, freeze dried faeces were spiked with approximately 3 mg of Ca-oleic acid soap, Na-laureate and free stearic acid.
[6]Sample 4: 50 mg fat free, freeze dried faeces were spiked with approximately 3 mg of phospholipids (PL).
[7]ND - not detected

3.4.5. Limitations of the soap determination assay

While the three-step method developed here to determine Ca-fatty acid soaps in faeces appears to offer promise, there are some important limitations that must be considered. Firstly, the method is an indirect method whereby interfering compounds are removed leaving the compounds of interest. Consequently, if non-Ca-bound fatty acid compounds are present in faeces that have not been considered in the present study, then Ca-fatty acid soaps may be overestimated. Secondly, the behaviour of soaps comprising divalent cations other than Ca has not been considered. However, the amount of "other" divalent cation soaps in faeces is expected to be negligible since the amount of dietary divalent cations is low in comparison with that of dietary Ca.

3.5. Conclusion

A new method for determining Ca-fatty acid soaps in faeces has been developed. The method provides an indirect determination of Ca-fatty acid soaps by extracting non-Ca bound fatty acids using three different solvents in a sequential extraction procedure that gives almost complete recovery of Ca-fatty acid soaps to the exclusion of all other fatty acid containing compounds. This method is the first indirect method that has considered the presence of monovalent cation-fatty acid salts in faeces.

CHAPTER FOUR: Effect of Fatty Acids from Different Fat Sources and Dietary Calcium Concentration on Soap Formation in the Growing Pig.

4.1. Abstract

The effect of dietary calcium (Ca) concentration on the absorption of major nutritional long-chain fatty acids (palmitic, stearic, oleic and linoleic acid) from different fat sources was investigated. Growing pigs were fed purified diets containing four different Ca concentrations (0, 2, 4 and 6 g kg^{-1} diet) and four different fat sources (tallow, palmolein oil, soya bean oil and olive oil). Increasing dietary Ca concentration led to increased total faecal fatty acid excretion for all the major fatty acids investigated when diets contained tallow and palmolein oil as the major fat source. This effect was not seen for olive oil and soya bean oil. Stearic acid supported the highest faecal fatty acid excretion and extent of fatty acid-soap formation. Tallow, containing the highest amount of saturated fatty acids tested, decreased faecal Ca excretion compared to the oils, presumably due to Ca-fatty acid soap formation. The results support the hypothesis that Ca-fatty acid soap formation leads to decreased fat absorption in the gastrointestinal tract.

4.2. Introduction

A considerable amount of scientific evidence supports a relationship between dietary calcium (Ca) intake and body weight in adult humans, where increased dietary Ca intake leads to reduced body weight. Several observational studies (Davies *et al.* 2000; Zemel *et al.* 2000; Jacqmain *et al.* 2001) and some clinical trials (Zemel *et al.* 2004; 2005a; 2005b) have

reported a correlation between dietary Ca intake and body weight, body fat, and weight loss in humans.

Several possible mechanisms, not mutually exclusive, have been proposed to explain the effect of dietary Ca on weight loss and body composition (Major *et al.* 2008). Some of the proposed mechanisms are based on the role of calcitriol in Ca homeostasis, where increased levels of calcitriol in circulation, such as that occurring when a diet low in Ca is consumed, increase intracellular Ca levels (Zemel *et al.* 2000) and inhibit the expression of uncoupling protein 2 (UCP2) in adipocytes (Shi *et al.* 2002; Sun and Zemel 2004b) resulting in greater lipid accumulation. High dietary Ca intake leads to suppressed calcitriol levels, which in turn prevent increased intracellular Ca levels and increase UCP2 expression which leads to inhibition of lipogenesis and stimulation of lipolysis, promoted adipocyte apoptosis and increased fatty acid oxidation. It has also been suggested that an inadequate dietary Ca intake negatively influences appetite control (Major *et al.* 2009). Yet another possible mechanism is the formation of indigestible Ca-fatty acid soaps within the gastrointestinal tract leading to reduced fat absorption (Drenick 1961). Fat is rich in calories and a reduced uptake of fat has a disproportionate effect upon calorie uptake.

Studies in rats and humans have shown that an increase in dietary Ca intake, either from dairy products or Ca supplements, leads to higher faecal fatty acid excretion (Saunders *et al.* 1988; Denke *et al.* 1993; Govers *et al.* 1996; Shahkhalili *et al.* 2001a; Bendsen *et al.* 2008). There is some evidence from both *in vitro* and *in vivo* studies (Gacs and Barltrop 1977; Graham and Sackman 1983) suggesting that this effect is more pronounced for long chain saturated fatty acids (Denke *et al.* 1993; Brink *et al.* 1995; Shahkhalili *et al.* 2001a) due to a lower solubility of saturated long-chain Ca-fatty acid soaps. More specifically, *in vitro* studies have shown that

the solubility of synthetic Ca-fatty acid soaps in simulated intestinal fluids is lower for the saturated long-chain fatty acid stearic acid when compared to shorter Ca-fatty acid soaps and soaps containing unsaturated fatty acids (Gacs and Barltrop 1977; Graham and Sackman 1983). Furthermore, in *in vivo* studies, where Ca-fatty acid soaps were infused into externalised duodenal loops of the rat, it has been shown that absorption is low for synthetic Ca-stearic acid soap, but increases with decreased chain length and decreased degree of saturation of the fatty acids present in the Ca-soaps (Gacs and Barltrop 1977). Moreover, fatty acids in the sn-1 and sn-3 positions of triglycerides may also be affected to a greater degree for soap formation compared to those in the sn-2 position (Mattson *et al.* 1979; Brink *et al.* 1995), since hydrolysis of triglycerides by pancreatic lipase in the gastrointestinal tract is specific for fatty acids in the sn-1 and sn-3 position.

The majority of studies that have reported increased faecal fatty acid output due to increased dietary Ca intake have made no attempt to determine the Ca-fatty acid soaps in the faeces. In general, total faecal fatty acid excretion was determined and the assumption made that the increase in faecal fat was due to soap formation. Furthermore, the impact of different dietary fats on soap formation *in vivo* has not been well explored. Consequently, the aim here was to investigate the effects of diets containing different dietary fat sources and varying Ca concentrations on the faecal output of fatty acids and fatty acid soaps using a newly developed assay (as described in Chapter Three) for determining fatty acid soaps in faecal samples.

4.3. Material & Methods

4.3.1. Materials

Extra Virgin olive oil, soya bean oil and palmolein oil were obtained from Davis Trading, Palmerston North, New Zealand. Tallow was obtained from JSB Wanganui, New Zealand. The Ca-free vitamin and mineral mix and Ca carbonate were purchased from Denver Stock Feeds, Palmerston North, New Zealand. All solvents and acids used for analysis were obtained from Merck, Darmstadt, Germany. Other chemicals for analysis were obtained from Sigma-Aldrich, St. Louis, MO.

4.3.2. Diets

Sixteen semi-synthetic diets varying in the fat source (tallow, palmolein oil, olive oil and soya bean oil) and in the concentration of Ca (0, 2, 4 and 6 g kg^{-1}) were prepared and stored at -18°C prior to use to minimise fat oxidation. The selected fat sources comprised mainly palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$), but they differed in the relative amounts of each of the latter fatty acids and their positional arrangement in the triglyceride molecule. The ingredient composition of the experimental diets is shown in Table 4-1 and the determined concentration of selected nutrients in the test diets is shown in Table 4-2. The diets met the nutrient requirements of the growing pigs for all nutrients except Ca in some of the diets (NRC, 2012) . The fat content of all diets was sufficient to supply 30% of the total energy requirement which is similar to the recommended fat intake for a typical western diet for humans. Titanium dioxide (TiO_2) was included in all the diets as an indigestible marker.

Table 4-1: Ingredient composition (g kg^{-1}air-dry weight) of the test diets fed to the growing pigs during the experimental period.

	Diet			
Ingredient	0% Ca	0.2% Ca	0.4% Ca	0.6% Ca
Wheat starch	517.7	510.3	502.8	495.4
Casein	177	177	177	177
Fat source[1]	130	130	130	130
Purified cellulose	51.4	51.4	51.4	51.4
Sucrose	105.9	105.9	105.9	105.9
Sodium chloride	3	3	3	3
Sodium phosphate	10	10	10	10
Calcium carbonate	-	7.4	14.9	22.3
Vit/Min mix[2]	2	2	2	2
Titanium dioxide	3	3	3	3

[1]The fat source consisted of either tallow, palmolein oil, olive oil or soya bean oil for each dietary Ca concentration respectively. The diets were formulated to contain 130 g kg^{-1} of fat.

[2]The vitamin/mineral mix was specially prepared to be devoid of Ca. The premix supplied per kg of diet: zinc (130 mg), iron (90 mg), manganese (45 mg), copper (10 mg), iodine (0.7 mg), selenium (0.3 mg), and cobalt (0.05 mg) , vitamin A ($10*10^6$ IU), vitamin D_3 ($2*10^6$ IU), vitamin E (30 mg), vitamin K (2 mg), vitamin B_1 (1.7 mg), vitamin B_2 (5 mg), vitamin B_6 (2.5 mg), vitamin B_{12} (0.03 mg), folic acid (0.6 mg), pantothenic acid (15 mg), biotin (0.01 mg), niacin (26 mg), choline (200 mg).

Table 4-2: Determined fatty acid, calcium and phosphorus contents (g kg^{-1} air dry weight) of the 16 experimental diets.

Fat source	Ca	Total fat	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	Ca	P
Olive oil	0	128	15.3	4.5	99.5	8.7	0.4	4.1
	2	132	15.2	4.5	99.7	9.7	2.0	3.8
	4	130	13.8	4.4	93.0	10.7	4.9	3.9
	6	132	14.1	4.3	94.2	11.7	6.6	4.0
Palmolein oil	0	130	46.5	4.9	54.4	14.9	0.1	4.0
	2	131	45.8	4.8	53.5	14.6	2.1	3.9
	4	119	44.9	4.7	51.9	14.1	5.7	4.0
	6	130	49.1	5.2	57.3	15.3	6.7	4.1
Soya bean oil	0	131	15.2	5.8	27.0	68.7	0.4	4.1
	2	131	14.9	5.7	26.7	67.5	2.4	4.0
	4	130	16.0	6.0	28.5	72.4	4.6	3.9
	6	134	16.0	5.8	28.2	71.8	6.5	4.0
Tallow	0	128	34.2	34.1	36.1	2.7	0.3	4.1
	2	136	36.0	36.5	38.5	2.8	3.0	4.2
	4	127	37.3	37.4	40.0	2.8	5.0	4.0
	6	128	33.1	33.7	35.6	2.4	7.7	3.9

4.3.3. Molecular distribution of fatty acids in the triglyceride molecule

The proportion of each fatty acid in the sn-2 and sn-1/3 positions of the glycerol backbone for the four fat sources was determined using pancreatic lipase hydrolysis as described by Luddy *et al.* (1964) with slight modifications. Briefly, 50 mg triglycerides were dispersed in 500 µL of 1 M tris hydroxymethyl-aminomethane (pH 8) plus 100 µL 22% $CaCl_2$ and 250 µL 0.1% bile salt solution using sonication at 40°C (10 sec sonication for the oils and 40 sec sonication for tallow). Pancreatic lipase was dissolved in 1 M tris hydroxymethyl-aminomethane (16 mg mL^{-1}) by mixing on a vortex and 500 µL were added to the triglyceride suspension. It has been reported that acyl migration (migration of the fatty acid from the sn-2 position to the sn-1/3 position) can occur during hydrolysis with pancreatic lipase.

Consequently, 1,3-dipalmitoyl-2-oleoylglycerol (POP) was used to quantify and correct for any fatty acid acyl migration that occurred when determining the positional distribution of the fatty acids. Thin layer chromatography (TLC) was used to isolate the lipolytic products after lipase hydrolysis. TLC was performed on Silica Gel 60 (F254 Aluminium sheets, 20 × 20 cm; Merck, Germany) using petroleum ether (boiling range 40–60°C) : diethyl ether : acetic acid (84.5:15:0.5) as the mobile phase. The different lipid fractions were visualized using iodine vapour. The band containing the monoglycerides was scraped off and collected into 12 cm screw cap tubes and then underwent methyl transesterification (section 4.3.6) to determine the fatty acids present in the sn-2 position. The amount of each fatty acid present in the sn-1 plus sn-3 positions was determined as the difference between the total amount of each fatty acid and the amount of each fatty acid present in the sn-2 position.

4.3.4. Animal trial

All procedures were approved by the Animal Ethics Committee, Massey University, New Zealand. One hundred and forty four entire male pigs (21.7 ± 0.15 kg body weight (mean ± SEM), PIC Camborough 46 sow x PIC boar 356 L, PIC New Zealand, Christchurch, New Zealand) were housed individually in metabolism crates in a room maintained at 21 ± 2°C with a 10 h/14 h light/dark cycle (Animal Physiology Unit, Massey University, Palmerston North, New Zealand). Tap water was available at all times. The Ca concentration of the tap water was determined to be 28.4 mg L^{-1} which represented approximately 1% of the daily Ca intake for the pigs receiving the 6 g Ca kg^{-1} diets. Animals were randomly assigned to the experimental diets such that there were nine pigs receiving each diet. The pigs underwent a five day acclimatization period during which time the pigs were fed a semi-synthetic basal diet which was the same as the experimental diets, except that the concentration of the basal

fat source (soya bean oil) was 100 g kg^{-1} and the dietary Ca concentration was 6 g kg^{-1}. At the outset of the study the pigs were weighed and feed intakes were calculated as 9% of metabolic body weight (0.09 x body $weight^{.75}$). The diets were freshly prepared as a slurry with water (approximately 50% w/w of diet) and given each day as three equal meals at 08:00 h, 12:00 h and 16:00 h. The experimental diets were then fed to the animals for a total of nine days. Faeces were collected on days seven to nine using ostomy bags as described by Rutherfurd *et al.* (2006). The faecal samples for each pig were pooled across sampling days, homogenized in a kitchen blender (Goldair) and freeze dried. The freeze dried samples were ground through a 1 mm mesh and stored at -18°C prior to analysis for fatty acids (either total fatty acids or those present as fatty acid soaps), Ca, P and titanium dioxide.

4.3.5. Determination of fatty acids in faeces present as soaps

The amount of faecal fatty acids present in the form of Ca-soaps was determined by the three step extraction protocol described in Chapter Three of this dissertation (see section 3.3.8). Ca-fatty acid soaps containing saturated and unsaturated fatty acids were determined separately via extraction of non-Ca bound fatty acid material at different temperatures due to the greater solubility of unsaturated Ca-fatty acid soaps in solvents at elevated temperature. Briefly, 50 mg of freeze dried faeces was weighed into screw cap tubes and 3 mL hexane was added to extract the non-polar lipids. The tubes were then mixed on a vortex and incubated at either 60°C for 10 min when determining the saturated fatty acids of the Ca-soaps or at room temperature (RT) for 10 min when determining the unsaturated fatty acids of the Ca-soaps. The tubes were centrifuged (1500 g, 5 min) and the supernatant, containing mainly free fatty acids and triglycerides, discarded. The hexane extraction step was repeated as described above. Thereafter, the precipitate was re-suspended in 3 mL of a

hexane-isopropanol (3:2) mixture to extract polar lipids, and incubation of 15 min at 37°C for determination of saturated Ca-fatty acid soaps as well as incubation at room temperature for determination of unsaturated Ca-fatty acid soaps followed. The material was then centrifuged, the supernatant discarded and the incubation step was repeated with another 3 mL of hexane-isopropanol mixture. The precipitate, which contained the fatty acid soaps, was dried under a flow of nitrogen to remove any remaining solvent. Any sodium or potassium fatty acid salts which may have formed during transit through the gastrointestinal tract were removed by aqueous filtration. Saturated Na- or K-fatty acid salts were separated from saturated Ca-fatty acid soaps by heating the tube content to 80°C with 6 mL distilled water for 30 min followed by a quick filtration under vacuum. For the determination of unsaturated Ca-fatty acid soaps room temperature was sufficient to dissolve unsaturated Na- or K-fatty acid salts. After filtration the filter discs containing faecal material including Ca-fatty acid soaps were dried in a desiccator and transferred to 12 cm screw cap tubes for methyl transesterification.

4.3.6. Fatty acid analysis

The fatty acid content of the diets and faeces (total fatty acids and soap-fatty acids) were determined using the saponification method as described by Zhu *et al.* (2011) with slight modifications. The modifications included sodium hydroxide being used in place of potassium hydroxide and the incubation temperature was 65°C instead of 55°C. Methyl-nonadecanoate ($C_{19:0}$, Fluka Sigma-Aldrich, Steinheim, Germany) in n-hexane served as an internal standard. Once saponification was complete, the hexane layer containing the fatty acid methyl esters (FAMEs) was transferred into glass tubes, evaporated under a stream of nitrogen and the FAMEs were re-dissolved in 1 mL hexane. The FAMEs were separated by

capillary gas chromatography (GC) on a Supelcowax® 10 (30 m x 0.20 mm x 0.20 μm) fused silica capillary column installed in an Agilent Technologies 7890A gas chromatograph equipped with a flame ionisation detector and split/splitless injector (Agilent Technologies, Victoria, Australia).

4.3.7. Ca and P analysis

The Ca and P content of the diets and the faecal samples were determined colorimetrically following sample preparation as described by AOAC 968.08D (2005). Ca was determined using the *o*-cresolphthalein complexone method on the Vitalab Flexor (Vital scientific, Netherlands) and P was determined on the spectrophotometer (Genesys 10uv, Madison County, USA) at a wavelength of 680 nm after reaction with ammonium molybdate and amino-naphtol sulphonic reagent.

4.3.8. Trace element analysis

Analysis of copper (Cu), iron (Fe) and zinc (Zn) in the diets and the faecal samples was performed using inductively coupled plasma mass spectrometry (ICP-MS). 100 mg of sample underwent nitric/hydrochloric acid digestion prior to dilution and analysis on an ICP-mass spectrometer (Hills Laboratory).

4.3.9. TiO_2 analysis

The TiO_2 content of the diets and faeces was determined based on the method of Short *et al.* (1996). Briefly, 100 to 500 mg of freeze dried faeces or diet was weighed into 100 mL glass beakers. Samples were ashed in a furnace overnight prior to digestion with H_2SO_4 (7.4 M) for 1.5 h at 210°C on a heating block. After cooling the samples were transferred to 10 mL volumetric flasks and the volume was adjusted with distilled water. Aliquots (1 to 4 mL)

were transferred to test tubes containing 1 mL hydrogen peroxide (H_2O_2); the volume was topped up to 5 mL with distilled water. The colourimetrical reaction between the acidic Ti(IV) and H_2O_2 was determined on a spectrophotometer (Genesys 10uv, Madison County, USA).

4.3.10. Data analysis

The flow of a compound as referred to here, is defined as the amount of the compound (fatty acid or mineral) present in faeces normalised for the dietary food intake, based on the ratio of TiO_2 in the diet to TiO_2 in faeces. The units of flow are mg kg^{-1} DMI, where DMI is dry matter intake.

Faecal flow of palmitic, stearic, oleic and linoleic acids, Ca, P, Zn, Fe and Cu were calculated as follows:

Faecal compound flow (mg kg^{-1} DMI) =

Faecal compound content (mg kg^{-1} DM) x dietary TiO_2 (mg kg^{-1} DM) /
faecal TiO_2 (mg kg^{-1} DM)

Apparent compound digestibility (%) was calculated using the following equation:

Apparent faecal compound digestibility (%) =

(Dietary compound flow (mg kg^{-1} DMI) - faecal compound flow (mg kg^{-1} DMI)) /
dietary compound flow (mg kg^{-1} DMI) x 100

Endogenous faecal Ca in the pig was predicted using a linear regression model where the total faecal Ca was plotted against the determined dietary Ca concentration where the y- intercept was endogenous faecal Ca. The endogenous faecal Ca values were used to calculate the true faecal Ca digestibility.

True faecal Ca digestibility (%) =

(Dietary Ca flow (mg g^{-1} DMI) – (faecal Ca flow (mg g^{-1} DMI) – endogenous faecal Ca flow (mg g^{-1} DMI))) / dietary Ca flow (mg g^{-1} DMI) x 100

4.3.11. Statistical analyses

All statistical analyses were performed using the PROC MIXED procedure of the Statistical Analysis Software (SAS/STAT version 9.2, 2008, SAS Institute Inc., Cary, NC, USA). A two-way analysis of variance (ANOVA) was conducted to test the effects of the dietary fat source, dietary Ca concentration and interaction between fat source and Ca concentration in a factorial arrangement (4x4) for a completely randomised design, with the pig as the experimental unit. The normal distribution of the residuals for each variable was tested using the PROC UNIVARIATE and the plot options of the ODS GRAPHS in SAS. When the residuals were not normally distributed, the data were transformed to achieve a normal distribution and the fitted mean values were back transformed. When the F-value of the ANOVA was significant ($P < 0.05$), the means were compared using the least significant difference (LSD) test. Orthogonal contrasts using the Tukey-Kramer t-test were conducted where appropriate.

4.4. Results

4.4.1. Molecular distribution of fatty acids in the triglyceride molecule

The four fat sources were analysed for the total amount of each fatty acid and the sn distribution of each fatty acid within the triglyceride molecules. The results are shown in Table 4-3. Based on the pancreatic lipase hydrolysis of 1,3-dipalmitoyl-2-oleoylglycerol, acyl migration appeared to be negligible (data not shown). Consequently, the method used here

was considered to be suitable for determining the molecular fatty acid distribution in the fat sources.

Tallow, the most saturated fat source used here contained three major fatty acids, palmitic, stearic and oleic acid, each comprising approximately 30% of the total fatty acids, with the saturated fatty acids predominately in the sn-1/3 position (40% stearic acid sn-1/3 and 30% palmitic acid sn-1/3). In contrast, olive oil and soya bean oil contained more than 70% of unsaturated fatty acids in the sn-1/3 position (Table 4-3).

Table 4-3: Fatty acids (FA) and their positional distribution in tallow, palmolein oil, olive oil and soya bean oil.

	Fatty acids (FA) (mol%)[1]						
Fat or oil	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	other
Tallow							
total[2]	3.6	28.0	28.6	30.8	1.2	0.7	7.2
sn-2[3]	1.4	20.6	8.9	61.6	0.3	1.2	6
sn-1/3[4]	4.7	31.7	38.4	15.3	1.7	0.4	7.8
Palmolein oil							
total[2]	0.9	38.4	3.3	45.0	11.1	tr	1.2
sn-2[3]	tr[5]	10.8	0.2	70.1	18.1	-	0.8
sn-1/3[4]	1.3	52.2	4.9	32.5	7.6	-	1.5
Olive oil							
total[2]	tr	10.6	3.1	78.3	5.3	tr	2.2
sn-2[3]	-	4.2	0.0	82.2	12.6	-	0.5
sn-1/3[4]	-	13.8	4.7	76.4	1.6	-	3.1
Soya bean oil							
total[2]	tr	11.4	4.3	21.4	55.1	6.3	1.4
sn-2[3]	-	1.4	0.2	22.9	68.5	6.8	0.2
sn-1/3[4]	-	16.4	6.3	20.7	48.4	6.1	2.0

[1]Each line presents the mol% of each fatty acid in the total triglyceride, the sn-2 position or sn-1/3 position. Lines add up to 100%; columns do not sum to 100%.
[2]total fatty acids present in triacylglycerol.
[3]fatty acids present as monoglycerides were determined after hydrolysis with pancreatic lipase which is specific for fatty acids in the sn-1/3 position.
[4]The proportion of each fatty acid in the sn-1 and 3 positions were calculated using the formula [FA in position sn-1/3 (mol%)] = (3 x [FA in triacylglycerol (mol%)] - [FA in position sn-2 (mol%)])/2
[5]trace amounts (< 0.05)

4.4.2. Animal trial

Faecal samples were obtained from most pigs during the collection period. However, a small number of pigs experienced loose stools during the collection period, probably due to the higher fat content than their normal diet (130 vs 50 g kg^{-1}), and for these pigs faecal samples were not included in the analysis.

4.4.3. Total faecal fatty acids and soap-fatty acids

Fatty acids were observed in the faeces of the pigs for all of the diets. Faecal fatty acids increased with increasing dietary Ca concentration for pigs receiving palmolein oil- and tallow-based diets but remained unchanged for the olive oil- and soya bean oil-based diets. The amount of fatty acid-soaps excreted with the faeces for the Ca-unsupplemented diets was between 35 and 60% of the total fatty acid excretion depending on the fatty acid. The highest total faecal fatty acid flows for all of the determined fatty acids ($P < 0.05$) were observed for pigs receiving the tallow-based diets (Tables 4-4 to 4-7). The total faecal fatty acid flows for pigs receiving the tallow-based diets were more than 3-times ($P < 0.05$) higher compared to the palmolein oil-based diets and up to 8-times higher ($P < 0.05$) than olive or soya bean oil-based diets.

There was an interaction ($P < 0.001$) between dietary Ca concentration and fat source for the faecal total palmitic acid and faecal soap-palmitic acid flows. Faecal total palmitic acid flows and faecal soap-palmitic acid flows increased ($P < 0.05$) with increasing dietary Ca concentration for pigs fed the tallow- and palmolein oil-based diets but not for the pigs fed the olive oil- or soya bean oil-based diets (Table 4-4). The highest excretion of total palmitic acid and soap-palmitic acid was observed for the tallow-based diet containing 6 g kg^{-1} Ca

with a total palmitic acid flow of 5.5 g kg^{-1} DMI and a soap-palmitic acid flow of 4.6 g kg^{-1} DMI. The faecal flows of palmitic acid across the tallow-based diets were 36% higher ($P < 0.05$) than for the palmolein oil-based diets, even though the palmitic acid content of tallow was 30% lower than that in palmolein oil (Table 4-4). The proportion of total palmitic acid present as soap increased from approximately 60 to 84% across the tallow- and palmolein oil-based diets as the dietary Ca concentration increased from 0 to 6 g kg^{-1} DMI. No increase ($P > 0.05$) in the flow of faecal soap-palmitic acid was observed for the pigs receiving the olive oil or soya bean oil-based diets. Approximately half of the total faecal palmitic acid was present in soaps for the olive oil- and soya bean oil-based diets.

There was an interaction ($P < 0.001$) between dietary Ca concentration and fat source for the faecal total stearic acid and faecal soap-stearic acid flows. Faecal stearic acid and faecal soap-stearic acid flows increased ($P < 0.05$) with increasing dietary Ca concentration for pigs fed the tallow-based diet only (Table 4-5). No equivalent effect was observed for the other fat sources. However, even though there was no increase in total faecal stearic acid for the latter fat sources the proportion of stearic acid present in soap in the faeces increased from approximately 60% when no Ca was added to the diet to almost 80% when the highest concentration of Ca was fed ($P < 0.05$; data not shown), suggesting that with increasing dietary Ca concentrations an increased amount of Ca-stearic acid soap formation occurred even though it did not affect the overall fat excretion.

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[3]
	Dietary total palmitic acid concentration (g kg^{-1} DM ± SE)[5]				
	35.2 ± 0.93	50.6 ± 0.93	15.9 ± 0.41	17.0 ± 0.33	
	Dietary sn-1/3 palmitic acid concentration (g kg^{-1} DM ± SE)[6]				
	26.1 ± 1.05	46.2 ± 1.13	12.8 ± 0.35	15.3 ± 0.77	
	Faecal total palmitic acid (mg kg^{-1} DMI)				
0	1170 (3.1)^{cx}	905 (3.0)^{dx}	422 (2.6)^{ay}	502 (2.7)^{ay}	(0.06)
2	3171 (3.5)^{bx}	1476 (3.2)^{cy}	474 (2.7)^{az}	509 (2.7)^{az}	(0.06)
4	2995 (3.5)^{bx}	2209 (3.3)^{bx}	431 (2.6)^{ay}	499 (2.7)^{ay}	(0.06)
6	5530 (3.7)^{ax}	3640 (3.6)^{ay}	409 (2.6)^{az}	521 (2.7)^{az}	(0.06)
Overall SE[4]	(0.06)	(0.06)	(0.06)	(0.06)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		
	Faecal palmitic acid present as soap (mg kg^{-1} DMI)				
0	745 (2.9)^{cx}	504 (2.7)^{cx}	248 (2.4)^{ax}	321 (2.5)^{ax}	(0.06)
2	1971 (3.3)^{bx}	1062 (3.0)^{cby}	260 (2.4)^{az}	291 (2.5)^{az}	(0.06)
4	2462 (3.4)^{bx}	1730 (3.2)^{by}	231 (2.4)^{az}	242(2.4)^{az}	(0.06)
6	4599 (3.7)^{ax}	3102 (3.5)^{ay}	261 (2.4)^{az}	323(2.5)^{az}	(0.06)
Overall SE[4]	(0.06)	(0.06)	(0.06)	(0.06)	
Statistical analysis			Significance[6]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil, 0 g kg^{-1} Ca; Soya bean oil, 0, 2 and 6 g kg^{-1} Ca; Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Data are back-transferred values from log values (presented in brackets) as statistical analysis required log transformation to achieve normal distribution.
[3]Overall standard error (SE) of the mean log data across fat sources
[4]Overall standard error (SE) of the mean log data across dietary Ca concentration
[5]mean values ± SE of total fatty acid on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[6]determined based on hydrolysis using a sn-1/3 specific lipase.
[7] *** $P < 0.001$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[3]
	Dietary total stearic acid concentration (g kg^{-1} DM ± SE)[5]				
	35.4 ± 0.91	5.3 ± 0.10	4.8 ± 0.06	6.4 ± 0.08	
	Dietary sn-1/3 stearic acid concentration (g kg^{-1} DM ± SE)[6]				
	31.9 ± 1.11	5.2 ± 0.34	4.8 ± 0.14	6.3 ± 0.21	
	Faecal total stearic acid (mg kg^{-1} DMI)				
0	2749 (3.4)^{cx}	904 (3.0)^{az}	1258 (3.1)^{ayz}	1468 (3.2)^{ay}	(0.07)
2	7022 (3.8)^{bx}	848 (2.9)^{az}	1450 (3.2)^{ay}	1279 (3.1)^{ayz}	(0.07)
4	6417 (3.8)^{bx}	853 (2.9)^{az}	1227 (3.1)^{ayz}	1322 (3.1)^{ay}	(0.07)
6	11114 (4.0)^{ax}	1027 (3.0)^{ay}	1116 (3.0)^{ay}	1337 (3.1)^{ay}	(0.07)
Overall SE[4]	(0.07)	(0.07)	(0.06)	(0.07)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			*		
Fat source*Ca concentration			**		
	Faecal stearic acid present as soap (mg kg^{-1} DMI)				
0	2015 (3.3)^{cx}	512 (2.7)^{az}	806 (2.9)^{az}	1027 (3.0)^{ay}	(0.07)
2	5089 (3.7)^{bx}	580 (2.8)^{aby}	1037 (3.0)^{ay}	888 (2.9)^{ay}	(0.07)
4	5687 (3.8)^{bx}	632 (2.8)^{aby}	875 (2.9)^{ay}	755 (2.9)^{ay}	(0.07)
6	9817 (4.0)^{ax}	828 (2.9)^{by}	834 (2.9)^{ay}	941 (3.0)^{ay}	(0.07)
Overall SE[4]	(0.07)	(0.07)	(0.07)	(0.07)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil, 0 g kg^{-1} Ca; Soya bean oil, 0, 2 and 6 g kg^{-1} Ca; Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Data are back-transferred values from log values (presented in brackets) as statistical analysis required log transformation to achieve normal distribution.
[3]Overall standard error (SE) of the mean log data across fat sources
[4]Overall standard error (SE) of the mean log data across dietary Ca concentration
[5]mean values ± SE of total fatty acid on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[6]determined based on hydrolysis using a sn-1/3 specific lipase.
[7] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

There was an interaction ($P < 0.001$) between dietary Ca concentration and fat source for the faecal total oleic acid and faecal soap-oleic acid flows. Faecal total oleic acid and faecal soap-oleic acid flows increased ($P < 0.05$) with increasing dietary Ca concentration for pigs fed the tallow- and palmolein oil-based diets but not for pigs fed the olive oil- or soya bean oil-based diets (Table 4-6). The lowest faecal flow of oleic acid was observed for the soya bean oil-based diets ($P < 0.05$). Dietary Ca concentrations of 4 and 6 g kg^{-1} led to a significantly higher total soap-oleic acid excretion for pigs receiving tallow- and palmolein oil-based diets compared to olive oil and soya bean oil-based diets. Oleic acid excreted in the faeces as soap was approximately 40% of the total faecal oleic acid for the pigs receiving olive oil-based diets. For the pigs fed the tallow- and palmolein oil-based diets containing 4 and 6 g kg^{-1} Ca, between 68-85% of the total oleic acid present in the faeces was in the form of soap.

There was an interaction ($P < 0.05$) between dietary Ca concentration and fat source for the faecal total linoleic acid and faecal soap-linoleic acid flows. Faecal total linoleic acid flows increased ($P < 0.05$) with increasing dietary Ca concentration for pigs fed the tallow- and palmolein oil-based diets but not for the pigs fed the olive oil- or soya bean oil-based diets (Table 4-7). Similarly, faecal soap-linoleic acid flow increased ($P < 0.05$) for the pigs fed the tallow- and the palmolein oil-based diets but no increase was observed for the pigs fed the olive oil- or soya bean oil-based diets (Table 4-7). When compared across all diets, total linoleic acid excretion was highest ($P < 0.05$) for the pigs receiving tallow-based diets containing 6 g kg^{-1} Ca.

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[3]
	Dietary total oleic acid concentration (g kg^{-1} DM ± SE)[5]				
	37.5 ± 1.04	59.0 ± 1.14	105.5 ± 1.84	30.2 ± 0.54	
	Dietary sn-1/3 oleic acid concentration (g kg^{-1} DM ± SE)[6]				
	12.6 ± 1.31	28.8 ± 0.94	71.0 ± 2.06	19.2 ± 0.47	
	Faecal total oleic acid (mg kg^{-1} DMI)				
0	84.8 (1.9)^{cxy}	60.9 (1.8)^{cxy}	109.8 (2.0)^{ax}	48.2 (1.7)^{ay}	(0.07)
2	263.3 (2.4)^{abx}	97.7 (2.0)^{by}	133.3 (2.1)^{ay}	50.7 (1.7)^{az}	(0.07)
4	173.5 (2.2)^{bx}	136.0 (2.1)^{bxy}	117.0 (2.1)^{ay}	62.9 (1.8)^{az}	(0.07)
6	338.8 (2.5)^{ax}	235.2 (2.4)^{axy}	165.5 (2.2)^{ay}	58.5 (1.8)^{az}	(0.07)
Overall SE[4]	(0.07)	(0.07)	(0.07)	(0.07)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		
	Faecal oleic acid present as soap (mg kg^{-1} DMI)				
0	48.1 (1.7)^{cx}	35.2 (1.5)^{cx}	48.5 (1.7)^{ax}	30.8 (1.5)^{ax}	(0.06)
2	69.6 (1.8)^{bcx}	66.2 (1.8)^{bcx}	57.9 (1.8)^{ax}	30.3 (1.5)^{ax}	(0.06)
4	117.1 (2.1)^{bx}	106.8 (2.0)^{bx}	48.2 (1.7)^{ay}	35.1 (1.5)^{ay}	(0.06)
6	213.5 (2.3)^{ax}	210.5 (2.3)^{ax}	68.5 (1.8)^{ay}	37.6 (1.6)^{ay}	(0.06)
Overall SE[4]	(0.06)	(0.06)	(0.06)	(0.06)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil, 0 g kg^{-1} Ca; Soya bean oil, 0, 2 and 6 g kg^{-1} Ca; Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Data are back-transferred values from log values (presented in brackets) as statistical analysis required log transformation to achieve normal distribution.
[3]Overall standard error (SE) of the mean log data across fat sources
[4]Overall standard error (SE) of the mean log data across dietary Ca concentration
[5]mean values ± SE of total fatty acid on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[6]determined based on hydrolysis using a sn-1/3 specific lipase.
[7] *** $P < 0.001$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[3]
	Dietary total linoleic acid concentration (g kg^{-1} DM ± SE)[5]				
	2.4 ± 0.01	16.0 ± 0.25	8.9± 0.70	76.8± 1.44	
	Dietary sn-1/3 linoleic acid concentration (g kg^{-1} DM ± SE)[6]				
	1.4± 0.01	6.7± 0.06	1.5± 0.04	45.0± 1.07	
	Faecal total linoleic acid (mg kg^{-1} DMI)				
0	31.0 (1.49)^{cx}	15.6 (1.19)^{by}	17.6 (1.25)^{ay}	33.1 (1.52)^{ax}	(0.066)
2	75.2 (1.88)^{abx}	20.5 (1.31)^{abz}	18.9 (1.28)^{az}	38.2 (1.58)^{ay}	(0.064)
4	59.8 (1.78)^{bx}	22.1 (1.34)^{aby}	16.7 (1.22)^{ay}	49.1 (1.69)^{ax}	(0.063)
6	104.5 (2.02)^{aw}	27.6 (1.44)^{ay}	15.8 (1.20)^{az}	44.7 (1.65)^{ax}	(0.064)
Overall SE[4]	(0.066)	(0.064)	(0.062)	(0.065)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			*		
	Faecal linoleic acid present as soap (mg kg^{-1} DMI)				
0	18.9 (1.28)^{cx}	7.1 (0.85)^{ay}	7.2 (0.86)^{ay}	13.9 (1.14)^{ay}	(0.070)
2	42.6 (1.63)^{bx}	9.3 (0.97)^{ay}	6.1 (0.79)^{ay}	11.9 (1.08)^{ay}	(0.069)
4	45.7 (1.66)^{bx}	11.2 (1.05)^{aby}	5.7 (0.76)^{ay}	16.2 (1.21)^{ay}	(0.068)
6	83.0 (1.92)^{ax}	18.8 (1.27)^{by}	7.8 (0.89)^{ay}	16.0 (1.20)^{ay}	(0.069)
Overall SE[4]	(0.070)	(0.069)	(0.067)	(0.070)	
Statistical analysis			Significance[7]		
Fat source			***		
Ca concentration			***		
Fat source*Ca concentration			***		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil, 0 g kg^{-1} Ca; Soya bean oil, 0, 2 and 6 g kg^{-1} Ca; Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Data are back-transferred values from log values (presented in brackets) as statistical analysis required log transformation to achieve normal distribution.
[3]Overall standard error (SE) of the mean log data across fat sources
[4]Overall standard error (SE) of the mean log data across dietary Ca concentration
[5]mean values ± SE of total fatty acid on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[6]determined based on hydrolysis using a sn-1/3 specific lipase.
[7] *** $P < 0.001$; * $P < 0.05$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

4.4.4. Apparent faecal fatty acid digestibility

The digestibility of oleic and linoleic acid was in excess of 99% for almost all of the dietary treatments (data not shown) and while statistically significant ($P < 0.05$) inter-treatment differences were observed for the two unsaturated fatty acids the actual differences were small (1-5 %units). For palmitic acid and stearic acid, there was an interaction ($P < 0.01$) between dietary fat source and dietary Ca concentration on the apparent faecal digestibility of both fatty acids (Table 4-8 and Table 4-9). The mean apparent faecal digestibility of palmitic acid across all four fat sources in the Ca-free diets was approximately 97% (Table 4-8). Apparent faecal palmitic acid digestibility decreased ($P <0.05$) with increasing dietary Ca concentration (from 0 to 6 g of Ca kg^{-1} diet) for palmolein oil (5% decrease) and tallow (15% decrease) but not for olive oil or soya bean oil. Apparent faecal stearic acid digestibility was higher ($P < 0.05$) for the Ca-free tallow-based diet (91.4%) compared to the Ca-free diets containing either olive oil, palmolein oil or soya bean oil (digestibility ranged from 72 to 82%) (Table 4-9). For the three oil-based diets, the apparent faecal digestibility of stearic acid did not change ($P > 0.05$) regardless of the dietary Ca concentration. However for the tallow-based diet, apparent faecal stearic acid digestibility decreased ($P < 0.001$) by 28% when the dietary Ca concentration increased from 0 to 6 g kg^{-1} (Table 4-9).

Table 4-8: Mean (n = 9)[1] apparent faecal palmitic acid digestibility (%) for pigs receiving experimental diets differing in fat source and Ca concentration.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[2]
	Dietary total palmitic acid concentration (g kg^{-1} DM ± SE)[4]				
	35.2 ± 0.93	50.6 ± 0.93	15.9 ± 0.41	17.0 ± 0.33	
	Dietary sn-1/3 palmitic acid concentration (g kg^{-1} DM ± SE)[5]				
	26.1 ± 1.05	46.2 ± 1.13	12.8 ± 0.35	15.3 ± 0.77	
	Apparent faecal palmitic acid digestibility (%)				
0	96.4^{ax}	98.1^{ax}	97.4^{ax}	96.8^{ax}	0.45
2	90.9^{by}	96.9^{abx}	97.0^{ax}	96.8^{ax}	0.44
4	91.8^{bz}	95.4^{by}	97.1^{axy}	97.1^{ax}	0.44
6	82.5^{cz}	92.8^{cy}	97.2^{ax}	97.0^{ax}	0.45
Overall SE[3]	0.45	0.44	0.44	0.46	

Statistical analysis	Significance[6]
Fat source	***
Ca concentration	***
Fat source*Ca concentration	***

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil 0 g kg^{-1} Ca, Soya bean oil 0, 2 and 6 g kg^{-1} Ca, Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Overall standard error of the mean across fat sources
[3]Overall standard error of the mean across dietary Ca concentration.
[4]mean values ± SE of total fatty acids on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[5]determined based on hydrolysis using a sn-1/3 specific lipase
[6] *** $P < 0.001$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

Table 4-9: Mean (n = 9)[1] apparent faecal stearic acid digestibility (%) for pigs receiving experimental diets differing in fat source and Ca concentration.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[2]
	Dietary total stearic acid concentration (g kg^{-1} DM ± SE)[4]				
	35.4 ± 0.91	5.3 ± 0.10	4.8 ± 0.06	6.4 ± 0.08	
	Dietary sn-1/3 stearic acid concentration (g kg^{-1} DM ± SE)[5]				
	31.9 ± 1.11	5.2 ± 0.34	4.8 ± 0.14	6.3 ± 0.21	
	Apparent faecal stearic acid digestibility (%)				
0	91.4^{cx}	81.6^{az}	72.3^{ayz}	73.2^{ay}	1.89
2	80.4^{bx}	82.0^{az}	68.6^{ay}	77.9^{ayz}	1.86
4	82.4^{bx}	82.3^{az}	73.2^{ayz}	79.1^{ay}	1.86
6	65.9^{ax}	81.1^{ay}	74.2^{ay}	77.7^{ay}	1.89
Overall SE[3]	1.89	1.86	1.84	1.92	
Statistical analysis			Significance[6]		
Fat source			***		
Ca concentration			NS		
Fat source*Ca concentration			***		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil 0 g kg^{-1} Ca, Soya bean oil 0, 2 and 6 g kg^{-1} Ca, Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Overall standard error of the mean across fat sources
[3]Overall standard error of the mean across dietary Ca concentration.
[4]mean values ± SE of total fatty acids on the triglyceride (TAG) across diets with the same fat source but different Ca concentration.
[5]determined based on hydrolysis using a sn-1/3 specific lipase
[6] *** $P < 0.001$; NS – not significant $P > 0.05$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

4.4.5. Faecal Ca and faecal Ca digestibility

Faecal Ca increased ($P < 0.001$) with increasing dietary Ca concentration and also differed ($P < 0.01$) across dietary fat sources (Table 4-10). However, there was no significant ($P > 0.05$) interaction between dietary Ca concentration and dietary fat source. Faecal Ca excretion was higher ($P < 0.05$) in pigs fed the tallow-based diets compared with the olive oil, palmolein oil or soyabean oil-based diets. The faecal Ca flow increased linearly ($P < 0.001$, $r^2 = 0.71$) with increasing dietary Ca concentration.

The determined endogenous faecal Ca was 399.7 (± 36.80), 323.5 (± 40.06), 338.2 (± 51.02), and 364.3 (± 55.44) mg kg^{-1} DMI for the tallow-, palmolein, olive, and soya bean oil-based diets respectively and were not different ($P > 0.05$) across the dietary fat sources.

The fat source given to the pigs did not significantly ($P > 0.05$) affect apparent or true faecal Ca digestibility (Table 4-10). Moreover, apparent faecal Ca digestibility was not affected ($P > 0.05$) by the dietary Ca concentration but true faecal Ca digestibility was significantly lower for pigs receiving diets containing 6 g kg^{-1} Ca in comparison to diets containing 2 g kg^{-1} Ca.

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.

Diets		Faecal Ca (mg kg^{-1} DMI)	Apparent faecal Ca digestibility (%)	True faecal Ca digestibility[2] (%)
		Main Effects		
Fat source	Olive oil	1193^{b}	64.9	73.4
	Palmolein oil[1]	1425^{b}	61.6	69.4
	Soya bean oil[1]	1291^{b}	64.4	71.8
	Tallow[1]	2040^{a}	55.6	64.9
Overall SE[3]		130.1	2.90	2.85
Ca level (g kg^{-1})	0[1]	322^{d}		
	2[1]	1015^{c}	60.9	74.4^{a}
	4[1]	1870^{b}	64.1	70.7^{ab}
	6[1]	2742^{a}	59.8	64.5^{b}
Overall SE[4]		130.1	2.51	2.47
		Significance[5]		
Fat source		**	NS	NS
Ca concentration		***	NS	*
Fat source*Ca concentration		NS	NS	NS

[1]n<36, pigs with loose stools during the 72 h faeces collection period were excluded from analysis:
n=35 for palmolein oil; n=33 for soya bean oil; n=33 for tallow
n=33 for 0 g kg^{-1} Ca; n = 35 for 2 g kg^{-1} Ca; n=35 for 4 g kg^{-1} Ca; n= 34 for 6 g kg^{-1} Ca
[2]Endogenous faecal Ca used to correct apparent faecal Ca digestibility to true faecal Ca digestibility was determined using the regression method.
[3]Overall standard error of the mean across fat sources
[4]Overall standard error of the mean across dietary calcium concentration.
[5] *** P < 0.001; ** P < 0.01; * P < 0.05; NS – not significant P > 0.05
[a,b] Within a column, means without a common superscript differ (P < 0.05).

4.4.6. Faecal P and apparent faecal P digestibility

Faecal P increased ($P < 0.001$) with increasing dietary Ca concentration and also differed ($P < 0.05$) across dietary fat sources (Table 4-11). However, there was no interaction ($P > 0.05$) between dietary Ca concentration and dietary fat source. Faecal P was not different ($P > 0.05$) for the pigs fed the olive oil-, soya bean oil-, and tallow-based diets but was lower ($P < 0.05$) for the palmolein-based diets compared with the other three diets. Faecal P increased ($P < 0.001$) by 70% with increasing dietary Ca concentration from 0 to 6 g Ca kg^{-1} of diet. Apparent faecal P digestibility decreased ($P < 0.001$) by 5% units with increasing dietary Ca concentration and also differed ($P < 0.05$) across dietary fat sources, but there was no ($P > 0.05$) interaction between dietary Ca concentration and fat source. The apparent faecal phosphorus digestibility was approximately 90% across the fat sources. Although the digestibility of phosphorus from palmolein oil-based diets was statistically higher ($P < 0.05$) the actual differences were less than 2%.

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.

Diets		faecal P (mg kg^{-1} DMI)	Apparent faecal P digestibility (%)
		Main Effects	
Fat source	Olive oil	356[a]	90.8[ab]
	Palmolein oil	296[b]	92.3[a]
	Soya bean oil	376[a]	90.4[b]
	Tallow	377[a]	90.2[b]
Overall SE[2]		23.3	0.58
Ca level (g kg^{-1})	0	268[c]	93.2[a]
	2	356[b]	90.9[b]
	4	337[b]	91.3[b]
	6	453[a]	88.2[c]
Overall SE[3]		23.3	0.58
		Significance	
Fat source		*	*
Ca concentration		***	***
Fat source*Ca concentration		NS	NS

[1]n=<36, pigs with loose stools during the 72 h faeces collection period were excluded from analysis:
n=35 for palmolein oil; n=33 for soya bean oil; n=33 for tallow-based diets
n=33 for 0 g kg^{-1} Ca; n = 35 for 2 g kg^{-1} Ca; n=35 for 4 g kg^{-1} Ca; n= 34 for 6 g kg^{-1} Ca
[2]Overall standard error of the mean across fat sources
[3]Overall standard error of the mean across dietary calcium concentration.
[4] *** P < 0.001; ** P < 0.01; * P < 0.05; NS – not significant P > 0.05
[a,b] Within a column, means without a common superscript differ (P < 0.05).

4.4.7. Faecal trace minerals

An interaction between fat source and Ca concentration was observed for faecal Fe (P < 0.001) and faecal Zn (P < 0.05) (Table 4-12 and Table 4-13). Faecal Fe flow increased with increasing dietary Ca concentrations from approximately 70 to above 200 mg kg^{-1} DMI. A strong correlation (r^2 > 0.80; P < 0.001) between faecal Fe flow and dietary Ca concentration for each fat source was observed. Faecal Fe excretion (P < 0.01) differed significantly (P < 0.05) across fat source, being higher for pigs receiving tallow-based diets containing

2 and 6 mg Ca kg^{-1} DMI compared to the oil-based diets at equal dietary Ca concentration. Furthermore, a correlation between faecal Fe and faecal total fatty acids was observed when pigs were fed the tallow based diet ($r^2 = 0.58$; $P < 0.001$; n=33) but no correlation was reported for pigs receiving olive oil or soya bean oil. The correlation between faecal Fe and faecal total fatty acids was weaker but still significant when pigs were fed the palmolein oil-based diet ($r^2 = 0.42$; $P < 0.01$; n=35). For Zn, faecal excretion increased with increasing dietary Ca concentration for palmolein oil-based diets. Faecal Zn excretion was higher for pigs receiving 6 g Ca kg^{-1} tallow- and olive oil-based diets compared to the equivalent diets with no Ca added. The correlation between faecal Zn flow and dietary Ca concentration was strongest for palmolein oil-based diets ($r^2 = 0.77$; $P < 0.001$, n = 35), and less profound for olive oil-based diets ($r^2 = 0.41$; $P < 0.01$; n = 36) and tallow-based diets ($r^2 = 0.34$; $P < 0.05$; n = 33). Faecal Zn flows were highest for the tallow-based diet. Furthermore, faecal Zn increased by more than 60 mg kg^{-1} DMI when pigs received the tallow- or palmolein oil-based diets ($P < 0.001$) but only by approximately 30 mg kg^{-1} DMI for olive oil-based diets ($P < 0.05$) and by approximately 20 mg kg^{-1} DMI for soya bean oil-based diets ($P > 0.05$). A correlation between faecal Zn and faecal total fatty acids was observed in pigs receiving the tallow-based diet ($r^2 = 0.63$, $P < 0.001$; n = 33) and the palmolein oil-based diet ($r^2 = 0.47$; $P < 0.01$; n = 35), whereas no correlation was reported for pigs receiving olive oil- and soya bean oil-based diets. Faecal Cu was not affected ($P > 0.05$) by dietary fat source and dietary Ca concentrations and were on average 20 mg kg^{-1} DMI (data not shown).

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[2]
	Faecal Fe (mg kg^{-1} DMI)				
0	79.4^{cx}	62.9^{dx}	68.4^{dx}	83.3^{cx}	4.23
2	162.6^{bx}	122.1^{cy}	129.1^{cy}	133.8^{by}	4.12
4	155.7^{b}	153.9^{b}	165.8^{b}	170.4^{b}	4.17
6	237.3^{ax}	209.1^{ay}	202.3^{ay}	212.4^{ay}	4.29
Overall SE[3]	4.24	4.11	4.16	4.30	
Statistical analysis			Significance[4]		
Fat source			**		
Ca concentration			***		
Fat source*Ca concentration			*		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil 0 g kg^{-1} Ca, Soya bean oil 0, 2 and 6 g kg^{-1} Ca, Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Overall standard error of the mean across fat sources
[3]Overall standard error of the mean across dietary calcium concentration
[4] *** P < 0.001; ** P < 0.01; * P < 0.05
[a,b] Within a column, means without a common superscript differ (P < 0.05).
[x,y] Within a row, means without a common superscript differ (P < 0.05).

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.

Ca level (g kg^{-1})	Tallow	Palmolein oil	Olive oil	Soya bean oil	Overall SE[2]
	Faecal Zn (mg kg^{-1} DMI)				
0	171^{bx}	111^{cz}	131^{ay}	160^{x}	4.37
2	218^{ax}	145^{bz}	154^{abzy}	163^{y}	4.26
4	185^{bx}	162^{abxy}	156^{aby}	183^{x}	4.32
6	236^{ax}	174^{ay}	163^{by}	179^{y}	4.44
Overall SE[3]	4.38	4.25	4.30	4.45	
Statistical analysis			Significance[4]		
Fat source			***		
Ca concentration			*		
Fat source*Ca concentration			**		

[1]n=8, pigs with loose stools during the 72 h faeces collection period were excluded from analysis (Palmolein oil 0 g kg^{-1} Ca, Soya bean oil 0, 2 and 6 g kg^{-1} Ca, Tallow 0, 4 and 6 g kg^{-1} Ca).
[2]Overall standard error of the mean across fat sources
[3]Overall standard error of the mean across dietary calcium concentration
[4] *** P < 0.001; ** P < 0.01; * P < 0.05
[a,b] Within a column, means without a common superscript differ (P < 0.05).
[x,y] Within a row, means without a common superscript differ (P < 0.05).

4.5. Discussion

The findings demonstrate that increasing dietary Ca intake increased faecal fat output by reducing fatty acid absorption in the gastrointestinal tract due at least in part to the formation of Ca-fatty acid soaps. Increased faecal fatty acid excretion was particularly pronounced for the diets containing either palmolein oil or tallow, both sources containing high concentrations of saturated fatty acids. These latter findings were consistent with previous reports from the literature, showing that fats high in saturated fatty acids are more prone to Ca-fatty acid soap formation (Apgar *et al.* 1987).

Faecal fatty acid excretion was higher for Ca-supplemented diets than for the unsupplemented diets. For example, increasing the dietary Ca intake for pigs by 6 g kg^{-1} diet increased the total fatty acid content excreted with the faeces from 2.1 to 5.9 g kg^{-1} DMI when pigs received the palmolein oil-based diets and from 4.7 to 20.2 g kg^{-1} DMI when pigs received the tallow-based diets. These findings are consistent with previously reported studies which have investigated the effect of dietary Ca on faecal fat excretion in the rat (Westerlund 1934; Cheng *et al.* 1949; Tadayyon and Lutwak 1969b; Mattson *et al.* 1979; Brink *et al.* 1995; Papakonstantinou *et al.* 2003) and in humans (Saunders *et al.* 1988; Van der Meer *et al.* 1990; Denke *et al.* 1993; Welberg *et al.* 1994; Govers *et al.* 1996; Shahkhalili *et al.* 2001a; Jacobsen *et al.* 2005; Boon *et al.* 2007; Bendsen *et al.* 2008; Hjerpsted *et al.* 2011) and reported a significant increase of faecal fat excretion with increasing dietary Ca. The exceptions were the studies of Boon *et al.* (2007) and Hjerpsted *et al.* (2011) where no significant increase was observed. The lack of an effect of dietary Ca on faecal fat excretion in those latter two studies may have been the result of a higher dietary protein intake (> 15 E%) (Jacobsen *et al.* 2005)

which may have increased Ca absorption (Gaffney-Stomberg *et al.* 2010) thereby leaving less Ca to bind to fatty acids in the intestine.

Digestibility of the saturated fatty acids palmitic and stearic acid within the fat sources tallow and palmolein oil was noticeably reduced with increasing dietary Ca intake. Previous studies conducted in pigs receiving different sources of dietary fat (100 - 150 g kg^{-1} diet) at a dietary Ca concentration of 6 g kg^{-1} (or higher) reported similar findings to those reported here. For example, Duran-Montge *et al.* (2007) reported apparent faecal palmitic and stearic acid digestibility values in pigs receiving tallow-based diets of 84.7% and 64.1% respectively, which are very similar to the comparable values observed in the presently reported study (82.5% and 65.9% respectively). When Ca was not present in the diets, apparent faecal stearic acid digestibility was 10-20 % higher for the tallow-based diet as compared to the oil-based diets. Furthermore, when Ca was added to the diets the apparent stearic acid digestibility of the tallow diet decreased markedly but did not change for the oil-based diets. The low stearic acid digestibility for the oil-based diets may have been because the stearic acid concentration was very low for the oil-based diets and that the digestibility values were apparent digestibility values, and therefore not corrected for endogenous stearic acid concentrations. It is also possible that the low stearic acid values were due to microbial biohydrogenation of unsaturated fatty acids, as biohydrogenation has been shown to occur in the large intestine of the pig (Carlson and Bayley 1968; Jørgensen *et al.* 1992). For example, in the study of Jorgensen *et al.* (2000) stearic acid digestibility values were found to be negative and it was postulated that the microflora of the large intestine may have increased the saturated fatty acid levels in the faecal content at the expense of the unsaturated fatty acids .

The findings reported here provide a basis which supports the proposed Ca-fatty acid soap hypothesis to explain the impact of dietary Ca on body weight. Overall, a considerable proportion of fatty acids present in faeces (between 35% and 90% across all diets) were in the form of fatty acid soaps with Ca-fatty acid soaps being the predominant type of soap present. Furthermore, the proportion of fatty acids present as soap in the faeces was higher when pigs received tallow- and palmolein oil-based diets compared to the olive oil- or soyabean oil-based diets. Interestingly, the oleic acid present in the tallow formed soaps with increasing dietary Ca concentration but hardly any soap formation occurred with oleic acid from olive oil in the gastrointestinal tract. It would also appear that soap formation is influenced by the degree of saturation of the dietary fatty acids. For example, Ca-stearic acid soap was excreted to a higher extent compared to Ca-oleic and Ca-linoleic acid soap of the same chain length across all fat sources.

The fatty acid predominantly found in fatty acid soaps was stearic acid followed by palmitic acid, both of which are saturated long chain fatty acids. In contrast, little soap formation was observed for the two unsaturated fatty acids, oleic and linoleic acid. Following hydrolysis from the triglyceride molecule, saturated-long chain fatty acids have been shown to have a longer incorporation time into biliar micelles compared to unsaturated fatty acids (Wilson *et al.* 1971). This may increase the opportunity for saturated-long chain fatty acids to complex with free Ca ions and form Ca-fatty acid soaps within the gastrointestinal tract. Moreover, Ca-fatty acid soaps containing saturated-long chain fatty acids have been shown to be highly insoluble under simulated gastrointestinal conditions (Graham and Sackman 1983) whereas mono- and polyunsaturated fatty acids of the same chain length have been found to be more soluble under gastrointestinal conditions when tested using *in vitro* models (Gacs and

Barltrop 1977; Graham and Sackman 1983) and when measured using pre-synthesised Ca-fatty acid soaps infused into the intestinal tract (Gacs and Barltrop 1977).

The faecal Ca was higher for pigs receiving the tallow-based diets, where the greatest formation of soaps was observed, compared to pigs fed any of the oil-based diets. While the Ca content of the soaps was not determined directly, theoretically, Ca has the capacity to bind two fatty acid molecules to form the Ca-fatty acid soap complex. The observed flows of 15 g kg^{-1} of faecal fatty acids present as soaps in pigs fed tallow-based diets at the highest dietary Ca concentration would therefore equate to 1 g of Ca. Pigs receiving the tallow-based diet at 6 g kg^{-1} dietary Ca concentration had a 1.2 g higher faecal Ca flow compared to pigs fed the oil based diets at the same dietary Ca intake. This higher faecal Ca excretion can be attributed to the higher presence of soaps in the faeces of tallow fed pigs. Given that there appeared to be a relationship between faecal fatty acid output and faecal Ca output, the results of the presently reported study would support the hypothesis that Ca-fatty acid soap formation in the gastrointestinal tract contributes to a greater fatty acid excretion in the presence of dietary Ca.

Findings regarding the trace minerals are consistent with findings of previous studies in animals which have shown that Ca competitively inhibits Zn and Fe absorption (Lynch 2000). The results suggest that the source of dietary fat also has an impact on essential mineral excretion. Previous studies have shown that a diet high in polyunsaturated fatty acids (linoleic acid) reduces Zn and Fe absorption and depletes body reserves of Zn and Fe in the rat (Amine and Hegsted 1975; Lukaski *et al.* 1986; Johnson *et al.* 1987; Johnson *et al.* 1992). A possible explanation why the absorption of polyvalent cations such as Fe might be affected by unsaturated fatty acids could be in regards to the nature of the minerals being catalysed

during fatty acid oxidation ($Fe^{2+} \rightarrow Fe^{3+}$). For example, ferrous iron is much more readily absorbed in the human gastrointestinal tract compared to ferric iron, which might form insoluble complexes with other compounds. Absorption of Zn and Fe has been shown to be improved in the presence of saturated fatty acids, but the majority of these studies were performed using coconut oil as the saturated fat source, which contains mainly medium chain saturated fatty acids such as lauric and myristic acid (Amine and Hegsted 1975; Lukaski *et al.* 1986; Johnson *et al.* 1987). Our findings on the other hand indicate that a high amount of saturated long-chain fatty acids in the diet increased the excretion of Zn and Fe. For example, an additional 65 mg of Zn was excreted when pigs were fed the 6 mg Ca kg^{-1} tallow-based diet compared to the unsupplemented Ca tallow-based diet, whereas only 20 mg of additional Zn was excreted with the soya bean oil-based diet when dietary Ca concentration increased from 0 to 6 g kg^{-1} diet. Furthermore, the correlation between faecal Zn and faecal total fatty acids ($r^2 = 0.6$) in pigs receiving the tallow-based diet was shown to be stronger than the correlation between faecal Zn and dietary Ca ($r^2 = 0.3$), which suggests that the additional excretion of Zn might be due to complexation of the divalent cation with saturated fatty acids rather than competition for absorption with Ca. The results indicate the possibility of the formation of Zn- and Fe-fatty acid soaps with saturated long-chain fatty acids such as stearic acid and palmitic acid.

The position of a fatty acid on the glycerol molecule is likely to play an important role during soap formation. Fatty acids in the sn-1 and sn-3 position of the triglyceride molecule are hydrolysed by gastrointestinal lipases, resulting in the formation of sn-2 monoglycerides and free fatty acids (Paltauf and Wagner 1976; Levy *et al.* 1984). The sn-2 monoglycerides are easily emulsified into biliar micelles and only a small percentage undergoes further

hydrolysis (Hofmann 1963). Therefore, it would be expected that the fatty acids present in the sn-1 and sn-3 positions are predominantly those that will complex with Ca in the gastrointestinal tract (Aoyama *et al.* 1995; Brink *et al.* 1995). The results of the presently reported study provide evidence for soap formation being influenced by the fatty acid's positional distribution. One example would be the unsaturated fatty acids in tallow. Oleic acid is predominately present in the sn-2 position on the triglycerides of tallow whereas linoleic acid occupies the sn-1/3 positions. Based on the chemistry (chain length and number of double bonds) of the fatty acids it would be expected that oleic acid would be incorporated into soaps to a greater degree than linoleic acid. However, for tallow, the amount of excreted oleic acid present in soaps was only 60%, whereas the amount of excreted linoleic acid present in soaps was up to 80%, a similar percentage to the two saturated fatty acids, stearic and palmitic acid. It would appear that the unavailability of the sn-2 oleic acid led to a reduced incorporation into soaps.

As expected, increasing dietary Ca concentrations led to increased faecal Ca, and reduced faecal Ca digestibility. The presently reported study showed that the fat source, also had an effect on Ca excretion as the pigs receiving the tallow-based diets excreted more Ca compared to pigs receiving any of the oil-based diets. Although this effect didn't translate to a significant statistical difference for faecal Ca digestibility, the difference approached statistical significance ($P = 0.059$). Moreover, when comparing the tallow-based diet with the other diets individual true faecal Ca digestibility was significantly different for olive oil ($P = 0.015$) and soyabean oil ($P = 0.047$) but not palmolein oil ($P > 0.05$).

4.6. Conclusion

Direct evidence is given that increases in dietary Ca content leads to increases in faecal fatty acid excretion due to the formation of indigestible soaps. Furthermore, saturated fatty acids, particularly stearic acid, are affected to a greater degree than unsaturated fatty acids. The type of fat source (solid fat vs liquid oils) as well as the position of the fatty acids on the triglyceride molecules also impact the formation of soaps in the gastrointestinal tract.

CHAPTER FIVE: The Ability of Divalent Cations to Form Fatty Acid-Soaps under Gastrointestinal Conditions.

5.1. Abstract

The formation of divalent cation fatty acid soaps in the gastrointestinal tract was investigated using *in vitro* simulated gastric and small intestinal digestion phases. Since calcium (Ca) can form poorly soluble fatty acid soaps in the gastrointestinal tract, the aim was to examine whether other nutritionally relevant divalent cations such as magnesium (Mg), zinc (Zn), iron (Fe), and copper (Cu) have the ability to complex with fatty acids and form fatty acid soaps. Medium-chain fatty acids (lauric and myristic acid), saturated long-chain fatty acids (palmitic and stearic acid) and unsaturated long-chain fatty acids (oleic and linoleic acid) were incubated at a concentration of 13.2 mM with divalent cations at a concentration of 6.6 mM, under simulated gastrointestinal conditions (10 mM mixed bile salts, 1.45 mM phospholipids, gastric pH = 2.5, small intestinal pH range = 4.5-7.5) but without the presence of digestive enzymes. For all divalent cations tested there was some precipitation of free fatty acids, but the saturated long-chain and medium-chain fatty acids were precipitated to a greater extent than the unsaturated fatty acids. The divalent-cation fatty acid soap formation was pH dependent, resulting generally in a greater extent of soap formation at higher pH and with no soaps precipitating at pH 2.5. Fatty acid precipitation occurred to the greatest extent for Ca and Zn (> 80% of saturated fatty acids were precipitated between pH 5.5-7.5) whereas fatty acid precipitation was generally below 50% for Cu and Fe. Fatty acid soap formation occurs with divalent cations other than Ca, which suggests that fatty acids have the capacity to impair the intestinal absorption of dietary essential minerals, and likewise several minerals may be able to bind fatty acids potentially affecting fatty acid absorption.

5.2. Introduction

Increased dietary calcium (Ca) intake has been associated with an increased faecal fat output in animals, infants and healthy adult humans (Cheng *et al.* 1949; Hanna *et al.* 1970; Denke *et al.* 1993; Aoyama *et al.* 1995; Shahkhalili *et al.* 2001a). The increased faecal fat excretion is accompanied by increased Ca losses suggesting the formation of insoluble Ca-fatty acid soaps in the intestine (Calverley and Kennedy 1949; Aoyama *et al.* 1995; Brink *et al.* 1995; Nelson *et al.* 1996). Ca is a divalent cation, and therefore when ionised has the ability to bind two free fatty acids forming a Ca-fatty acid complex that is likely to be insoluble at the pH of the intestinal fluids, and therefore should pass through the gut unabsorbed (Gacs and Barltrop 1977). Studies in animals have indicated that consuming high-fat diets may adversely affect bone health (Atteh and Leeson 1983) by impairing Ca absorption, most likely due to Ca-fatty acid soap formation. Consequently, concern has risen over recent years that dietary fat can impair Ca absorption in humans (Corwin *et al.* 2006). Considering that dietary fat has the ability to impair the absorption of Ca, it is possible that the absorption of other dietary indispensable divalent cations (Mg, Zn, Fe, or Cu) may also be impaired due to the formation of divalent cation soaps. Conversely, several minerals may have the potential to contribute to the binding of fatty acids thus impairing fatty acid absorption. However, little work has been conducted examining the ability of divalent cations other than Ca to form soaps. The aim of this study was to investigate the ability of nutritionally relevant divalent cations to form fatty acid soaps, using an *in vitro* model that simulated conditions in the gastrointestinal lumen.

5.3. Material & Methods

5.3.1. Materials

Several fatty acids (lauric, myristic, palmitic, stearic, oleic and linoleic acid, as well as methyl-nonadecanoate), glycocholic acid, L-α-phosphatidylcholine and the minerals $CaCl_2x2H_2O$, $MgCl_2x6H_2O$, $ZnSO_4x7H_2O$, $CuSO_4x5H_2O$, $FeSO_4x7H_2O$ and $FeCl_3x6H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sodium salts of the bile acids (glycodeoxycholic acid, taurodeoxycholic acid, and taurocholic acid) were obtained from EMD Chemicals (San Diego, CA, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

5.3.2. Determining divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

The formation of fatty acid soaps was examined for a range of divalent cations (calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), and iron (Fe)) and several different fatty acids (lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and linoleic acid ($C_{18:2}$)) using an *in vitro* digestion model at a range of pH values (2.5, 4.5, 5.5, 6.5, 7.5). The model comprised a gastric phase (15 ml of 140 mM NaCl and 0.66 mM phospholipids) which aimed to simulate the chemical environment of the human stomach lumen and a sequential small intestinal phase (30 mL of 140 mM NaCl, 10 mM mixed bile salts and 1.45 mM phospholipids) which aimed to simulate the chemical environment of the lumen of the human jejunum, and was based on the model used by Gacs and Barltrop (1991). Results were obtained for simulated gastric digestion and simulated gastric + small intestinal digestion. Digestive enzymes were not included in the mixtures as they are not relevant to soap formation in the case of using free fatty acids.

Approximately 80 – 110 mg of each free fatty acid (equating to 396 μmol fatty acid) was weighed individually into 15 mL Falcon™ tubes and dissolved in 2 mL ethanol (100%) at 37°C. To solubilise the stearic acid the solution was initially heated to 50°C before adjusting the temperature to 37°C. The tubes containing the dissolved fatty acids were sonicated in an ultrasound bath maintained at 37°C and a saline phosphatidylcholine (PC) solution (1.52 mg PC mL^{-1}) was added drop-wise over 30 seconds to the solubilised fatty acids. The Falcon™ tubes were sealed and the contents mixed by inverting the tubes for 5 seconds. Thereafter, the samples were sonicated for another 30 seconds. For stearic and palmitic acids, sonication was performed for 45 sec each time instead of 30 sec. The sonication step was used to homogeneously disperse the water insoluble fatty acids in the saline solution with the aid of phospholipids as emulsifiers. The fatty acid-phospholipid emulsions were individually transferred into a pH stat incubator (pre-warmed at 37°C). Salts of $CaCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$ or $FeCl_2$ (equating to 198 μmol of each divalent cation) were individually weighed out (to give a molar ratio of fatty acid-to-cation of 2:1 when added to the fatty acid solution) and solubilised in 8 mL acidic NaCl solution (pH 2). The salt solutions were each slowly transferred into the pH stat with constant stirring and the pH was adjusted to 2.5. The final concentration of the gastric phase reaction mixture was 140 mM NaCl, 0.66 mM phospholipids, 26.4 mM fatty acids, and 13.2 mM divalent cations. After 0.5 h incubation the pH of the gastric phase reaction mixture was increased to around 4.0 by adding NaOH (0.05 - 0.5 M), and 15 mL of 140 mM NaCl solution at 37°C containing 45 mM mixed bile salts, and 1.72 mg mL^{-1} phosphatidylcholine was slowly added. The pH was carefully adjusted to 4.5, 5.5, 6.5 or 7.5 and the material incubated for a further 2 h at 37°C. The final concentration of the simulated gastric + intestinal phase reaction mixture was 13.2 mM fatty acids and 10 mM mixed bile salts, resulting in a fatty acid to bile salt ratio of 1.32 (Shiau *et al.* 1990). The final

concentration of the phospholipids and divalent cations was 1.45 mM and 6.6 mM, respectively.

After incubation, the divalent cation fatty acid soaps were separated from the unreacted divalent cations and fatty acids by filtering separate duplicate samples, for the gastric and gastric + intestinal phase incubations, through a pre-weighed Wattman filter disc using a Buchner funnel. The material remaining on the filter disc (soaps) was washed with 150 ml of distilled water and 150 ml of 100% ethanol to remove any residual divalent cations and unbound fatty acids, respectively. To quantitatively remove any unbound saturated long-chain fatty acids (palmitic and stearic acid), the ethanol was heated to 50°C prior to use. The filter discs containing the soaps were then placed in a desiccator and dried overnight at 37°C. The amount of soap present on the filter discs was determined gravimetrically. The fatty acid and divalent cation concentrations of the precipitate present on the filter discs was also determined as a means of predicting the purity of the soaps, where it was assumed that a molar ratio of fatty acids to cations of two, indicated pure soaps while a ratio less than two indicated the presence of cation salts in addition to the soap. To determine the fatty acid and divalent cation concentrations, the precipitates were acidified with 6 M HCl and incubated for 30 min either at room temperature, to dissociate soaps comprised of unsaturated fatty acids, or at 80°C, to dissociate soaps comprised of saturated fatty acids. Free fatty acids were extracted as described in Chapter Three section 3.3.3. The cations were determined as described for Ca in Chapter Three section 3.3.4.

5.3.3. Investigating the kinetics of divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

For most of the fatty acids, only one intestinal incubation time was used to examine soap formation. However, one medium-chain fatty acid (lauric acid), one saturated long-chain fatty acid (stearic acid) and one unsaturated fatty acid (oleic acid) were selected along with three divalent cations (Ca, Mg and Zn), to examine the effect of different fatty acids and different divalent cations on the kinetics of soap formation. The experiment was performed as described above but soap formation was determined after 30 min (gastric phase of incubation) followed by either 10 min, 30 min or 120 min incubation at pH 7.5 (simulating the approximate time of transit through the duodenum (10 min), jejunum (30 min) and ileum (120 min) (Kim 1968). The kinetics of soap formation with Cu and Fe were not examined since the latter cations were found to readily form hydroxide salts.

5.3.4. Investigating the impact of cation concentration on divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

This experiment tested whether the divalent cation (Ca, Zn and Cu) concentration of the reaction mixture influenced soap formation. The single divalent cation concentration (6.6 mM) discussed above was based on the reported exogenous and endogenous Ca concentration in the gastrointestinal tract of humans (Bronner and Pansu 1999). Given that Zn and Cu are normally found in the intestinal lumen at lower concentrations compared to Ca (Lonnerdal 2008), decreasing concentrations of the minerals (below 6.6 mM) were also included in the study. The six concentrations of the three cations examined (Ca, Zn and Cu) were 0.21 mM, 0.41 mM, 0.83 mM, 1.7 mM, 3.3 mM, and 6.6 mM. A reaction mixture pH of

7.5 was chosen for Ca and Zn whereas a pH of 5.5 was chosen for Cu, since a significant amount of $Cu(OH)_2$ precipitation has been reported at pH 7.5 (Albrecht *et al.* 2011).

5.3.5. Fatty Acid Analysis

Fatty acid analysis was conducted as described in Chapter Three section 3.3.5.

5.3.6. Divalent Cation Analysis

The divalent cations were determined using atomic absorption spectrometry (AAS) by diluting the reaction mixture samples as required. For Ca and Mg analysis, $SrCl_2$ was added to the samples, standards and blanks as a releasing agent (to avoid interference with any phosphates or sulphates present) to provide a strontium concentration of 1000 ppm. The cation concentration of the test solutions was determined using absorbance at a wavelength of 422.7 nm for Ca, 202.5 nm for Mg, 213.9 nm for Zn, 324.8 nm for Cu and 248.3 nm for Fe.

5.3.7. Statistical Analyses

Statistical analyses were performed using the Mixed Model procedure of SAS (SAS/STAT Version 9.4, SAS Institute Inc., Cary, NC, USA). For the first analysis, completely randomised factorial arrangements of treatments were tested for the effects of different factors (pH, time, fatty acid, divalent cation) on fatty acid precipitation. For the second analysis, a one-way ANOVA including orthogonal polynomial contrasts was performed to test the effect of divalent cation concentration on divalent cation and stearic acid recovery. The model diagnostics of each response variable were tested using the ODS GRAPHICS options of SAS. When the F-value of the analysis of variance was significant ($P < 0.05$), selected means were compared using the adjusted Tukey test.

5.4. Results

5.4.1. Non soap specific precipitation under *in vitro* simulated gastric and small intestinal conditions

To determine the extent to which fatty acids or divalent cations formed precipitates other than cation fatty acid soaps, either fatty acids or the divalent cations where incubated alone using the *in vitro* gastrointestinal conditions described above. Almost no precipitation (less than 0.1%) occurred when free fatty acids were incubated in the absence of divalent cations (data not shown). For the incubation of divalent cations in the absence of fatty acids a significant ($P < 0.001$; overall SEM = 1.24) interaction was found between pH and divalent cation (Table 5-1). For the cations, Ca and Mg, no precipitation occurred with incubation in the absence of free fatty acids. However, a considerable degree of precipitation occurred when Zn, Cu and Fe were incubated at pH 4.5-7.5 in the absence of fatty acids, with increasing amounts of precipitation being observed with increasing pH. A small degree of Fe precipitation (8%) occurred at pH 4.5 and precipitation increased steadily with increasing pH, such that 99% of Fe was precipitated at pH 7.5. Cu precipitation (80%) occurred at pH 5.5 and increased to > 96% at pH 6.5 and 7.5. Approximately 40% of Zn precipitated at pH 6.5 and this increased to 92% at pH 7.5 (Table 5-1). The precipitates observed for Zn, Cu and Fe were likely hydroxide salts which would form in the presence of NaOH, which was used to adjust the pH of the reaction mixture after the gastric phase incubation. The difference observed between the total weights of the precipitates and the weights of the cations alone (based on AAS determination) was consistent with the presence of hydroxide salts (data not shown).

Table 5-1: Divalent cation[1] precipitation (µmol) after incubation in the absence of free fatty acids[2].

	Divalent cations				
pH	Ca	Mg	Zn	Cu	Fe
2.5	0.0	0.0	0.0^{c}	0.0^{c}	0.0^{c}
4.5	0.0^{y}	0.0^{y}	0.0^{cy}	0.0^{cy}	23.4^{dz}
5.5	0.0^{x}	0.0^{x}	0.0^{cx}	158.2^{bz}	55.2^{cy}
6.5	0.0^{w}	0.0^{w}	76.4^{bx}	190.6^{az}	140.3^{by}
7.5	0.0^{x}	0.0^{x}	181.8^{ay}	191.9^{az}	195.9^{az}

[1]Divalent cation (198 µmol) was incubated in the absence of free fatty acids in a reaction mixture comprising 15 mL of 140 mM NaCl and 0.66 mM phospholipids at pH 2.5 or 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts at pH 4.5-7.5.
[2]There was a statistically significant ($P < 0.001$; overall SEM = 1.24) interaction between pH and divalent cation.
[ab]Within a column, means without common superscript differ
[zy]Within a row, means without common superscript differ

5.4.2. Divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

To predict the behaviour of nutritionally relevant divalent cations in the presence of different free fatty acids in the upper gastrointestinal tract, different combinations of selected dietary fatty acids and divalent cations were incubated *in vitro* under simulated gastrointestinal conditions. While cation salts may also be present with cation soaps in the precipitate after incubation, the molar ratio of fatty acids to divalent cations (which is 2:1 for soaps) can be used as a predictor of the purity of the soaps, where a ratio of two suggests that soaps only are present and a ratio lower than two suggests that cation salts (most likely hydroxides) are also present. The molar ratio of fatty acids to divalent cations observed here ranged from 0.2 to 2.1 depending on the cation (Table 5-2). For example, lower ratios were observed for Zn at pH 6.5 and 7.5, particularly for the unsaturated fatty acids, for Cu at pH 5.5 to 7.5 and for Fe at pH 4.5 to 7.5. The lower molar ratios of fatty acid to divalent cation observed for Zn, Cu and Fe are consistent with the presence of divalent cation salts and this is in line with the results obtained when the cations were incubated in the absence of fatty acids. Consequently,

it was deemed that for the subsequently reported studies the amount of soap present should be estimated based on the amount of fatty acid present rather than the amount of cation present.

The amounts of cation fatty acid soaps for lauric, myristic, palmitic, stearic, oleic and linoleic acid formed in combination with Ca, Mg, Zn, Cu and Fe and found in the precipitates after incubation at pH 2.5 (gastric phase) and incubation at pH 4.5, 5.5, 6.5 and 7.5 (small intestinal phase) are presented in Table 5-2 and shown graphically in Figure 5-1. Soaps are given as amounts of fatty acids precipitated. Upon statistical analysis a significant ($P < 0.001$, overall SEM = 10.72) three-way interaction was found between pH, fatty acid and divalent cation. This means that there was an effect of fatty acid on soap formation but the extent of the effect was dependent on both pH and the divalent cation.

No soaps were present after incubation under gastric conditions (pH 2.5) for any of the fatty acids tested in the presence of any of the divalent cations. Moreover, little or no soaps were present after incubation at pH 2.5 followed by incubation at pH 4.5 but this was dependent on the fatty acid-cation combination. For Ca, Mg, Cu and Fe, soap formation after incubation at pH 4.5 was either zero or not statistically significantly different from soap formation at pH 2.5. For Zn, approximately 20% of the initial amount of saturated fatty acid (lauric, myristic, palmitic, and stearic acid) was recovered as soap after incubation at pH 4.5. For the unsaturated fatty acids, oleic and linoleic acid, no soap formation was observed for any of the tested cations at pH 4.5.

Precipitation of fatty acids (equal to soap formation) generally increased with increasing pH beyond the gastric phase for all fatty acids when incubated together with Ca, Zn and Fe and for lauric, myristic, palmitic and stearic acid when incubated together with Mg and Cu. Fatty

acid precipitation was highest in the presence of Zn. Incubation with Zn at pH 5.5 resulted in between 76 and 88% of lauric, myristic, palmitic and stearic acids forming soaps. Any further increase in pH (pH 6.5 and 7.5) did not result in a significantly higher degree of precipitation of the latter saturated fatty acids when incubated with Zn. A practically significant degree of precipitation of oleic acid was observed at pH 5.5 for the incubation with Zn, whereby 18% of the oleic acid was precipitated as soap. Incubation at pH 6.5 and 7.5 resulted in increasing oleic acid precipitation with Zn, where 41% and 64% of the oleic acid precipitated as soap. A similar trend was observed for linoleic acid when incubated with Zn.

A significant amount of lauric, myristic, palmitic and stearic acid (between 51 and 76%) was precipitated when incubated with Ca at pH 5.5. Incubation at pH 6.5 and 7.5 resulted in increasing lauric and stearic acid precipitation with Ca, whereby 66% and 83% of lauric and stearic acid respectively, were precipitated as soap. No significant changes were observed for the precipitation of myristic and palmitic acid in the presence of Ca with pH 6.5 and 7.5. The precipitation of saturated long-chain fatty acids (palmitic and stearic acid) in the presence of Ca was similar to the fatty acid precipitation of palmitic and stearic acid in the presence of Zn, but the precipitated amount of medium chain fatty acids (lauric and myristic acid) was significantly lower in the presence of Ca compared to Zn. In the presence of Ca, soap formation with the unsaturated fatty acids oleic and linoleic acid occurred only when incubated at pH 7.5 and was significantly lower compared to soap precipitation with Zn.

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.

Fatty acid (FA)	pH	Ca-fatty acid soaps			Mg-fatty acid soaps		
		FA[1] µmol	Ca[1] µmol	Ratio[2]	FA[1] µmol	Mg[1] µmol	Ratio[2]
Lauric acid[5]	2.5[3]	$0.0^{cz\alpha}$	0.0z	-	$0.0^{bz\alpha}$	0.0z	-
	4.5[4]	$0.0^{cy\alpha}$	0.0	-	$0.0^{by\alpha}$	0.0	-
	5.5[4]	$200.8^{by\beta}$	102.4	2.0	$0.0^{bw\beta}$	0.0	-
	6.5[4]	$244.9^{aby\beta}$	127.0	1.9	$2.6^{bw\chi}$	1.3	2.0
	7.5[4]	$260.9^{ay\beta}$	137.3	1.9	$110.7^{ax\beta}$	52.8	2.1
Myristic acid[5]	2.5[3]	$0.0^{bz\alpha}$	0.0	-	$0.0^{cz\alpha}$	0.0	-
	4.5[4]	$0.0^{by\alpha}$	0.0	-	$0.0^{cy\alpha}$	0.0	-
	5.5[4]	$265.7^{ay\alpha}$	124.7	2.1	$10.6^{cw\beta}$	5.6	1.9
	6.5[4]	$278.8^{ay\beta}$	136.3	2.0	$126.3^{bw\beta}$	70.0	1.8
	7.5[4]	$295.7^{azy\alpha\beta}$	140.4	2.1	$251.8^{ay\alpha}$	126.4	2.0
Palmitic acid[5]	2.5[3]	$0.1^{bz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-
	4.5[4]	$34.9^{by\alpha}$	18.3	1.9	$0.0^{by\alpha}$	0.0	-
	5.5[4]	$301.0^{az\alpha}$	167.3	1.8	$33.4^{bx\beta}$	17.1	1.9
	6.5[4]	$341.7^{azy\alpha}$	179.7	1.9	$312.2^{ay\alpha}$	147.2	2.1
	7.5[4]	$323.9^{azy\alpha}$	170.4	1.9	$291.1^{ay\alpha}$	136.7	2.1
Stearic acid[5]	2.5[3]	$0.1^{cz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-
	4.5[4]	$43.0^{cz\alpha}$	21.5	2.0	$7.8^{by\alpha}$	4.0	1.9
	5.5[4]	$263.6^{bzy\alpha}$	124.8	2.1	$255.0^{ay\alpha}$	132.2	1.9
	6.5[4]	$335.5^{az\alpha}$	179.1	1.9	$289.6^{az\alpha}$	138.2	2.1
	7.5[4]	$317.9^{az\alpha}$	177.1	1.8	$285.6^{az\alpha}$	136.3	2.1
Oleic acid[5]	2.5[3]	$0.0^{bz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-
	4.5[4]	$0.0^{bz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-
	5.5[4]	$0.0^{by\chi}$	0.0	-	$0.0^{ay\beta}$	0.0	-
	6.5[4]	$13.0^{bx\chi}$	7.7	1.7	$0.0^{ax\chi}$	0.0	-
	7.5[4]	$146.4^{ay\chi}$	80.0	1.8	$0.6^{aw\chi}$	n.d.	-
Linoleic acid[5]	2.5[3]	$0.0^{bz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-
	4.5[4]	$0.0^{bz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-
	5.5[4]	$0.0^{bz\chi}$	0.0	-	$0.0^{az\beta}$	0.0	-
	6.5[4]	$6.8^{by\chi}$	4.4	1.6	$0.0^{ay\chi}$	0.0	-
	7.5[4]	$91.4^{ay\delta}$	59.2	1.5	$0.0^{ax\chi}$	0.0	-

[1]Free fatty acid (396 µmol) and divalent cation (198 µmol) were added to the reaction mixture each time

[2]Molar ratio of fatty acid to divalent cation = µmol FA / µmol divalent cation

[3]Gastric phase: 15 mL of 140 mM NaCl, 0.66 mM phospholipids, 26.4 mM fatty acids, 13.2 mM divalent cation; incubation time 30 min at pH 2.5

[4]Gastric plus sequential intestinal phase: 15 mL gastric phase plus 15 mL bile salt mix: 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM fatty acids, 6.6 mM divalent cations; incubation time 2h.

Table 5-2 continued

Zn-fatty acid soaps			Cu-fatty acid soaps			Fe-fatty acid soaps		
FA[1] µmol	Zn[1] µmol	Ratio[2]	FA[1] µmol	Cu[1] µmol	Ratio[2]	FA[1] µmol	Fe[1] µmol	Ratio[2]
$0.0^{cz\alpha}$	0.0z	-	$0.0^{bz\alpha}$	0.0	-	$0.0^{cz\alpha}$	0.0z	-
$60.7^{bz\alpha}$	30.6	2.0	$0.0^{by\alpha}$	0.0	-	$12.4^{czy\alpha}$	28.9	0.4
$299.5^{az\alpha}$	157.6	1.9	$61.1^{ax\beta}$	161.6	0.4	$107.4^{bx\alpha}$	101.1	1.1
$327.5^{az\alpha}$	191.5	1.7	$40.0^{abw\beta\chi}$	196.1	0.2	$157.8^{ax\alpha}$	196.6	0.8
$313.1^{az\alpha}$	194.9	1.6	$24.5^{abw\beta\chi}$	196.5	0.1	$152.4^{abx\alpha}$	183.9	0.8
$0.1^{cz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-	$0.0^{cz\alpha}$	0.0	-
$85.3^{bz\alpha}$	44.0	1.9	$0.0^{by\alpha}$	0.0	-	$14.3^{cyx\alpha}$	29.5	0.5
$346.9^{az\alpha}$	172.1	2.0	$94.1^{ax\beta}$	196.5	0.5	$124.7^{bx\alpha}$	113.2	1.1
$356.0^{az\alpha}$	190.8	1.9	$75.5^{av\beta}$	195.6	0.4	$176.4^{ax\alpha}$	185.4	1.0
$340.0^{az\alpha}$	196.4	1.7	$61.7^{aw\beta}$	196.1	0.3	$160.3^{abx\alpha}$	189.7	0.8
$0.2^{cz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-
$86.9^{bz\alpha}$	44.2	2.0	$0.0^{by\alpha}$	0.0	-	$24.9^{by\alpha}$	34.6	0.7
$335.3^{az\alpha}$	168.0	2.0	$156.0^{ay\alpha}$	195.5	0.8	$146.3^{ay\alpha}$	116.9	1.3
$364.0^{az\alpha}$	193.3	1.9	$138.9^{ax\alpha}$	195.8	0.7	$187.7^{ax\alpha}$	183.1	1.0
$342.8^{az\alpha}$	196.6	1.7	$125.6^{aw\alpha}$	195.6	0.6	$184.1^{ax\alpha}$	196.8	0.9
$0.1^{cz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-
$68.2^{bz\alpha}$	34.7	2.0	$0.0^{by\alpha}$	0.0	-	$24.0^{bz\alpha}$	33.9	0.7
$305.8^{az\alpha}$	150.9	2.0	$152.0^{ax\alpha}$	195.5	0.8	$150.0^{ax\alpha}$	122.8	1.2
$326.9^{az\alpha}$	191.9	1.7	$129.9^{ax\alpha}$	195.8	0.7	$182.7^{ay\alpha}$	193.0	0.9
$314.4^{az\alpha}$	196.6	1.6	$121.5^{ay\alpha}$	196.4	0.6	$168.9^{ay\alpha}$	196.4	0.9
$0.0^{dz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-	$0.0^{cz\alpha}$	0.0	-
$0.0^{dz\beta}$	0.0	-	$0.0^{az\alpha}$	0.0	-	$0.0^{cz\alpha}$	24.9	-
$72.8^{cz\beta}$	40.0	1.8	$45.9^{azy\beta\chi}$	160.7	0.3	$22.1^{bcy\beta}$	81.8	0.3
$164.2^{bz\beta}$	167.1	1.0	$22.2^{ax\chi\delta}$	196.6	0.1	$80.1^{ay\beta}$	189.8	0.4
$252.5^{az\beta}$	195.2	1.3	$0.0^{ay\chi}$	195.8	-	$61.5^{abx\beta}$	183.3	0.3
$0.0^{cz\alpha}$	0.0	-	$0.0^{az\alpha}$	0.0	-	$0.0^{bz\alpha}$	0.0	-
$0.0^{cz\beta}$	0.0	-	$0.0^{az\alpha}$	0.0	-	$0.0^{bz\alpha}$	22.4	-
$26.7^{cz\beta}$	16.2	1.6	$0.0^{az\chi}$	161.5	-	$16.2^{abz\beta}$	68.9	0.2
$110.5^{bz\chi}$	149.1	0.7	$0.0^{ay\delta}$	196.6	-	$34.6^{aby\beta}$	191.1	0.2
$204.1^{az\beta}$	195.6	1.0	$0.0^{ax\chi}$	195.8	-	$27.2^{ax\beta}$	182.9	0.1

[5]A significant (P< 0.001; overall SEM = 10.72) three-way interaction was found between pH, fatty acid and divalent cation. The effects of pH, fatty acid and divalent cation were analysed for FA only, as the amount of soaps were estimated by means of precipitated FAs.

[abc]Across pH within fatty acid (column) for a given divalent cation, means without common superscript differ.

[zyx]Across divalent cation within fatty acid (row) at a given pH, means without a common superscript differ.

[αβχ]Across fatty acids at a given pH (column) for a given divalent cation, means without common superscript differ.

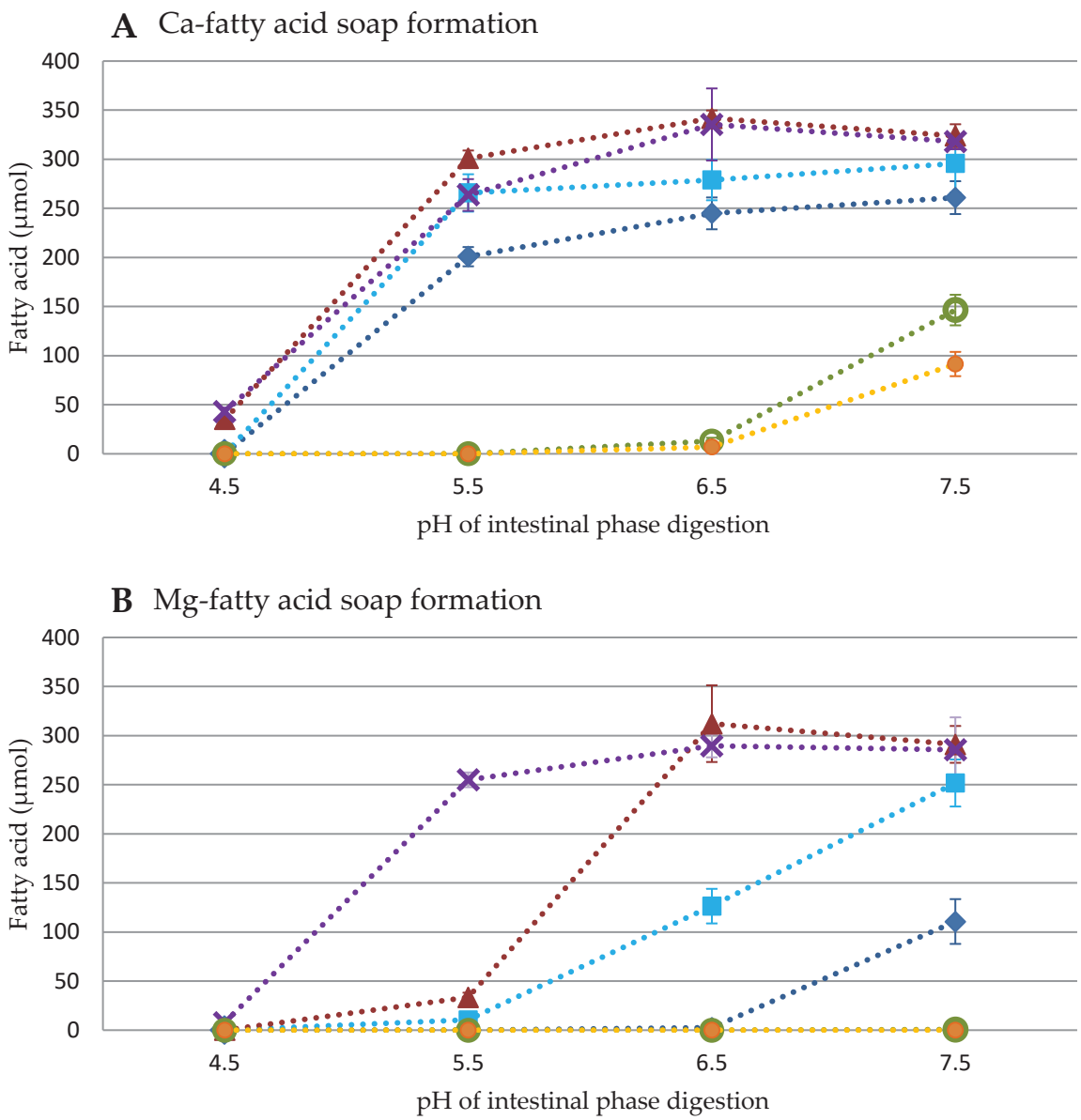
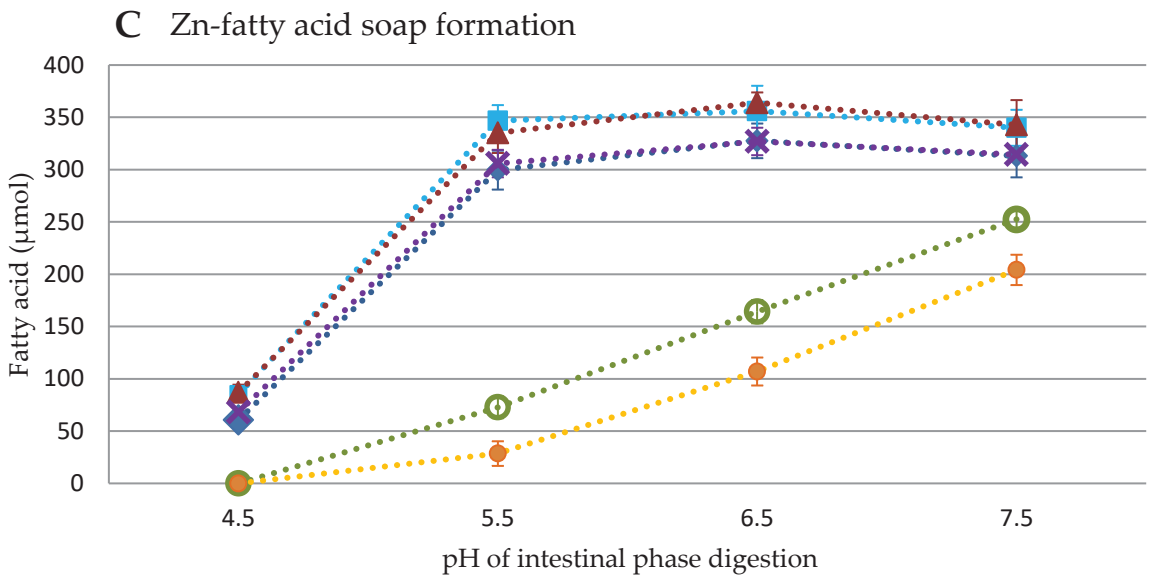
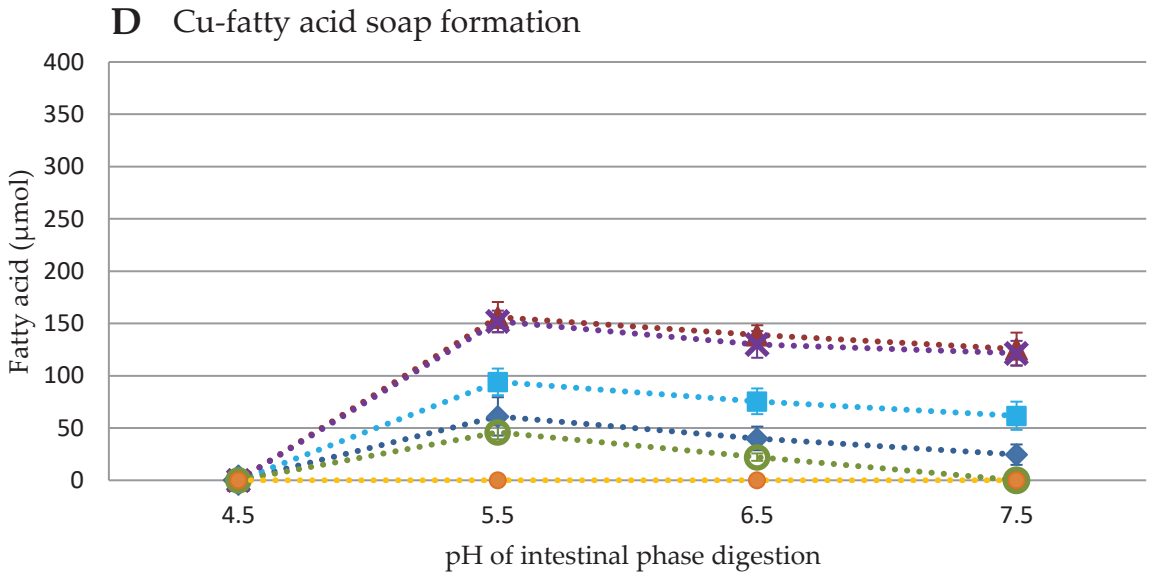


Figure 5-1: Amount of lauric acid (◆), myristic acid (■), palmitic acid (▲), stearic acid (×), oleic acid (○) and linoleic acid (●) that precipitated after incubation with Ca (**A**), Mg (**B**), Zn (**C**), Cu (**D**) or Fe (**E**) at increasing pH under *in vitro* simulated gastrointestinal conditions. For statistical differences between pH and fatty acids tested refer to Table 5-2.





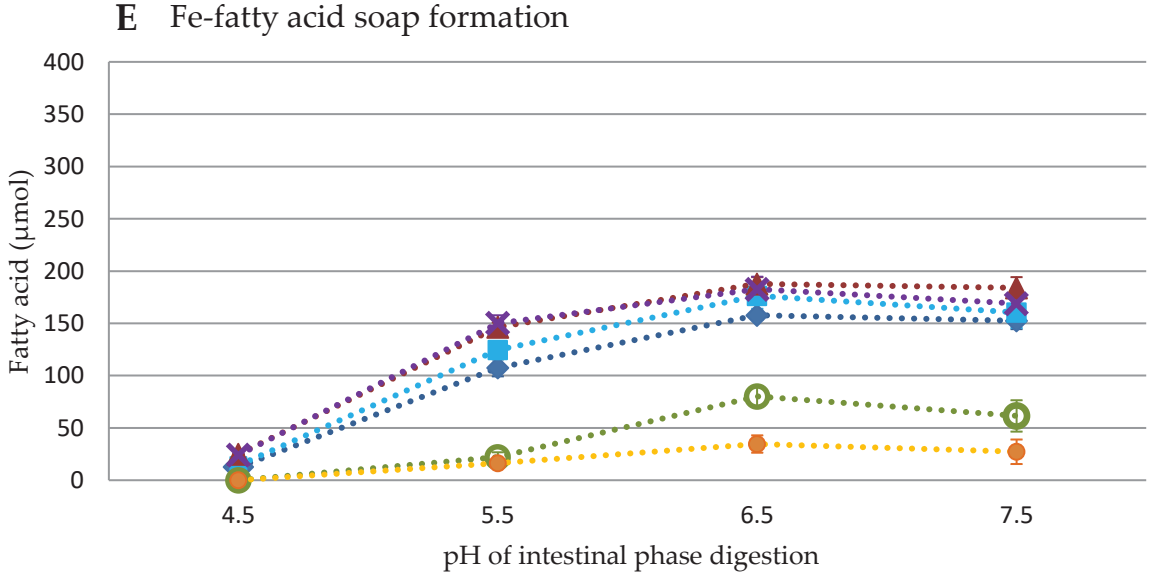


Figure 5-1 continued.

Incubation of fatty acids with Mg resulted in 64% of stearic acid precipitation as soap at pH 5.5 (no significant changes with further increasing pH), whereas no or little soap formed with lauric, myristic, and palmitic acid at pH 5.5. Significant myristic and palmitic acid (32% and 79%, respectively) precipitation in the presence of Mg occurred at pH 6.5. Lauric acid precipitation as Mg-soap only occurred at pH 7.5 (28%). The amount of precipitated stearic acid with Mg was comparable to stearic acid precipitation with Ca and at higher pH (pH 6.5 and 7.5) with Zn. For the unsaturated fatty acids, oleic and linoleic acid, no soap formation was observed with Mg at any pH tested.

The lowest soap formation was observed for incubations in the presence of Cu and Fe. Significant soap formation in the presence of Cu was only observed for the saturated fatty acids, whereas the unsaturated fatty acid precipitation was either zero or not significantly different from zero. On average, 35% of stearic acid and palmitic acid, 19% of myristic acid and 11% of lauric acid were found as soap after incubation with Cu across pH 5.5 to 7.5. Fatty acid precipitation in the presence of Fe exceeded that of Cu with increasing pH. The precipitation of saturated fatty acids was not significantly different at pH > 5.5 when fatty acids were incubated with Fe, and averaged 40% at pH 5.5 to 7.5.

5.4.3. The kinetics of divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

The kinetics of divalent cation fatty acid soap formation were investigated using lauric acid, stearic acid and oleic acid which represented examples of saturated medium-chain, saturated long-chain, and unsaturated long-chain fatty acids respectively. Divalent cations that formed cation salts (those that were found to have a molar fatty acid to divalent cation ratio significantly less than two, for example Cu and Fe) were not included in this study. Precipitation of the three latter fatty acids with the divalent cations Ca, Mg and Zn was investigated for intestinal incubation times of either 10, 30 or 120 minutes. The results are given in Table 3 and are shown graphically in Figure 5-2. There was a significant ($P < 0.01$; overall SEM = 9.40) three-way interaction between time, fatty acid and divalent cation. This means that there was a statistically significant effect of time but that the magnitude of the effect differed statistically, depending upon the cation and the fatty acid. Statistical significance is shown for the effect of time, determined for each cation and fatty acid, individually. After gastric phase incubation plus 10 min of small intestinal phase incubation 77%, 55% and 84% of the lauric acid present in the reaction mixture had formed soaps with Ca, Mg, and Zn, respectively. Incubation for longer than 10 min led to a numerical reduction in the concentration of all three soaps such that after 120 min incubation, approximately 66%, 28% and 79% of the original fatty acid was found in the precipitate. After gastric phase incubation plus 10 min of small intestinal phase digestion, an average of 71% of the stearic acid present had formed soaps with the three tested divalent cations. After a longer small intestinal phase incubation (30 min), the concentration of Ca and Mg soaps increased significantly, resulting in, on average, 81% of stearic acid precipitating as soap for the two divalent cations. At 120 min small intestinal phase incubation, no further increase in the

concentration of any of the three stearic acid soaps (77%) was observed. After gastric phase incubation plus 10 min small intestinal phase incubation 53%, 4% and 71% of oleic acid present in the reaction mixture had formed soaps with Ca, Mg, and Zn, respectively. Small intestinal phase incubation for longer than 10 min led to a significant reduction in the concentration of Ca and Mg soaps, such that after 120 min incubation, approximately 37% and > 1% of the original fatty acid were present for Ca and Mg.

Table 5-3: Kinetics of divalent-cation fatty acid soap formation under *in vitro* simulated upper gastrointestinal tract conditions[1] at pH 7.5

Fatty acid (FA)	Time[2] (min)	Ca-fatty acid soaps			Mg-fatty acid soaps			Zn-fatty acid soaps		
		FA[3] (µmol)	Ca[3] (µmol)	Ratio[4]	FA[3] (µmol)	Mg[3] (µmol)	Ratio[4]	FA[3] (µmol)	Zn[3] (µmol)	Ratio[4]
Lauric acid[5]	10	303.8	155.6	2.0	216.2[a]	112.6	1.9	333.2	165.6	2.0
	30	299.2	147.4	2.0	199.0[a]	107.3	1.9	329.9	193.9	1.7
	120	260.9	137.3	1.9	110.7[b]	52.8	2.1	313.1	194.9	1.6
Stearic acid[5]	10	286.5[b]	142.9	2.0	261.5[b]	131.2	2.0	295.0	159.2	1.9
	30	335.0[a]	177.8	1.9	309.0[a]	160.6	1.9	322.2	192.0	1.7
	120	317.9[ab]	177.1	1.8	285.6[ab]	136.3	2.1	314.4	196.6	1.6
Oleic acid[5]	10	209.9[a]	111.4	1.9	16.0[a]	8.8	1.8	282.2	161.4	1.7
	30	200.8[ab]	104.8	1.9	12.9[a]	6.8	1.9	276.9	194.2	1.4
	120	146.4[b]	80.0	1.8	0.6[b]	0.0	-	252.5	195.2	1.3

[1]The reaction mixture comprised 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM fatty acids, 6.6 mM divalent cations.
[2]Time of intestinal phase digestion which followed gastric digestion. There was no gastric precipitation of soap after 30 min incubation for any of the fatty acids or divalent cations tested.
[3]Free fatty acid (396 µmol) and divalent cation (198 µmol) were added to the reaction mixture.
[4]Molar ratio of fatty acid to divalent cation = µmol FA / µmol divalent cation
[5]A significant three-way interaction ($P < 0.01$; overall SEM = 9.40) was found between time, fatty acid and divalent cation. The effect of time for each fatty acid and divalent cation on fatty acid-soap formation was analysed statistically.
[abc]Mean values across time points within each fatty acid for each cation without common superscript were significantly different ($P < 0.05$).

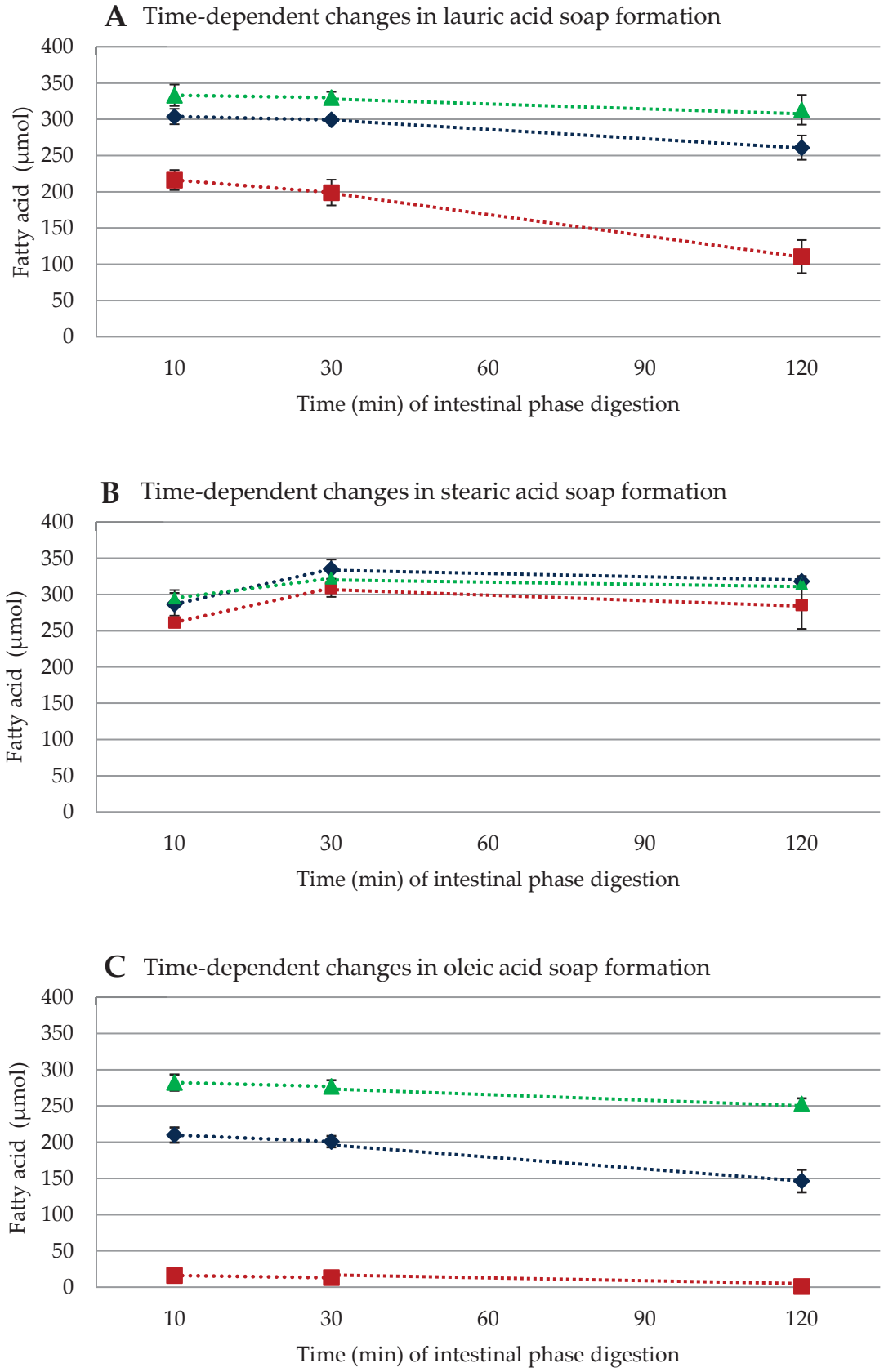


Figure 5-2: Kinetics of fatty acid concentration (marker of divalent cation fatty acid soaps)
Fatty acid soap formation of lauric acid (**A**), stearic acid (**B**) and oleic acid (**C**) with Ca (◆), Mg (■) or Zn (▲) was observed at three different time points (10, 30 and 120 min) under simulated gastrointestinal conditions.
For statistical analysis refer to Table 5-3.

5.4.4. The impact of cation concentration on divalent cation fatty acid soap formation under *in vitro* simulated gastric and small intestinal conditions

The formation of stearic acid soap was examined as a function of increasing concentrations (from 0.21 to 6.6 mM) of Ca or Zn at pH 7.5, or of Cu at pH 5.5 and the results are given in Tables 5-4, 5-5 and 5-6 and are shown graphically in Figure 5-3. The recovery (%) of the divalent cation in the precipitate and the predicted recovery (%) of stearic acid as soap were calculated as was the actual ratio of divalent cations to stearic acid in the precipitate. The predicted recovery of stearic acid in the form of stearic acid-soap was based on the maximum amount of stearic acid that could potentially form divalent cation fatty acid soaps with the amount of divalent cation present in the reaction mixture. The ratio provides information as to the composition of the precipitate. A ratio of two indicates that only soap was present in the precipitate while a ratio lower than two would suggest that cation salts were also present. In general, high proportions of stearic acid were recovered as soaps. Only for the reaction between stearic acid and Cu were lower recoveries (38 - 49%) found and only at the higher concentrations of Cu (Table 5-6 and Figure 5-3).

Increasing the concentration of divalent cation (Ca, Zn or Cu) in the reaction mixture led to increased precipitation of the individual divalent cation itself and to increased precipitation of stearic acid. In the case of Ca, the molar ratio of stearic acid to Ca was approximately two across all Ca concentrations tested. The predicted recovery of Ca and of stearic acid in the precipitate after incubation were not statistically significantly different ($P > 0.05$) for all Ca concentrations tested (mean values of 90% for Ca and 83% for stearic acid (Table 5-4 and Figure 5-3)).

Table 5-4: The effect of Ca concentration on Ca-stearic acid soap formation under simulated gastro-intestinal conditions[1] at pH 7.5

Ca	Total Ca present in reaction mixture	Ca in precipitate		Stearic acid (SA) in precipitate			Ratio[5]
		Determined	Recovery of Ca in precipitate[2]	Potential maximum of SA in soap[3]	Determined	Predicted recovery of SA as soap[4]	
mM	µmol	µmol	%[6]	µmol	µmol	%[6]	
0.21	6.2	5.62	91	12.4	10.60	85.6	1.9
0.41	12.4	11.14	90	24.8	21.27	85.6	1.9
0.83	24.8	21.79	88	49.6	40.52	81.9	1.9
1.7	49.5	44.59	90	99.0	79.60	80.4	1.8
3.3	99.0	89.93	91	198.0	167.58	84.6	1.9
6.6	198.0	177.09	89	396.0	317.88	80.3	1.8

[1]The reaction mixture comprised 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM stearic acid (396 µmol) and 0.21 - 6.6 mM Ca. The stearic acid to bile salt ratio was 1.32.

[2]Recovery of Ca in the precipitate calculated with reference to the added amount of Ca in the reaction mixture = (µmol Ca in precipitated / amount of Ca present in the reaction mixture) x 100

[3]Potential maximum amount of stearic acid that can precipitate with the Ca present in the reaction mixture in the form of Ca-stearic acid soap based on the known stoichiometry of the soap formation
= µmol Ca present in reaction mixture times 2

[4]Calculated proportion of the potential maximum of stearic acid precipitated as soap
= µmol detected amount of stearic acid in precipitate / µmol maximum potential stearic acid in precipitate x100

[5]molar ration of fatty acid to divalent cation = µmol FA / µmol divalent cation

[6]None of the differences were statistically significant ($P > 0.05$).

When stearic acid was incubated with increasing concentrations of Zn, the molar ratio of stearic acid to Zn in the precipitate decreased from 2.0 at 0.21 mM Zn, to 1.6 at 6.6 mM Zn, suggesting that as the Zn concentration of the reaction mixture increased the amount of non-soap precipitation of Zn (most likely in the form on $Zn(OH)_2$) also increased. The predicted recovery of stearic acid in the precipitate decreased numerically with increasing Zn concentration from 91% to 79%, but the decrease was only significant ($P < 0.05$) for the highest concentration of Zn. The recovery of Zn in the precipitate at different Zn concentrations did not differ significantly ($P > 0.05$).

Table 5-5: The effect of Zn concentration on Zn-stearic acid soap formation under simulated gastro-intestinal conditions[1] at pH 7.5

Zn	Total Zn present in reaction mixture	Zn in precipitate		Stearic acid (SA) in precipitate			Ratio[5]
		Determined	Recovery of Zn in precipitate[2]	Potential maximum of SA in soap[3]	Determined	predicted recovery of SA as soap[4]	
mM	µmol	µmol	%[6]	µmol	µmol	%[6]	
0.21	6.2	5.59	90	12.4	11.28	91.2[a]	2.0
0.41	12.4	11.33	92	24.8	22.35	90.3[ab]	2.0
0.83	24.8	23.08	93	49.6	44.73	90.4[ab]	1.9
1.7	49.5	47.46	96	99.0	87.47	88.4[ab]	1.8
3.3	99.0	97.10	98	198.0	162.12	81.9[ab]	1.7
6.6	198.0	194.55	98	396.0	314.37	79.4[b]	1.6

[1]The reaction mixture comprised 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM stearic acid (396 µmol) and 0.21 - 6.6 mM Zn. The stearic acid to bile salt ratio was 1.32.
[2]Recovery of Zn in the precipitate calculated with reference to the added amount of Zn in the reaction mixture = (µmol Zn in precipitated / amount of Zn present in the reaction mixture) x 100
[3]Potential maximum amount of stearic acid that can precipitate with the Zn present in the reaction mixture in the form of Zn-stearic acid soap based on the known stoichiometry of the soap formation
= µmol Zn present in reaction mixture times 2
[4]Calculated proportion of the potential maximum of stearic acid precipitated as soap
= µmol detected amount of stearic acid in precipitate / µmol maximum potential stearic acid in precipitate x100
[5]molar ration of fatty acid to divalent cation = µmol FA / µmol divalent cation
[6]There was a significant ($P < 0.01$; SEM = 5.38) effect of Zn concentration on the recovery of Zn in the precipitate and on the predicted recovery of SA as soap.
[a,b]Across Zn concentration (column); means without common superscript differ

The molar ratio of stearic acid to Cu, reveals that only at Cu concentrations below 1 mM pure Cu-stearic acid soap precipitated. The predicted recovery of stearic acid in the precipitate decreased numerically with increasing Cu concentration from 72 to 38%, and the recoveries for the two lowest Cu concentrations were significantly ($P< 0.01$) higher than the two highest Cu concentrations. Concomitantly, the recovery of Cu in the precipitate increased numerically from 72 to 99%, leading to more non-soap precipitation of Cu with increasing Cu concentrations. Statistically significant ($P < 0.05$) differences for Cu recovery in the precipitate were found with values for the Cu concentrations below 1 mM being significantly different from those above 1 mM (Table 5-6).

Table 5-6: The effect of Cu concentration on Cu-stearic acid soap formation under simulated gastro-intestinal conditions[1] at pH 5.5

Cu.	Total Cu present in reaction mixture	Cu in precipitate		Stearic acid (SA) in precipitate			Ratio[5]
		Determined	Recovery of Cu in precipitate[2]	Potential maximum of SA in soap[3]	Determined	Predicted recovery of SA as soap[4]	
mM	µmol	µmol	%[6]	µmol	µmol	%[6]	
0.21	6.2	4.45	72[b]	12.4	8.91	72.0[a]	2.0
0.41	12.4	9.20	74[b]	24.8	17.94	72.5[a]	2.0
0.83	24.8	18.58	75[b]	49.6	33.74	68.2[ab]	1.8
1.7	49.5	47.98	97[a]	99.0	48.19	48.7[ab]	1.0
3.3	99.0	96.80	98[a]	198.0	78.98	39.9[b]	0.8
6.6	198.0	195.49	99[a]	396.0	151.97	38.4[b]	0.8

[1]The reaction mixture comprised 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM stearic acid (396 µmol) and 0.21 - 6.6 mM Cu. The stearic acid to bile salt ratio was 1.32.
[2]Recovery of Cu in the precipitate calculated with reference to the added amount of Cu in the reaction mixture = (µmol Cu in precipitated / amount of Cu present in the reaction mixture) x 100
[3]Potential maximum amount of stearic acid that can precipitate with the Cu present in the reaction mixture in the form of Cu-stearic acid soap based on the known stoichiometry of the soap formation
= µmol Cu present in reaction mixture times 2
[4]Calculated proportion of the potential maximum of stearic acid precipitated as soap
= µmol detected amount of stearic acid in precipitate / µmol maximum potential stearic acid in precipitate x100
[5]molar ration of fatty acid to divalent cation = µmol FA / µmol divalent cation
[6]There was a significant ($P < 0.01$; SEM = 5.38) effect of Cu concentration on the recovery of Cu in the precipitate and on the predicted recovery of SA as soap.
[a,b]Across Cu concentration (column); means without common superscript differ

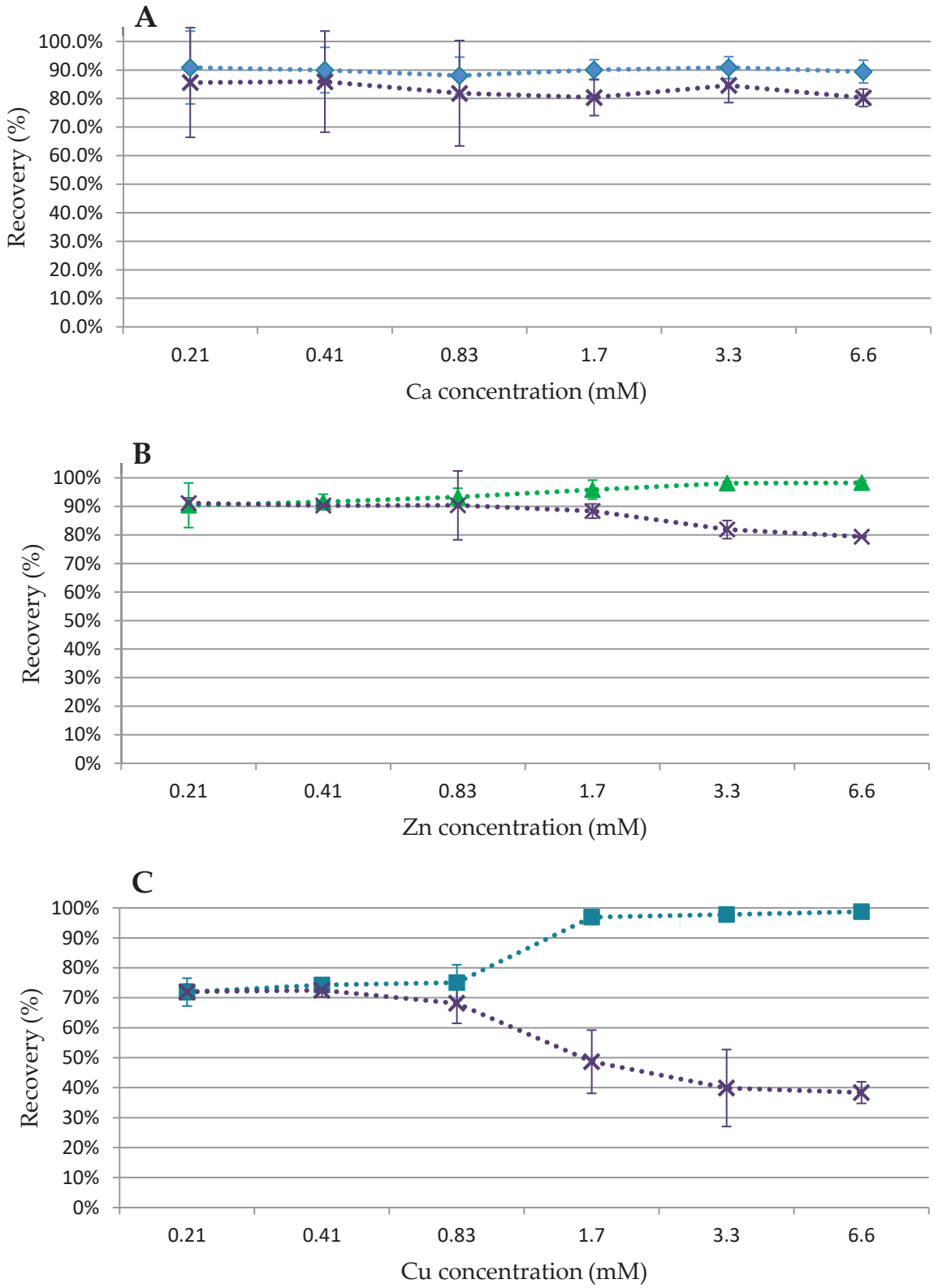


Figure 5-3: Effect of divalent cation concentration on divalent cation and stearic acid precipitation under simulated gastrointestinal conditions[1]

Precipitation of ◆ Ca and × stearic acid (**A**), ▲ Zn and × stearic acid (**B**) and ■ Cu and × stearic acid (**C**) were tested as a function of increasing concentrations of the respective divalent cation.

[1]Gastrointestinal conditions: 30 mL of 140 mM NaCl, 1.45 mM phospholipids, 10 mM bile salts, 13.2 mM fatty acids (stearic acid (SA) concentrations were used at unchanged concentration to maintain the fatty acid to bile salt ratio of 1.32), and 0.21 - 6.6 mM divalent cations; pH 7.5 for Ca and Zn, pH 5.5 for Cu

For statistically significant differences refer to Tables 4, 5, 6.

5.5. Discussion

Interactions between dietary fat and Ca ions have been hypothesised to occur during digestion within the gastrointestinal tract leading to increased faecal fatty acid output (Cheng *et al.* 1949; Denke *et al.* 1993; Shahkhalili *et al.* 2001a; and see Chapter Four) and reduced Ca absorption (Westerlund 1934; Calverley and Kennedy 1949; Nelson *et al.* 1996; and see Chapter Four) , supposedly due to the formation of indigestible Ca-fatty acid soaps (Gacs and Barltrop 1977). In contrast, very little similar research has been reported for other divalent cations which, like Ca, may also be able to bind to fatty acids and form insoluble indigestible divalent cation fatty acid soaps in the gastrointestinal tract. In the presently reported study, the behaviour of nutritionally relevant divalent cations was investigated in the presence of free fatty acids, when incubated *in vitro* under conditions that simulated the chemical environment of the upper gastrointestinal tract.

The first important finding is that divalent cations other than Ca, namely Mg, Zn, Fe and Cu are able to form soaps with a range of fatty acids in the upper gastrointestinal tract. The molar ratio of fatty acids to divalent cations observed for Ca and Mg, as well as for Zn for most of the fatty acids tested, supports the hypothesis that the precipitated fatty acids and minerals were largely in the form of divalent cation fatty acid soaps. The amounts of divalent cation fatty acid soaps formed under the simulated gastrointestinal conditions over the pH range examined were dependent on the chain length of the fatty acid and the degree of saturation of the fatty acid. Incubation with the saturated long chain fatty acids, stearic and palmitic acids, resulted in the greatest amount of soap formation across all of the minerals tested and throughout the pH range tested. Soap formation for saturated medium chain fatty acids (namely myristic and lauric acids) was slightly lower compared to stearic and palmitic

acids when incubated with Ca, Mg and Cu but was similar for Zn and Fe. Unsaturated fatty acids formed significantly less soaps with all of the divalent cations across all pH ranges. The difference in soap formation between fatty acids of different chain length and degree of saturation may relate to the solubility of the free fatty acids themselves and to the solubility of the soaps comprising those fatty acids in gastrointestinal fluids. Certainly the solubility of fatty acids and their Ca-soaps in bile salt solutions has been shown to increase with decreasing chain length and increasing degrees of unsaturation of the fatty acid (Graham and Sackman 1983). In our study, soap formation also occurred in a pH dependent manner, where in general, precipitation of saturated fatty acids with most of the divalent cations tested was observed for incubations at pH 5.5 and above. Above pH 5.5, the amount of divalent cation fatty acid soap formation increased markedly as the reaction mixture pH increased, for stearic, palmitic, myristic and lauric acids when incubated with Ca or Zn, and for stearic acid when incubated with Mg, and reached a maximum soap concentration between pH 6.5 and 7.5. Mg-fatty acid soap formation with palmitic, myristic and lauric acids only occurred at pH 6.5-7.5. Zn-oleic and Zn-linoleic acid soap formation increased linearly between pH 5.5 and pH 7.5 whereas Ca-oleic and Ca-linoleic acid soaps only formed at pH 7.5. In the presence of Fe, fatty acid soap formation was approximately half that observed for Zn. A reddish-brown precipitate was present when the pH of the reaction mixture containing Fe was increased, suggesting that the initial ferrous (Fe^{2+}) form was oxidised to its ferric (Fe^{3+}) state and precipitated most likely as $Fe(OH)_3$. From the results of the current study it was not possible to draw any conclusions as to whether fatty acid soap formation occurred with either ferrous or ferric iron. An additional experiment (results not presented here) was undertaken where ferric iron was incubated with several fatty acids (lauric, stearic and oleic acids). Interestingly, a precipitate was observed in the reaction

mixture at pH 4.5 and higher, but it did not contain any fatty acids. Consequently, the precipitate was likely to be $FeOH_3$. The formation of Cu-fatty acid soaps was maximal at pH 5.5 and decreased only slightly when the pH was increased above 5.5. This was presumably as a result of competition between Cu-fatty acid soap formation and $Cu(OH)_2$ formation which also occurred at pH values above 5.5 (Table 5-1).

The most striking observation was that the divalent cations behaved very differently from each other with respect to their ability to precipitate different free fatty acids. Ca and Zn were most similar in their behaviour and formed soaps with the saturated fatty acids to the greatest extent in comparison to the other cations examined. However, Zn generally formed soaps with the unsaturated fatty acids to a much greater extent than did Ca or any of the other cations. The formation of insoluble cation salts (probably with hydroxide ions) may be the reason for the lower amounts of Cu- and Fe- soaps as evidenced by the much lower fatty acid-to-cation ratio for Cu and Fe. For Cu and Fe, and for Zn when unsaturated fatty acids were incubated at pH 6.5 and above, a molar ratio of fatty acids to divalent cations close to and below 1 was observed. The formation of fatty acid soaps and hydroxide salts appeared to occur simultaneously when the pH was raised by adding NaOH. The competitive formation of $Zn(OH)_2$ over Zn-fatty acid soaps with higher pH was evidenced by the observed decrease in the molar ratio of saturated fatty acids to Zn (from 2 at pH 4.5 to 1.65 at pH 7.5) when the pH of the reaction mixture was increased. However, the affinity of Zn for the saturated fatty acids appeared to be stronger compared to that for hydroxide ions. The precipitation of the divalent cations in the reaction mixture to form either fatty acid soap or hydroxide salts is likely to be related to differential solubility of the soaps versus the salts (see Table 5-7). For example, cations with a low solubility product for their hydroxide salts

(such as Fe and Cu) are more likely to form hydroxides rather than fatty acid-soaps, whereas cations with a solubility product for the hydroxide salt which is higher than that for the soap (such as Ca and Mg) will favour soap precipitation over hydroxide precipitation.

Table 5-7: The solubility product for fatty acid soaps and hydroxide salts of different minerals according to the literature.

Solubility Product Constant (Ksp)							
Fatty acid soap		Hydroxide salt[1]					
SA-soap[2]	OA-soap[3]	$Ca(OH)_2$	$Mg(OH)_2$	$Zn(OH)_2$	$Cu(OH)_2$	$Fe(OH)_3$	$Fe(OH)_3$
$1x10^{-15}$–$2x10^{-17}$	$2x10^{-11}$	$6x10^{-6}$	$2x10^{-11}$	$3x10^{-17}$	$2x10^{-20}$	$1x10^{-15}$	$5x10^{-37}$

[1]Solubility products for hydroxide salts as found in the literature (Ayres 1969)

[2]Solubility product range for Ca-stearic acid soap, Mg-stearic acid soap and Zn-stearic acid soap as found in the literature (Patai and Rappoport 1972)

[3]Solubility product for Ca-oleic acid soap as found in the literature.

Magnesium ions did not react to form $Mg(OH)_2$, but significant amounts of Mg did form fatty acid soaps when saturated long-chain fatty acids were present at high pH. No Mg-fatty acid soap formation occurred with the unsaturated fatty acids, oleic and linoleic acids. Mg-fatty acid soap precipitation for lauric and myristic acid occurred only at higher pH (e.g. 6.5 and 7.5) and was significantly lower compared to Ca-fatty acid soap formation. It has been reported that Ca binds preferentially with carboxyl groups of molecules due to its affinity for carboxylate oxygen. However, Mg has very little affinity for carboxyl groups and due to its electron number (12 e^-) is a weak binder, as its d orbitals are less accessible.

When soap formation was investigated as a function of incubation time there was less soap containing lauric acid and oleic acid present after the longer incubation times as compared to the shorter incubation times of 10 or 30 min. *In vitro* studies, using synthesised Ca-fatty acid soaps of different chain lengths (C_6-C_{18}) and degree of saturation ($C_{18:0}$ – $C_{18:2}$), have shown a higher solubility of Ca-fatty acid soaps comprised of shorter chain length and greater degrees of unsaturation after 2 h incubation in bile salt solutions (Gacs and Barltrop 1977).

Ca-stearic acid soaps were reported to be the least soluble soaps, and this is also reflected by results in the presently reported study, where stearic acid soaps that formed within the first 30 min were barely soluble over the 2 h incubation in the reaction mixture. The results suggest that soap formation occurs rapidly and early on in the digestive process. In some cases the initial amount of precipitated soaps reduced over time, particularly for lauric and oleic acid soaps, most likely due to the higher solubility of lauric and oleic acid soaps in the presence of bile salts (Gacs and Barltrop 1977; Graham and Sackman 1983). On the other hand, even though a substantial amount of stearic acid soap was present after 10 min incubation time, the formation of stearic acid soaps with several minerals (Ca, Mg and Zn) appeared to occur less rapidly when compared to oleic and lauric acid soaps, as the peak of stearic acid soaps was detected after 30 min of incubation. The slower precipitation of stearic acid with divalent cations may be due to a lower saturation ratio (the maximum amount of fatty acid that a bile salt solution can hold) of stearic acid in biliary micelles. Freeman (1969) reported a saturation ratio for stearic acid in glycodeoxycholate solutions of 0.07, compared to oleic and lauric acid of 1.04 and 1.86 respectively. Observations from preliminary experiments in our laboratory showed that free fatty acids need to be in solution to interact with ionized divalent cations. In this work insoluble stearic acid did not form soaps with the presently used minerals. In the presence of bile salts, stearic acid will be solubilised but to a lower extent compared to oleic and lauric acid, due to the difference in saturation ratio. When solubilized stearic acid precipitates with the divalent cation, bile salts would become available again to solubilize more stearic acid which then could interact further with the divalent cation, and this might result in a delayed stearic acid soap formation.

The impact of different Ca, Zn and Cu concentrations on the formation of Ca-, Zn- and Cu-fatty acid soaps was investigated. Intraluminal Ca concentrations of 10 mM have been reported after a high Ca meal (Allen 1982), whereas even with high oral doses of Zn, the intraluminal Zn concentration does not exceed 1 mM (Steinhardt and Adibi 1984) and for Cu, even lower intraluminal concentrations (20 uM) have been predicted (Linder *et al.* 1998). In the presently reported study, at Zn and Cu concentrations below 1 mM, little or no hydroxide salts appeared to form. Albrecht *et al.* (2011) showed that the precipitation of $Zn(OH)_2$ and $Cu(OH)_2$ as a function of increasing pH is a concentration dependent process, such that in the presence of higher Zn or Cu concentrations, hydroxide salts formed at lower pH (7.5 for Zn; 5.5 for Cu). The latter research group reported that at concentrations greater than 1 mM, Zn and Cu precipitated as their hydroxide salts at pH 7 and pH 5.5, respectively, at incubation temperatures of 35°C (Albrecht *et al.* 2011). The presently reported study showed that incubation of stearic acid in the presence of Zn or Cu led predominantly to soap formation at divalent cation concentrations below 1 mM, whereas at Zn and Cu concentrations of 1.7 mM and above hydroxide salts formed along with soaps as was corroborated by a lower stearic acid-to-divalent cation ratio. It appears that at around 1 mM Zn or Cu, hydroxide formation occurs which leads to competition with fatty acid soap formation for the available cations.

The findings reported here suggest that under simulated gastrointestinal conditions, fatty acid soaps can form with divalent cations other than just Ca. If these results are extrapolated to the gastrointestinal tract, then it is possible that the absorption of essential minerals may be reduced by the formation of fatty acid soaps in the gastrointestinal tract. The experiments focussing on reaction kinetics suggest that soap formation occurred rapidly (although only

one reaction mixture pH was examined), suggesting that the formation of divalent cation fatty acid soaps may occur rapidly once the minerals and free fatty acids have entered the small intestine. The latter may be important since if soap formation occurred slowly, then fatty acids and divalent cations may be absorbed before they had the opportunity to form soap complexes (Lee *et al.* 1989). Based on the results of the present study, the latter would appear to not be the case and it is likely that soap formation would compete significantly with the mineral absorption process.

Only a small number of studies have investigated the absorption of dietary Mg in the presence of diets containing high or low amounts of fat. An increase in the amount of butterfat from 5% to 20% in the diet of mature rats led to a 15-20% decrease in the apparent faecal Mg absorption. An indication that the additionally excreted Mg in the presence of high dietary fat was due to Mg-fatty acid soap formation, was given by the presence of Mg in the extracted lipid fraction of the faeces (Kaup *et al.* 1990). Studies in rats using either triolein, tripalmitin or tristearin as the sole dietary fat source showed that apparent faecal Mg digestibility decreased by approximately 26% when the fat content was increased from 5% to 25% in the case of tripalmitin and tristearin but not for triolein (Tadayyon and Lutwak 1969b). Comparisons between fat-free diets and diets containing 25% of one of the latter triglycerides showed that faecal apparent Mg digestibility decreased by more than 40% for tripalmitin and tristearin but did not change for triolein (Tadayyon and Lutwak 1969b). These results are in agreement with the present findings, where almost no soap formation occurred when Mg and oleic acid were incubated together, but a substantial amount of Mg was precipitated in the presence of palmitic and stearic acid when the pH of the reaction mixture was 6.5 or above. Considering that the *in vitro* Mg-fatty acid soap formation was

lower compared to that of Zn, the possibility that Zn forms soaps with fatty acids *in vivo* is very likely. The trace minerals Zn, Cu and Fe were found to precipitate with fatty acids at pH 5.5 (the pH approximating that of the duodenum and proximal jejunum, the main absorption sites for many minerals). The latter result suggests that fatty acid soap formation with divalent cations can occur prior to the site of absorption, thereby leading to impaired absorption of Zn, Cu and Fe which may in turn lead to mineral deficiency. Moreover, Fe might behave differently from Zn or Cu as it can exist as ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions in the diet and in the gastrointestinal tract. To further investigate interactions of fatty acids and divalent cations in the gastrointestinal tract, *in vivo* studies will need to be performed.

5.6. Conclusion

Evidence is given that several nutritionally relevant divalent cations precipitate with fatty acids under *in vitro* simulated gastrointestinal conditions. Soap formation is greater in the presence of saturated fatty acids compared to unsaturated fatty acids, which may indicate that a diet high in saturated fat may impair divalent cation absorption from the gastrointestinal tract and thereby contribute to nutritional deficiency for some minerals.

CHAPTER SIX: Mapping Fatty Acid Soap Formation Throughout the Gastrointestinal Tract of the Growing Pig.

6.1. Abstract

High dietary calcium (Ca) intake has been shown to increase faecal fat excretion due to the formation of indigestible Ca-fatty acid soaps in the gastrointestinal tract which are then excreted in the faeces. However, the gastrointestinal location where soaps are formed is not known. Consequently, this study aimed to map fatty acid-soap formation in the gastrointestinal tract. Semi-synthetic diets, containing either a mixture of free fatty acids (diet A), an intact triglyceride (tallow; diet B) or no added fat (diet C) and containing supplementary calcium in the form of calcium carbonate were fed to growing pigs (n = 8) for a nine day period. Digesta were collected from ten different locations in the gastrointestinal tract and soaps of palmitic, stearic and oleic acid were determined. Diet A resulted in significantly ($P < 0.05$) greater soap formation throughout the small intestine compared to diet B (26.1 vs 12.1 g kg^{-1} DMI at the terminal ileum for diet A and B, respectively). The presence of fatty acid soaps in the stomach and duodenum was negligible (0.3 g kg^{-1} DMI) and increased ($P > 0.05$) for the saturated fatty acids from the distal jejunum (4.7 and 6.0 g kg^{-1} DMI for palmitic and stearic acid, respectively) to the terminal ileum (10.3 and 13.9 g kg^{-1} DMI for palmitic and stearic acid, respectively) for diet A. For diet B, the flows of stearic acid soap increased ($P < 0.05$) from the ileum (7.2 g kg^{-1} DMI) to the proximal colon (9.7 g kg^{-1} DMI), whereas ileal palmitic acid soap flows (5 g kg^{-1} DMI) were higher than gastric soap flows but did not increase beyond the ileum. The flow of oleic acid soap increased ($P < 0.05$) between the proximal and distal jejunum (3.0 and 1.7 g kg^{-1} DMI in distal jejunum for diets A and B respectively) but decreased ($P < 0.05$) from the terminal ileum

onwards. Apparent mineral digestibility for the divalent cations Ca, Mg, Zn and Fe was significantly ($P < 0.05$) lower for pigs receiving diet A or B compared to pigs fed diet C, suggesting that the formation of indigestible divalent cation-fatty acid soaps reduced the digestibility of the latter minerals.

Soaps did not form in quantitatively significant amounts anterior to the proximal jejunum, but a significant amount of saturated fatty acid soap did form from the distal jejunum to the terminal ileum. Soaps did not appear to form in the hind gut when the free fatty acid diet was given, but some stearic acid soap formation occurred in the large intestine for pigs receiving the tallow based diet. The presence of stearic and palmitic acid soaps at the major sites of fatty acid absorption suggests that soap formation might be a mechanism for reduced saturated fatty acid absorption.

6.2. Introduction

The formation of indigestible calcium-fatty acid soaps in the gastrointestinal tract after the consumption of fat-containing diets high in calcium (Ca) is believed to increase the excretion of dietary fatty acids in the faeces (Denke *et al.* 1993; Shahkhalili *et al.* 2001a; Bendsen *et al.* 2008). Moreover, Ca-fatty acid soap formation has been postulated as a mechanism by which high dietary Ca intake leads to weight loss in humans (Major *et al.* 2008). The latter hypothesis originated in the early 2000's when several observational studies (Davies *et al.* 2000; Zemel *et al.* 2000; Jacqmain *et al.* 2001) and a smaller number of clinical trials (Zemel *et al.* 2004; 2005a; 2005b) reported a correlation between dietary Ca intake and body weight, body fat, and weight loss in humans.

Previous *in vivo* studies have shown that saturated fatty acids in particular, are excreted to a greater extent in the presence of dietary Ca compared to unsaturated fatty acids (Denke *et al.* 1993; Shahkhalili *et al.* 2001a, and refer to Chapter Four). These observations are supported by findings from *in vitro* studies, where synthetic Ca-fatty acid soaps comprised of saturated long-chain fatty acids (stearic acid) were less soluble in simulated gastrointestinal fluids than soaps comprised of unsaturated long-chain fatty acids (oleic and linoleic acid) (Graham and Sackman 1983).

Because ionized Ca can only complex with free fatty acids, dietary triglycerides must first be hydrolysed by lipase before the liberated fatty acids can form soaps. Given that triglyceride digestion in the gastrointestinal tract results in the cleavage of fatty acids in the sn-1 and sn-3 positions only, it is likely that the positional distribution of the saturated fatty acids on the triglyceride molecule influences the interaction between Ca and fatty acids. Saturated fatty acids in the sn-1/3 position of triglycerides have been shown to be complexed to a greater extent by dietary Ca compared to the same fatty acids located in the sn-2 position (Aoyama *et al.* 1995; Brink *et al.* 1995). In our previous study (refer to Chapter Four) in pigs, the consumption of tallow, a fat high in saturated fatty acids with palmitic acid and stearic acid distributed mainly in the sn-1/3 position, led to the greatest amount of excreted fatty acid-soaps when ingested with Ca compared to the consumption of oils low in saturated fatty acids. These observations led to the conclusion that tallow is a suitable model fat to observe fatty acid soap formation and therefore tallow was chosen as the intact fat source in the presently reported work.

Previous studies have mainly focused on the effect of Ca on faecal fat excretion, as Ca is the quantitatively most important divalent mineral in the diet and therefore would likely have

the greatest impact on fatty acid soap formation. In our previous study in pigs (refer to Chapter Four) however, we observed a correlation between faecal fatty acid-soap excretion and faecal Zn or Fe excretion for the tallow-based diet. Evidence from our previous *in vitro* study (refer to Chapter Five) also suggested that nutritionally relevant divalent cations other than Ca have the ability to form soaps. The presently reported study, therefore, aimed to further investigate if fatty acid soap formation might have an effect on mineral absorption.

Most of the studies focussing on the effect of dietary Ca on fat digestion in humans have been limited to examining fatty acid digestion across the total gastrointestinal tract (ie. faecal fatty acid digestibility). No studies have examined the effect of dietary Ca on fat digestibility to the end of the small intestine (ileal digestibility) and none have attempted to determine the amount of soaps formed either to the end of the small intestine or at locations across the entire gastrointestinal tract. To our knowledge, only one study has examined the behaviour of Ca-fatty acid soaps directly in the small intestine (Gacs and Barltrop 1977), but rather than studying the formation of soaps in the gastrointestinal tract, synthetic soaps were introduced into rats by means of a duodenal ligation. There is no information available about how and where Ca-fatty acid soaps form in the gastrointestinal tract.

Consequently, the main aim of this study was to map Ca-fatty acid soap formation throughout the gastrointestinal tract. To achieve this, diets comprising either free fatty acids (palmitic, stearic and oleic acids) or the same fatty acids present in the form of a triglyceride (tallow) were fed to growing pigs, and soap formation was determined at ten different locations throughout the gastrointestinal tract.

6.3. Material & Methods

6.3.1. Materials

Tallow was obtained from JSB Wanganui, New Zealand. The Ca-free vitamin and mineral mix and pure Ca carbonate were purchased from Denver Stock Feeds, Palmerston North, New Zealand. The fatty acids myristic, palmitic, stearic, and oleic acid and methyl-nonadecanoate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and acids used for analysis were obtained from Merck, Darmstadt, Germany. All chemicals used were of analytical grade.

6.3.2. Diets

Three semi-synthetic diets containing either individual free fatty acids (Diet A), an intact triglyceride (tallow, Diet B), or no lipid material (Diet C) were prepared and stored at -18°C prior to use. The fatty acid composition of diet A was formulated to be the same as the fatty acid profile of diet B with respect to the fatty acids palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and oleic ($C_{18:1}$) acid. Diet C was formulated to be virtually devoid of fat for the purposes of determining the amount of any endogenous (non-dietary) fatty acids present in the gastrointestinal tract and to determine if soaps form with endogenous fatty acids. The ingredient composition of the experimental diets is given in Table 6-1 and the determined concentration of selected nutrients in the test diets in Table 6-2. The diets met the requirements of the growing pig for all nutrients (NRC, 2012). Titanium dioxide (TiO_2) was included in each diet as an indigestible marker.

Table 6-1: Ingredient composition (g kg^{-1} air dry weight) of the basal and experimental diets.

Ingredient	Diet			
	Basal	A (Free fatty acid-based diet)	B (Tallow-based diet)	C (Fat-free diet)
Wheat starch	591.1	555.0	541.1	641.1
Casein	155.6	155.6	155.6	155.6
Soyabean oil	50	0	0	0
Tallow	0	0	100	0
Free fatty acids (total)		86.1		
Myristic acid ($C_{14:0}$)		3.3		0
Palmitic acid ($C_{16:0}$)		26.0		0
Stearic acid ($C_{18:0}$)		26.4		0
Oleic acid ($C_{18:1}$)		26.4		0
Linoleic acid ($C_{18:2}$)		1.0		0
Purified cellulose	51.4	51.4	51.4	51.4
Sucrose	105.0	105.0	105.0	105.0
Sodium chloride	3	3	3	3
Potassium phosphate	12.8	12.8	12.8	12.8
Calcium carbonate	22.4	22.4	22.4	22.4
Magnesium oxide	0.7	0.7	0.7	0.7
Vit/Min mix[1]	5	5	5	5
Titanium dioxide	3	3	3	3

[1]The vitamin/mineral mix was specially prepared to be devoid of Ca. The premix supplied per kg of diet: zinc (325 mg), iron (225 mg), manganese (112 mg), copper (25 mg), iodine (1.7 mg), selenium (0.75 mg), and cobalt (0.13 mg), vitamin A ($25*10^6$ IU), vitamin D_3 ($5*10^6$ IU), vitamin E (75 mg), vitamin K (5 mg), vitamin B_1 (4.3 mg), vitamin B_2 (12.5 mg), vitamin B_6 (6.3 mg), vitamin B_{12} (0.08 mg), folic acid (1.5 mg), pantothenic acid (37.5 mg), biotin (0.03 mg), niacin (65 mg), choline (500 mg).

Table 6-2: Determined selected nutrient composition (g kg^{-1} air dry weight) of the three experimental diets.

	Diet A[1]	Diet B[2]	Diet C[3]
Nutrient			
Total fat[4]	89	109	3
Myristic acid ($C_{14:0}$)	1.3	3.3	0.13
Palmitic acid ($C_{16:0}$)	28.5	25.9	1.04
Stearic acid ($C_{18:0}$)	28.2	26.4	0.21
Oleic acid ($C_{18:1}$)	24.4	27.9	0.40
Linoleic acid ($C_{18:2}$)	1.8	0.8	0.94
Calcium	9.50	9.00	9.55
Phosphorus	4.30	4.45	4.00
Magnesium (mg kg^{-1})	565	540	550
Zinc (mg kg^{-1})	320	330	340
Iron (mg kg^{-1})	280	285	295
Copper (mg kg^{-1})	20	25	25

[1]Diet A: experimental diet including free fatty acids as the lipid source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as the lipid source
[3]Diet C: fat free diet; no fat was added to the diet, the presence of fat in the diet resulted from small amounts of fat in the other ingredients.
[4]Total fat content of the diets determined by Soxhlet extraction (AOAC 991.36)

6.3.3. Animal trial

All procedures were approved by the Animal Ethics Committee, Massey University, New Zealand. Twenty four entire male pigs (21.9 ± 1.28 kg body weight, mean ± SD; PIC Camborough 46 sow x PIC boar 356 L, PIC New Zealand, Christchurch, New Zealand) were housed individually in metabolism crates in a room maintained at 21 ± 2°C with a 10 h/14 h light/dark cycle (Animal Physiology Unit, Massey University, Palmerston North, New Zealand). Water was available at all times. Animals were randomly assigned to the three experimental diets such that there were 8 pigs receiving each diet. At the outset of the study the pigs were weighed and the daily feed ration was calculated as 9% of the metabolic body weight (0.09 x body weight$^{.75}$). The pigs underwent a five day acclimatization period during which time the pigs were fed the basal diet which included 50 g kg^{-1} of soya bean oil as the sole fat source. The daily ration was fed as three meals a day and the diets were freshly prepared for each meal and given as a water-based slurry (approximately 50% w/w). After the acclimatization period the pigs were fed the test diets for a further nine days receiving five discrete meals a day between 08^{00} h and 16^{00} h from day 2-6 of the nine day experimental period. Samples of faeces were collected on days 7-9. The faeces samples for each pig were pooled across sampling days, homogenized in a kitchen blender (Goldair, Melbourne, Austrialia) and freeze dried. On the final three days of the experiment, pigs were fed using a frequent feeding regimen with nine meals (each comprising 1/9 of their daily ration) fed at hourly intervals between 08^{00} h and 16^{00} h. Six hours after the start of feeding on day 9 the pigs were anaesthetised with an intramuscular injection of an anaesthetic cocktail (0·04 ml/kg BW of Zoletil 100 (50 mg/ml), Ketamine (50 mg/ml) and Xylazine (50 mg/ ml); Provet, New Zealand). Immediately after sedation, the pigs were euthanized by an intra-cardial injection

of sodium pentobarbitone (0·3 ml/kg BW of Pentobarb 300; Provet, New Zealand). The stomach and the small and large intestine were dissected out. The stomach was clamped at the oesophageal and duodenal ends, carefully removed, washed with deionized water and dried with absorbent paper. The pH of the chyme from the proximal and distal regions of the stomach was measured with an IQ150 portable IFSET pH meter (Hach Co., Loveland, CO, USA). The entire stomach contents were collected in zip lock bags and frozen on dry ice. The intestine was divided into nine sections (duodenum, proximal jejunum, distal jejunum, ileum and terminal ileum, proximal, middle and distal colon, and rectum) and digesta were collected separately from each section by flushing out the intestinal contents with distilled water into zip lock bags. The pH of the digesta from each region was measured prior to freezing the samples on dry ice. Chyme and digesta from the ten gastrointestinal regions were freeze dried, ground through a 1 mm mesh and stored at -18°C prior to analysis for fatty acids present in the soap component, Ca, P, Mg, Zn, Fe, Cu and titanium dioxide.

6.3.4. Chemical analyses

The amount of fatty acids present as soaps in the diets, stomach chyme, intestinal digesta and faeces were determined as described in section 4.3.5. Analysis of Ca, P, Mg, Zn, Fe and Cu in the diets, chyme, digesta and the faecal samples was performed using inductively coupled plasma mass spectrometry (ICP-MS). The TiO_2 content of the diets and faeces was determined based on the method of Short *et al.* (1996).

6.3.5. Data Analysis

The flow of a compound as referred to here, is defined as the amount of the compound (fatty acid or mineral) present in chyme, digesta or faeces normalised for the dietary food intake, based on the ratio of TiO_2 in the diet to TiO_2 in chyme, digesta or faeces. The units of flow are mg kg^{-1} DMI, where DMI is dry matter intake.

The flows of total, endogenous and diet related palmitic, stearic, and oleic acid soaps in the chyme, digesta or faeces were calculated as follows:

Total fatty acid-soap flow (mg kg^{-1} DMI)

= Total fatty acid-soap content in chyme, digesta or faeces (mg kg^{-1} DM) x dietary TiO_2 (mg kg^{-1} DM)/ TiO_2 (mg kg^{-1} DM) determined in chyme, digesta or faeces

Endogenous fatty acid-soap flow (mg kg^{-1} DMI)

= Endogenous fatty acid-soap content[‡] in chyme, digesta or faeces (mg kg^{-1} DM) x dietary TiO_2 (mg kg^{-1} DM)/ TiO_2 (mg kg^{-1} DM) determined in chyme, digesta or faeces

Diet related fatty acid soap flow (mg kg^{-1} DMI)

= Total fatty acid soap flow (mg kg^{-1} DMI) – endogenous fatty acid soap flow (mg kg^{-1} DMI)

[‡]Where endogenous fatty acid-soap content was determined by feeding a fat-free diet to the pigs.

The flows and digestibility (%) of the divalent cations (Ca, Mg, Zn, Fe, Cu) and P in the chyme, digesta or faeces were calculated as follows:

Mineral flow (mg kg^{-1} DMI)

= mineral content in chyme, digesta or faeces (mg kg^{-1} DM) x dietary TiO_2 (mg kg^{-1} DM)/ TiO_2 (mg kg^{-1} DM) determined in chyme, digesta or faeces

Apparent Mineral Digestibility (%)

= (Dietary mineral flow (mg kg^{-1} DMI) - mineral flow in chyme, digesta or faeces (mg kg^{-1} DMI) / Dietary mineral flow (mg kg^{-1} DMI) x 100

6.3.6. Statistical analysis

Statistical analyses were performed using the Mixed Model procedure of SAS. Different covariance structures were tested to model repeated measures for each pig. The smallest value for the Akaike's and Bayesian's information criteria was used to select the most appropriate covariance structure. The full statistical model included the effect of diet (free fatty acids versus intact triglycerides when testing for the effect of fatty acid; free fatty acids versus intact triglyceride versus fat-free diet when testing for the effect of mineral), gastrointestinal location (locations from stomach to rectum) and their interactions as fixed effects, and the pig as a random effect. Each response variable was tested for normality of distribution. The model diagnostics for each response variable were tested after combining the PROC UNIVARIATE and the ODS GRAPHICS procedures of SAS before comparing the means. When the F-value of the analysis of variance was significant ($P < 0.05$), the means were compared using the adjusted Tukey test.

6.4. Results

6.4.1. Animal trial

The pigs adapted readily to the diets and environment and all animals gained weight (3.1 ± 0.5 kg) during the experimental period.

6.4.2. Gastrointestinal pH

There was a significant interaction ($P < 0.001$) between diet and gastrointestinal location for pH (Table 6-3). Dietary treatment had no effect on the pH at each gastrointestinal region, except for the proximal colon where the pH for the pigs receiving the free fatty acid-based diet (Diet A; pH 5.8) was significantly lower ($P < 0.05$) than that for pigs receiving the fat-free diet (Diet C; pH 6.5). The pH of the gastric chyme did not vary ($P > 0.05$) between the proximal (fundus) and distal (antral) regions of the stomach and was on average 3.6 for the stomach across all diets. A marked increase in pH ($P < 0.001$) was observed when the chyme entered the duodenum where the mean pH across the three diets was 5.0. The pH of the intestinal digesta was lowest in the proximal regions of the small intestine (pH 5.0 across duodenum and proximal jejunum) and increased ($P < 0.001$) throughout the small intestine, reaching a pH of 6.9 (across all diets) at the terminal ileum. Lower pH values were found for the large-intestinal contents in comparison to the terminal ileum for the two fat containing diets.

Table 6-3: Mean (n = 8) pH throughout the gastrointestinal (GI) tract of the growing pig for the three test diets.

GI location	Diet A[1]	Diet B[2]	Diet C[3]	Overall SE
		pH		
Stomach				
- fundus	3.5^{e}	3.9^{e}	3.6^{e}	0.10
- antral	3.4^{e}	3.7^{e}	3.7^{e}	0.11
Duodenum	4.8^{d}	5.3^{d}	4.9^{d}	0.15
Prox. Jejunum	5.2^{d}	5.1^{d}	4.9^{d}	0.13
Dist. Jejunum	6.0^{c}	5.9^{c}	5.9^{c}	0.06
Ileum	6.5^{ab}	6.6^{ab}	6.4^{b}	0.08
Term. Ileum	6.8^{a}	7.0^{a}	6.9^{a}	0.06
Prox. Colon	5.8^{cdx}	6.1^{cxy}	6.5^{aby}	0.11
Mid Colon	6.4^{ab}	6.2^{c}	6.6^{ab}	0.13
Dist. Colon	6.3^{ac}	6.3^{bc}	6.6^{ab}	0.12
Rectum	6.2^{bc}	6.3^{bc}	6.6^{ab}	0.14

Statistical analysis	Significance[4]
Diet	*
GI location	***
Diet × location interactions	***

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: fat-free diet
[4] *** $P < 0.001$; ** $P < 0.05$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

6.4.3. Formation of fatty acid-soaps

The total amounts of fatty acid-soaps formed for the predominant fatty acids (palmitic, stearic and oleic acids) for the ten gastrointestinal regions and in the faeces are given in Table 6-4 and are shown graphically in Figure 6-1. There was an interaction ($P > 0.001$) between diet and gastrointestinal location for the fatty acid-soaps. No quantitatively significant amounts of fatty acid-soaps were found in the stomach or duodenum. Saturated

fatty acid-soap flows increased ($P < 0.05$) from the distal jejunum to the terminal ileum when free fatty acids were included in the diet. Stearic acid soap flow peaked in the terminal ileum and then declined ($P < 0.05$) in the proximal colon, but the stearic acid soap flow from the mid colon to the rectum (or in the faeces) was not significantly different ($P < 0.05$) from the ileal stearic acid soap flow when the free fatty acid diet was fed. No significant change ($P > 0.05$) in the palmitic acid-soap flow was observed from the ileum to the rectum (or in the faeces). Introducing stearic acid in the form of tallow also led to increased ($P < 0.05$) soap flow throughout the gastrointestinal tract with the highest soap flow occurring in the large intestine. Palmitic acid soap flow followed a similar trend to stearic acid soap, however, palmitic acid soap flow was significantly higher ($P < 0.05$) in the ileum compared to the anterior regions of the gastrointestinal tract but did not increase ($P > 0.05$) from the ileum to the rectum (or in the faeces).

Introducing fatty acids in the form of a triglyceride (tallow) resulted in lower ($P < 0.05$) amounts of saturated fatty acid soaps in the distal jejunum, ileum and terminal ileum compared to pigs receiving the free fatty acid diet. The saturated fatty acid-soap flows at the distal small intestine of the pigs receiving the free fatty acid-based diet were double those observed for the pigs receiving the tallow-based diet. Furthermore, the flow of palmitic acid soaps in the medial and distal colon, the rectum and in the faeces were significantly lower ($P < 0.05$) when pigs received the tallow-based diets, whereas no differences ($P > 0.05$) in the flow of stearic acid soaps in the hindgut locations were observed between the two diets.

For the pigs receiving the free fatty acid-based diet the flow of oleic acid soap increased ($P < 0.05$) from the proximal jejunum to the distal jejunum and then decreased ($P < 0.05$) along the small intestine to the terminal ileum. There was no further change ($P > 0.05$) in the

oleic acid soap flow from the terminal ileum to the rectum. For the pigs receiving the tallow-based diet, oleic acid soap flow increased ($P < 0.05$) from the stomach/duodenum to the distal jejunum, but the oleic acid soap flow did not change ($P > 0.05$) along the remainder of the intestinal tract. No net oleic acid soap formation occurred in the large intestine and the oleic acid soap flow in the hindgut did not differ ($P > 0.05$) between the free fatty acid- and tallow-based diets. However, the oleic acid soap flow was higher ($P < 0.05$) in the proximal and distal jejunum, ileum and terminal ileum for pigs receiving the free fatty acid-based diet compared to the tallow-based diet.

A dependency of saturated fatty acid soap formation on the pH of the gastrointestinal tract was observed. There was a significant correlation between palmitic acid soap flow ($r^2 = 0.67$; $P < 0.001$; $n = 159$) as well as stearic acid soap flow ($r^2 = 0.72$; $P < 0.001$; $n = 159$) and pH (pH data are given in Table 6-3). There were greater amounts of saturated fatty acid soaps present in the more distal regions of the gastrointestinal tract where the pH was higher, compared to the stomach and proximal regions of the small intestine where the pH was lower. Furthermore, there was a significant but weak correlation ($r^2 = 0.26$; $P < 0.05$; $n = 159$) between oleic acid soap flow and pH.

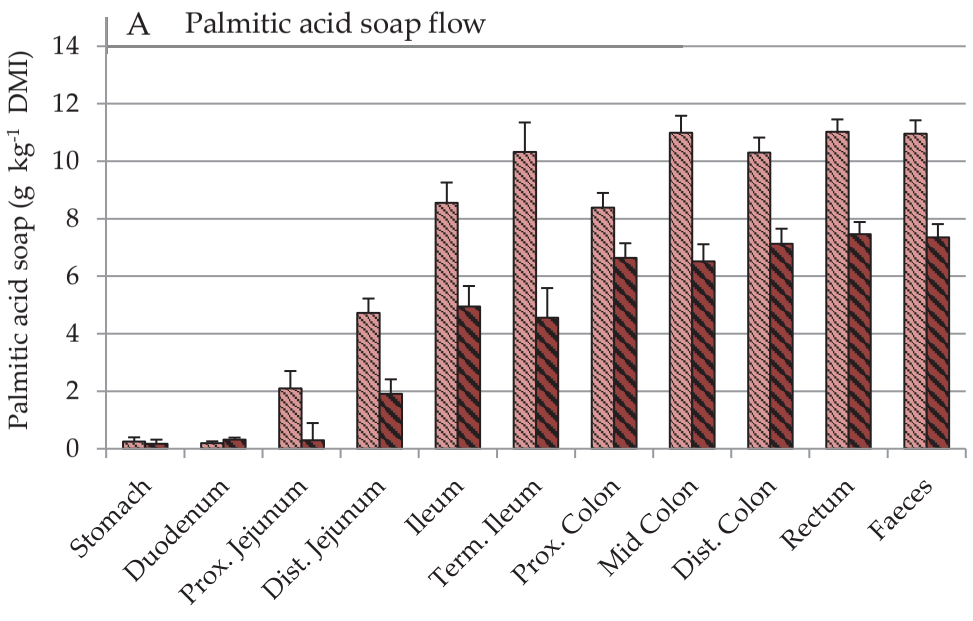
The amount of soaps formed with endogenous fatty acids (data not shown) was very minor. Endogenous fatty acid soap present at the terminal ileum was approximately 100 mg kg^{-1} DMI and comprised of 55 mg kg^{-1} of palmitic acid soap, 24 mg kg^{-1} DMI of stearic acid soap and 30 mg kg^{-1} DMI of oleic acid soap. The presence of endogenous fatty acid soaps increased throughout the hind gut, presumably due to fatty acid soap formation with endogenous fatty acids arising from microbes and endothelial cells. The flow of endogenous

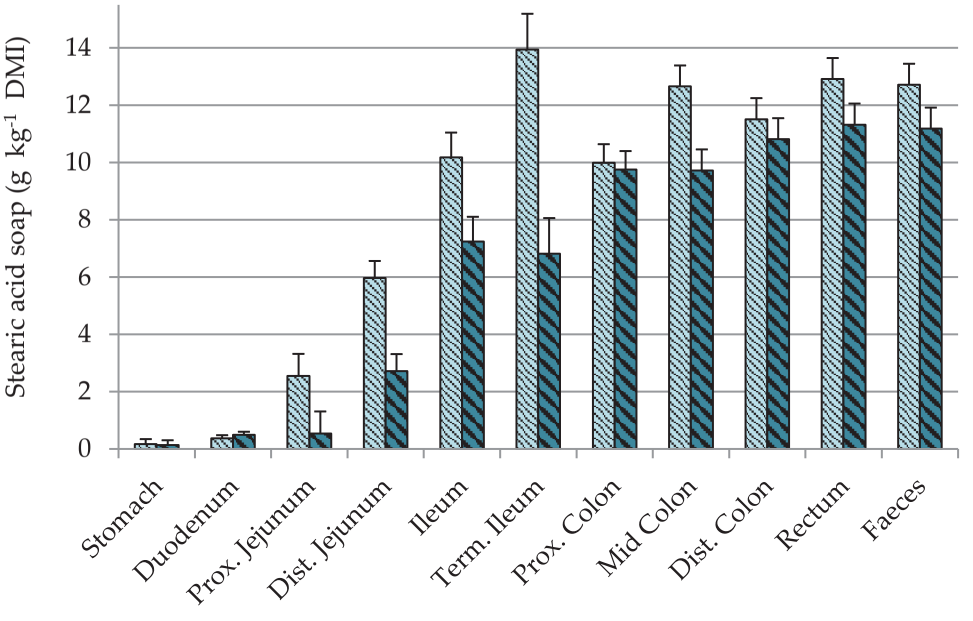
fatty acid soaps in the hindgut was approximately 440 mg kg^{-1} DMI with 210 mg of palmitic acid, 190 mg of stearic acid and 40 mg of oleic acid making up the endogenous soaps.

Table 6-4: Mean (n = 8) total[1] fatty acid soap flows for the experimental diets as determined throughout the gastrointestinal (GI) tract.

	Diet			Diet			Diet		
GI location	A[2]	B[3]	Overall SE	A[2]	B[3]	Overall SE	A[2]	B[3]	Overall SE
	Palmitic acid-soap (g kg^{-1} DMI)			Stearic acid-soap (g kg^{-1} DMI)			Oleic acid-soap (g kg^{-1} DMI)		
Stomach	0.25^{c}	0.17^{c}	0.146	0.09^{d}	0.06^{d}	0.173	0.54^{d}	0.15^{b}	0.061
Duodenum	0.19^{c}	0.32^{c}	0.067	0.27^{d}	0.39^{d}	0.110	0.40^{d}	0.29^{b}	0.106
Prox Jejunum	2.10^{bc}	0.29^{c}	0.603	2.55^{cd}	0.54^{d}	0.768	1.82^{bcx}	0.49^{aby}	0.403
Dist Jejunum	4.72^{bx}	1.91^{bcy}	0.504	5.97^{cx}	2.72^{cdy}	0.590	3.00^{ax}	1.71^{ay}	0.292
Ileum	8.55^{ax}	4.95^{aby}	0.710	10.18^{abx}	7.24^{by}	0.862	2.13^{abx}	1.25^{ay}	0.208
Term Ileum	10.32^{ax}	4.56^{aby}	1.029	13.94^{ax}	6.81^{by}	1.248	1.93^{bcx}	0.73^{aby}	0.264
Prox Colon	8.39^{a}	6.64^{a}	0.508	9.99^{b}	9.75^{a}	0.647	1.05^{cd}	0.93^{ab}	0.116
Mid Colon	10.99^{ax}	6.52^{ay}	0.593	12.65^{a}	9.72^{a}	0.734	1.30^{cd}	0.88^{ab}	0.142
Dist Colon	10.30^{ax}	7.13^{ay}	0.524	11.51^{ab}	10.81^{a}	0.816	1.12^{cd}	0.94^{ab}	0.122
Rectum	11.03^{ax}	7.46^{ay}	0.426	12.91^{a}	11.32^{a}	0.568	1.37^{bc}	1.03^{ab}	0.141
Faeces	10.96^{ax}	7.35^{ay}	0.464	12.71^{a}	11.18^{a}	0.593	1.36^{bc}	1.02^{ab}	0.144
Statistical Analysis	Significance[4]			Significance[4]			Significance[4]		
Diet	***			***			***		
GI location	***			***			**		
Diet × location interaction	***			**			**		

[1]Total fatty acid soap flow regardless of divalent cation
[2]Diet A: experimental diet including free fatty acids as its fat source
[3]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[4] *** $P < 0.001$; ** $P < 0.01$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).





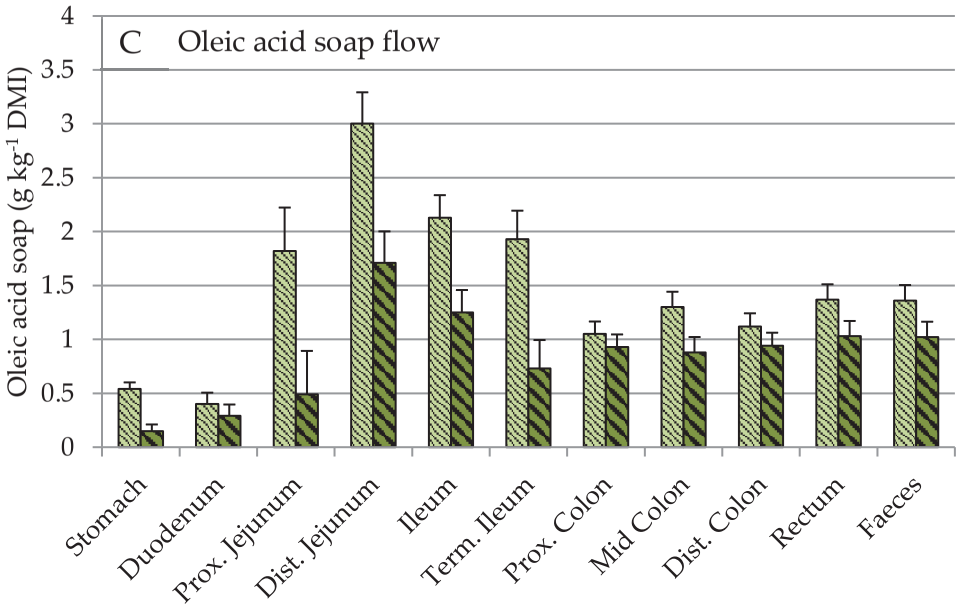


Figure 6-1: Mean total fatty acid soap flow (n=8) for ■ palmitic (A), ■ stearic (B) and ■ oleic (C) acid determined throughout the gastrointestinal tract of the growing pig receiving experimental diets containing free fatty acids (FFA) ▨ or tallow ▨.
For statistical differences between gastrointestinal location and dietary treatment refer to Table 4.

6.4.4. Ca flow and apparent Ca digestibility along the gastrointestinal tract

There was no significant interaction ($P > 0.05$) between diet and gastrointestinal location for Ca flow or apparent Ca digestibility (Table 6-5). For Ca flow and the apparent Ca digestibility there was a significant ($P < 0.01$) effect of diet and gastrointestinal location. The Ca flow did not differ ($P > 0.05$) between the diet containing free fatty acids (Diet A) or the intact triglyceride tallow diet (Diet B). However, the Ca flow in the gastrointestinal tract was higher ($P < 0.05$) for the fatty acid containing diets (Diet A and B) compared to that for the fat-free diet (Diet C). Similarly, apparent Ca digestibility was approximately 20% lower ($P < 0.05$) for the pigs receiving the fat containing diets compared to the pigs receiving the fat-free diet. The disappearance of dietary Ca from the gastrointestinal tract occurred predominantly in the proximal regions of the small intestine (duodenum and proximal jejunum) resulting in an overall apparent digestibility of approximately 70% in the proximal jejunum. An increase ($P < 0.05$) in Ca flow was observed when digesta passed from the terminal ileum into the hind gut but the Ca flow of the rectum/faeces was not significantly different ($P > 0.05$) from the Ca flow determined at the terminal ileum across the three experimental diets. The apparent ileal and faecal Ca digestibility did not differ significantly ($P > 0.05$) and was approximately 60%.

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.

	Ca ($g\ kg^{-1}$ DMI)	Overall SE	Apparent Ca digestibility (%)	Overall SE
Diet	Main Effect of GI location			
A[1]	5.21[a]	0.175	45.2[b]	2.31
B[2]	4.93[a]	0.177	46.0[b]	2.32
C[3]	4.12[b]	0.179	56.7[a]	2.32
GI location	Main Effect of diet			
Stomach	9.32[a]	0.468	0.3[g]	4.71
Duodenum	5.39[bc]	0.537	42.4[bef]	5.31
Prox Jejunum	2.57[f]	0.208	72.4[a]	2.06
Dist. Jejunum	2.86[f]	0.377	69.4[ac]	3.76
Ileum	3.43[def]	0.195	63.8[acd]	2.60
Term. Ileum	3.06[ef]	0.254	67.4[ac]	2.78
Prox Colon	6.77[b]	0.509	27.5[f]	5.06
Mid Colon	4.92[c]	0.238	48.1[e]	2.39
Dist. Colon	4.84[cd]	0.228	49.9[e]	2.27
Rectum	4.49[cde]	0.283	51.8[de]	2.86
Faeces	4.67[cde]	0.400	50.6[cde]	3.87
Statistical Analysis	Significance[4]			
Diet	**		**	
GI location	***		***	
Diet × location interaction	NS		NS	

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] *** $P < 0.001$; ** $P < 0.01$; NS – not sigificant
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

6.4.5. P flow and apparent P digestibility along the gastrointestinal tract

For the flow of P in the gastrointestinal tract, a significant interaction ($P < 0.01$) between the experimental diet and gastrointestinal location was observed (Table 6-6). Total P decreased ($P < 0.001$) throughout the gastrointestinal tract of the pigs for each of the experimental diets. The P flow did not differ between the proximal regions of the gastrointestinal tract nor across the three experimental diets in the stomach, duodenum and proximal jejunum. However, the P flow in the ileum was statistically significantly lower for the pigs receiving the fat containing diets (diet A and B) compared to pigs receiving the fat-free diet (Diet C). The P flow in the hindgut was not different ($P > 0.05$) between the three experimental diets with the exception of the proximal colon, where the P flow was lower ($P < 0.05$) for the pigs receiving the free fatty acid diet (Diet A) compared to pigs receiving the fat-free diet (Diet C). Faecal P flow for the pigs receiving the free fatty acid diet was also significantly lower ($P < 0.05$) compared to pigs fed the fat-free diet.

There was an interaction ($P < 0.001$) between the experimental diet and gastrointestinal location for apparent P digestibility (Table 6-6). Apparent P digestibility increased ($P < 0.05$) throughout the small intestine, but no further P absorption occurred in the hind gut. Moreover, the latter increase in digestibility from the duodenum to the distal jejunum was not different ($P > 0.05$) across all three experimental diets but P digestibility was significanty ($P < 0.05$) higher in the ileum for pigs receiving fat containing diets (Diets A and B). Furthermore, apparent P digestibility was generally higher ($P < 0.05$) throughout the hind gut when pigs received the fat containing diets (Diets A and B) compared to pigs fed the fat-free diet (Diet C).

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.

GI location	Diet			Overall SE	Diet			Overall SE
	A[1]	B[2]	C[3]		A[1]	B[2]	C[3]	
	P (mg kg^{-1} DMI)				Apparent P Digestibility (%)			
Stomach	3241^{a}	3580^{a}	3744^{a}	146.3	24.6^{d}	19.5^{d}	6.4^{d}	3.42
Duodenum	3178^{a}	3440^{a}	3617^{ab}	238.3	26.1^{d}	22.7^{d}	9.6^{cd}	5.78
Prox. Jejunum	2329^{ab}	2604^{ab}	2359^{bc}	274.6	45.8^{cd}	41.5^{cd}	41.0^{bc}	6.53
Dist. Jejunum	1207^{b}	1330^{bc}	1147^{cd}	172.2	71.9^{bc}	70.1^{bc}	71.3^{ab}	4.00
Ileum	572^{bx}	658^{cdx}	985^{dy}	111.7	86.7^{aby}	85.2^{aby}	75.3^{az}	2.71
Term. Ileum	560^{bc}	537^{cd}	732^{d}	126.5	87.0^{ab}	87.9^{ab}	81.7^{a}	2.98
Prox. Colon	404^{cx}	435^{dxy}	678^{dy}	45.7	90.6^{ay}	90.2^{ay}	83.0^{az}	2.98
Mid Colon	437^{c}	460^{d}	648^{d}	48.3	89.8^{ab}	89.7^{a}	83.8^{a}	1.17
Dist. Colon	424^{c}	448^{d}	645^{d}	53.1	90.1^{aby}	89.9^{ay}	83.9^{az}	1.30
Rectum	412^{c}	425^{d}	607^{d}	74.7	90.4^{aby}	90.4^{ay}	84.8^{az}	1.85
Faeces	381^{cx}	447^{dxy}	667^{dy}	74.9	91.1^{ay}	90.0^{ay}	83.3^{az}	1.83
Statistical Analysis	Significance				Significance			
Diet	*				**			
GI location	***				***			
Diet × location interaction	**				***			

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).
[x,y] Within a row, means without a common superscript differ ($P < 0.05$).

6.4.6. Mg flow and apparent Mg digestibility along the gastrointestinal tract

The flow and apparent digestibility of Mg had a similar trend to that observed for Ca. There was no significant interaction ($P > 0.05$) between diet and gastrointestinal location (Table 6-7). The Mg flow and the apparent Mg digestibility differed ($P < 0.01$) across the three experimental diets and also differed ($P < 0.001$) across the ten gastrointestinal locations. The Mg flow across the different gastrointestinal locations and faeces did not differ ($P > 0.05$) between pigs fed the diets containing free fatty acids (Diet A) or the intact triglyceride tallow (Diet B). The Mg flow in the gastrointestinal tract was significantly higher ($P < 0.01$) for the fatty acid containing diets (Diet A and B) compared to that for the fat-free diet (Diet C). Similarly, apparent Mg digestibility was approximately 40% lower ($P < 0.05$) in the pigs receiving the fat containing diets compared to the pigs receiving the fat-free diet. The Mg flow did not differ ($P > 0.05$) across the small intestine for the three experimental diets but the Mg flow at the distal hind gut (distal colon, rectum) and faeces was significantly lower ($P < 0.05$) compared to the Mg flow found at the terminal ileum. The apparent digestibility of Mg reached a low point at the end of the ileum, but increased throughout the hindgut, to reach a value at the faecal level similar to values obtained in the upper gut. Faecal apparent Mg digestibility was approximately 23% for the three experimental diets.

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.

	Mg (mg kg^{-1} DMI)	Overall SE[4]	Apparent Mg digestibility (%)	Overall SE
Diet	Main Effect of GI location			
A[1]	475.8^{a}	13.37	15.8^{b}	2.31
B[2]	475.3^{a}	13.43	12.0^{b}	2.32
C[3]	412.1^{b}	13.41	25.1^{a}	2.32
GI location	Main Effect of diet			
Stomach	460.8^{abc}	12.18	16.4^{abc}	4.71
Duodenum	435.9^{abc}	29.80	21.0^{abc}	5.31
Prox. Jejunum	441.8^{abc}	37.87	19.9^{abc}	2.06
Dist. Jejunum	466.8^{abc}	36.72	15.4^{abc}	3.76
Ileum	495.4^{a}	16.82	10.2^{c}	2.60
Term. Ileum	527.8^{ab}	28.82	4.3^{bc}	2.78
Prox. Colon	463.6^{abc}	7.75	15.9^{abc}	5.06
Mid Colon	431.5^{bc}	8.43	21.8^{ab}	2.39
Dist. Colon	428.9^{c}	7.61	22.2^{a}	2.27
Rectum	420.9^{c}	11.75	23.7^{a}	2.86
Faeces	425.0^{c}	11.01	22.9^{a}	3.87
Statistical Analysis	Significance[4]			
Diet	**		**	
GI location	***		***	
Diet × location interaction	NS		NS	

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] *** $P < 0.001$; ** $P < 0.01$; NS – not significant
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

6.4.7. Trace mineral (Fe, Zn and Cu) flow and apparent digestibility along the gastrointestinal tract

The Fe, Zn and Cu flows at each location of the gastrointestinal tract did not differ ($P > 0.05$) between the three experimental diets, however apparent Fe and Zn digestibility did differ ($P < 0.05$) across the experimental diets (Table 6-8, 6-9 and 6-10). Furthermore, the flows of Fe, Zn and Cu and apparent Fe, Zn and Cu digestibility differed ($P < 0.001$) across the gastrointestinal locations. Apparent Fe digestibility was higher ($P < 0.01$) for the fat-free diet compared to the free fatty acid diet. A similar trend was observed for Zn, where the apparent Zn digestibility was higher ($P < 0.05$) for the fat-free diet compared to both fat containing diets. There were no significant interactions ($P > 0.05$) between diet and gastrointestinal location for any of the trace minerals. The mean trace mineral flow across gastrointestinal locations for the three experimental diets was 279 mg, 334 mg and 23 mg kg^{-1} DMI for Fe, Zn and Cu, respectively. The disappearance of dietary Fe, Zn and Cu from the gastrointestinal tract occurred in the proximal regions of the small intestine, resulting in an apparent digestibility of approximately 14% for Fe in the proximal jejunum and of approximately 23% and 15% for Zn and Cu in the duodenum, calculated across all three experimental diets. However, no further disappearance of the trace minerals was observed but rather an increase in Fe, Zn and Cu flow was found in the further distal regions of the intestinal tract. Neither flow nor apparent digestibility of the trace minerals differed ($P > 0.05$) between the gastrointestinal locations of the stomach and the faeces.

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.

	Fe (mg kg^{-1} DMI)	Overall SE	Apparent Fe digestibility (%)	Overall SE
Diet	Main Effect of GI location			
A	279.8	4.61	0.06[b]	1.29
B	280.5	4.69	1.56[ab]	1.34
C	276.8	4.67	6.19[a]	1.34
GI location	Main Effect of diet			
Stomach	284.4[a]	7.12	0.7[c]	2.66
Duodenum	253.3[bc]	8.53	11.6[ab]	3.15
Prox. Jejunum	246.1[c]	8.21	14.0[a]	3.05
Dist. Jejunum	265.1[abc]	14.18	7.4[abc]	5.28
Ileum	306.9[ab]	12.10	-6.4[bc]	4.70
Term. Ileum	301.0[abc]	14.29	-2.6[abc]	5.00
Prox. Colon	283.1[abc]	5.82	1.2[abc]	2.19
Mid Colon	282.1[ab]	3.89	1.5[bc]	1.45
Dist. Colon	283.9[ab]	5.00	0.9[bc]	1.85
Rectum	278.4[abc]	5.47	2.7[abc]	2.00
Faeces	285.2[a]	3.74	0.4[c]	1.40
Statistical Analysis		Significance[4]		
Diet		NS	*	
GI location		***	***	
Diet × location interaction		NS	NS	

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] *** $P < 0.001$; * $P < 0.05$; NS – not significant
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

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.

	Zn (mg kg^{-1} DMI)	Overall SE	Apparent Zn digestibility (%)	Overall SE
Diet	Main Effect of GI location			
A	341.6	7.80	-3.9^{b}	1.59
B	280.5	4.69	-3.8^{b}	1.62
C	276.8	4.67	3.9^{a}	1.61
GI location	Main Effect of diet			
Stomach	327.5^{a}	7.87	0.7^{bc}	2.35
Duodenum	254.5^{c}	9.73	22.9^{a}	3.53
Prox. Jejunum	288.5^{bc}	11.21	12.5^{ab}	3.87
Dist. Jejunum	357.6^{ab}	27.66	-8.6^{c}	8.47
Ileum	392.1^{a}	23.05	-18.7^{c}	6.88
Term. Ileum	380.6^{a}	19.15	-16.7^{c}	5.91
Prox. Colon	327.3^{ab}	6.28	0.8^{bc}	1.91
Mid Colon	333.9^{ab}	7.71	-1.3^{bc}	2.31
Dist. Colon	337.7^{ab}	9.43	-2.4^{bc}	2.83
Rectum	328.6^{abc}	10.00	0.3^{bc}	2.97
Faeces	339.7^{a}	7.28	-3.1^{bc}	2.19
Statistical Analysis	Significance[4]			
Diet	NS		*	
GI location	***		***	
Diet × location interaction	NS		NS	

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] *** $P < 0.001$; * $P < 0.05$; NS – not significant
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

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.

	Cu (mg kg^{-1} DMI)	Overall SE	Apparent Cu digestibility (%)	Overall SE
Diet	Main Effect of GI location			
A	22.1	0.68	-0.2	2.75
B	23.7	0.69	3.3	2.76
C	23.5	0.69	6.9	2.76
GI location	Main Effect of diet			
Stomach	23.0^{abc}	0.73	0.9^{bc}	3.43
Duodenum	19.8^{c}	1.05	14.9^{a}	5.00
Prox Jejunum	23.6^{abc}	1.26	-1.8^{bc}	5.95
Dist Jejunum	21.8^{abc}	1.12	5.2^{abc}	5.23
Ileum	22.1^{bc}	0.64	2.7^{bc}	2.91
Term. Ileum	22.3^{abc}	1.43	3.9^{abc}	6.75
Prox Colon	23.3^{abc}	0.76	6.6^{abc}	3.06
Mid Colon	24.4^{ab}	0.50	2.1^{bc}	1.94
Dist Colon	24.3^{ab}	0.45	2.4^{bc}	1.85
Rectum	23.6^{ab}	0.67	5.2^{abc}	2.80
Faeces	25.2^{a}	0.42	-1.4^{c}	1.83
Statistical Analysis		Significance[4]		
Diet	NS		NS	
GI location	**		**	
Diet × location interaction	NS		NS	

[1]Diet A: experimental diet including free fatty acids as its fat source
[2]Diet B: experimental diet including an intact triglyceride (tallow) as its fat source
[3]Diet C: experimental control diet devoid of fat
[4] ** $P < 0.01$; NS – not significant
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

6.5. Discussion

The present finding that there is no difference in the pH of the proximal and distal regions of the stomach is in accordance with the conclusions of Merchant *et al.* (2011) who studied the gastrointestinal pH in male pigs fed a mixed diet, but is in contrast to the work of Bornhorst *et al.* (2013a; 2013b) where a significant difference in pH between the gastric proximal and distal site was reported when pigs were fed rice or almonds. The different values for pH in different regions of the stomach found in the latter studies may be a result of the particle size of the diet. The synthetic diet used in the presently reported study was a fine powder diet whereas the rice and almond diets contained much larger particles. In a previously reported study (Maxwell *et al.* 1970) comparing diets containing coarse or finely ground corn meal, a pH gradient from the proximal to distal stomach sites was observed for pigs receiving the coarse corn meal diet. The pH of the stomach in the pigs fed the finely ground corn meal was uniform over three measuring sites at all observed postprandial time points (Maxwell *et al.* 1970).

The pH of the small intestine in our study ranged from 5, for the proximal regions of the intestinal tract, to 7, for the distal regions. A small decrease in pH was observed in the proximal regions of the hind gut as compared to the distal regions of the small intestine. A similar trend has been reported previously in pigs (Merchant *et al.* 2011), as well as in other animal species and humans (Evans *et al.* 1988). The lower pH of the hind gut has been explained by the bacterial breakdown of cellulose and proteins into short chain fatty acids (Merchant *et al.* 2011). The pH of the remaining large intestine (distal colon and rectum) was on average 6.4 across the three diets.

Fatty acid soap formation occurred throughout the upper gastrointestinal tract when diets containing either free fatty acids or intact triglycerides were fed to the growing pigs. However, the fatty acid soap flow was greater when free fatty acids were present in the diet compared to when the fatty acids were present as part of a triglyceride molecule (tallow), especially in the small intestine. This would be expected since in the intact triglycerides the carboxyl groups of the fatty acids involved in the Ca interactions are bound to the glycerol molecule. Consequently, in the gastrointestinal tract, triglycerides must first be hydrolysed, before the fatty acids can form soaps. Even though lipid hydrolysis begins in the stomach where gastric lipase cleaves fatty acids (preferably of short and medium chain length) from the sn-3 position of the triglyceride molecule, it is only a relatively small percentage (between 10 to 30%) of the molecule that enters the duodenum in the form of free fatty acids. The majority of fat digestion occurs in the small intestine by the action of pancreatic lipase, which cleaves fatty acids from both the sn-1 and sn-3 positions of the triglyceride. The fatty acids in the free fatty acid-based diet would have entered the small intestine (where the pH favours fatty acid soap formation) in their free form and would therefore have been available for soap formation immediately. In contrast esterified fatty acids, such as those present in the tallow-based diet, would need to undergo lipid hydrolysis prior to forming complexes with ionised Ca, which explains the delayed increase in fatty acid soap flows observed for the tallow-based diet.

The flow of fatty acid soaps containing saturated fatty acids but not unsaturated fatty acids was correlated with the pH of the gastrointestinal contents. As the pH increased along the small intestine so did the flow of the palmitic and stearic acid soaps. Palmitic and stearic acid soap formation reached its peak at the terminal ileum (the gastrointestinal region with the

highest pH) and did not change throughout the large intestine, when the free fatty acid-based diet was fed to the pigs. The presence of saturated fatty acid soaps increased throughout the small intestine when pigs received the tallow-based diet, however the fatty acid soap flow increased further in the hind gut and this increase was statistically significant for stearic acid. Oleic acid soap formation followed a quite different pattern compared to palmitic and stearic acid. Oleic acid soap flow, though increasing in the first half of the small intestine, did not increase further with rising pH. Luminal pH has been addressed as a major factor playing a role in soap formation in the gastrointestinal tract but is not the only affecting factor. Correlation analyses suggest that saturated fatty acid soap formation depends on pH to a greater degree than the formation of oleic acid soaps. Other factors, such as the presence of bile salts and the solubility of Ca-fatty acid soaps, need to be taken into consideration.

Fatty acid soap formation was greater for the saturated fatty acids (palmitic and stearic acid) than for the unsaturated fatty acid (oleic acid) included in this study. Saturated fatty acids formed soaps throughout the small intestine and to a certain extent throughout the large intestine while unsaturated fatty acids formed soaps only in the first half of the small intestine, with no further soap formation observed in the hind gut. The different behaviour of saturated and unsaturated fatty acids for soap formation in the small intestine is likely due to the difference in the solubility of fatty acid soaps depending on the degree of saturation of the fatty acid involved. It has been reported that under *in vitro* simulated gastrointestinal conditions, Ca-fatty acid soaps containing saturated-long chain fatty acids are much more insoluble than soaps containing unsaturated fatty acids of the same chain length (Gacs and Barltrop 1977; Graham and Sackman 1983). Similar results have also been observed with pre-

synthesised Ca-fatty acid soaps infused directly into the intestinal tract (Gacs and Barltrop 1977). An initial formation of oleic acid soap was observed for the proximal and distal jejunum, which indicates the capability of oleic acid to form soaps *in vivo* but the declining oleic acid soap flow from the ileum to the hind gut suggests that the initally formed oleic acid soaps are soluble in the intestinal environment. The increased solubility of oleic acid soaps in the more distal regions of the small intestine might be due to the increasing bile salt concentration of the digesta (as a result of nutrient absorption from the ileum) because unsaturated fatty acid soaps have been reported to be highly soluble in the presence of bile salts. A possible formation of Ca-oleic acid soaps has been reported in bile duct ligated rats consuming olive oil where the absorption of Ca was reduced by 22% compared to that in non-bile duct ligated rats. The major fatty acid of olive oil, namely oleic acid, appeared to have the ability to complex with Ca in the absence of bile salts in the gastrointestinal tract of the bile duct ligated rats.

Ca-fatty acid soap formation has been discussed as a mechanism to reduce fat absorption. If soap formation is responsible for reducing fatty acid absorption, the Ca-fatty acid soaps need to complex prior to the absorption site of fatty acids. Once fatty acids are cleaved from the triglyceride they are integrated into mixed micelles and transported to the brush border where fatty acids are absorbed into the epithelial cells of the small intestine. The formation of insoluble Ca-fatty acid soaps is suspected to impair micelle formation and the fatty acids complexed with Ca would therefore not be transported to the epithelial cells, and fat absorption would be reduced. Fatty acid soap formation in pigs receiving the free fatty acid-based diet peaked at the terminal ileum, which would suggest that fatty acids complexed with Ca were not fully absorbed, resulting in reduced fatty acid absorption. On the other

hand, stearic acid soaps in pigs receiving the tallow-based diet increased posterior to the terminal ileum, indicating soap formation occurred in the hind gut. Soap formation past the absorption site of fat, namely the small intestine, will not reduce fat absorption (because the fatty acids would not be absorbed anyway) and therefore not contribute to reduced energy absorption or weight loss.

The indirect method of fatty acid-soap detection used here did not determine which component the fatty acids were complexed with, however, Ca-fatty acid soaps have been reported to be present in human faeces in previous studies whereas other divalent cation-fatty acid soaps have not been isolated in the past. Analysis of the luminal mineral content might give further insight in to which divalent cations are involved in fatty acid soap formation. The divalent cations Ca, Mg, Zn, Fe and Cu and P were determined throughout the ten locations of the gastrointestinal tract and in the faeces. The minerals Ca and Mg showed similar behaviours in response to the three experimental diets, whereby pigs receiving the fat-free diet had lower flows of the latter minerals in the intestinal tract and concomitantly a higher mineral digestibility when compared to the pigs receiving the diets containing either free fatty acids or tallow. Furthermore, the apparent digestibility of the trace minerals Fe and Zn was higher when pigs were fed the fat free diets. Gastrointestinal Cu flows or apparent Cu digestibility did not differ across the diets. The lower apparent mineral digestibility for Ca, Mg, Fe and Zn in conjunction with fat containing diets indicates that divalent cation-fatty acid soap formation does occur within the gastrointestinal tract and the formation of insoluble soaps may have led to the observed lower digestibility of the minerals. Apparent Ca digestibility was approximately 20% lower in pigs receiving the free fatty acid-based diet and the tallow-based diet compared to pigs receiving the fat-free diet.

Reduced Ca digestibility in the presence of fat has been reported previously in mature rats (Calverley and Kennedy 1949; Tadayyon and Lutwak 1969b; Kaup *et al.* 1990). For example, the addition of 5 g 100 g^{-1} fat to the experimental diets of rats increased faecal Ca by 35% compared to the fat-free diet. The latter fat source contained approximately 25% of saturated long-chain fatty acids, whereas adding a less saturated fat (> 10% saturated long-chain fatty acids) to the diets did not increase faecal Ca excretion (Calverley and Kennedy 1949). As observed in the presently reported study, the unsaturated fatty acid oleic acid was not a major contributor to fatty acid-soap formation, whereas the saturated long-chain fatty acids, palmitic and stearic acid, were present as soaps in digesta and faeces.

Apparent Mg digestibility was 44% lower across the ten gastrointestinal locations and faeces when pigs received the free fatty acid-based diet or the tallow-based diet compared to pigs receiving the fat-free diet. These results are in agreement with findings in rats, where the apparent faecal Mg digestibility was reduced by more than 40% when diets containing 25 g of fat per 100 g (in form of tripalmitin or tristearin) were compared to fat-free diets (Tadayyon and Lutwak, 1997).

Similar to Ca and Mg, the higher apparent Zn digestibility as observed for pigs receiving the fat free diet compared to pigs receiving the fat containing diets indicates that the presence of fat reduces the apparent digestibility of Zn throughout the gastrointestinal tract. Furthermore, apparent Fe digestibility was significantly higher for pigs receiving the fat free diet compared to pigs receiving the free fatty acid containing diet. Our results regarding the trace elements Fe and Zn are in disagreement with previous studies, which suggest that Fe and Zn absorption is enhanced in the presence of dietary fat (Johnson, 1987). Furthermore, it has been suggested that polyunsaturated fatty acids rather than saturated fatty acids increase

faecal Fe and Zn excretion (Lukaski, 2001). Our experimental diets contained mainly saturated long chain fatty acids and monounsaturated long chain fatty acids, but low amounts of polyunsaturated fatty acids. Further research will be necessary to shed light on Fe and Zn digestibility in conjunction with dietary fat. *In vitro* Fe- and Zn-fatty acid soap formation has been shown to occur under gastrointestinal simulated conditions particularly with saturated fatty acids (refer to Chapter Four). It is possible, therefore, that the presence of stearic and palmitic acid lowered the apparent digestibility of Fe and Zn via fatty acid soap formation.

Of interest, P behaved in a contradictory manner in comparison with the divalent cations and the apparent P digestibility was higher in the presence of dietary fat compared to the fat-free diet. Apparent P digestibility increased throughout the digestive tract for all three experimental diets but was lower from the ileum onwards for pigs receiving the fat free diet. These results indicate that dietary fat had a positive influence on P digestibility which may be the result of less Ca being available to complex in the form of calcium phosphate, as some Ca was complexed with fatty acids. The presence of Ca-fatty acid soaps has been suggested previously to increase P absorption (Telfer 1921). Insoluble calcium phosphate precipitation occurs between pH 6 to 7 (Van der Meer and De Vries 1985) which reflects the pH observed for the ileum (pH 6.5) in the presently reported study. Ca-fatty acid soap formation had already occurred from the jejunum onwards (as suggested by the fatty acid soap data) which might leave less Ca available to precipitate with P in the ileum.

Overall, it can be concluded that the apparent digestibility of most of the tested divalent cations was depressed in the presence of dietary fat. This was most likely due to fatty acid soap formation which occurs from the distal jejunum onwards. Active absorption of dietary

minerals has been reported to occur predominantly in the proximal regions of the small intestine, as was also indicated by our results for most of the minerals tested (disappearance of Ca, Mg, Fe, Zn and Cu from the duodenum). Fatty acid soap formation might not greatly interfere with the active absorption of minerals in the duodenum and jejunum but may reduce passive transport of minerals through the tight junctions which occur at more distal regions of the small intestine. Furthermore, reabsorption of endogenously secreted minerals may be reduced due to divalent-cation fatty acid soap formation and thereby deplete the storage of minerals (particularly Fe and Zn) in the body.

6.6. Conclusion

To our knowledge this is the first study investigating the formation of fatty acid soaps throughout the gastrointestinal tract. Fatty acid soaps were generally present from the distal jejunum onwards and increased throughout the small intestine to the terminal ileum for saturated fatty acids. Stearic acid soap formation increased along the large intestine when pigs were fed the tallow-based diet, whereas no further palmitic and oleic acid soap formation occurred in the hind gut. Different behaviours for saturated and unsaturated fatty acids in regards to soap formation were observed, whereby the saturated fatty acids palmitic and stearic acid formed substantially more soaps. Furthermore, the form of dietary fat (free fatty acids or triglycerides) had an impact on the amount of fatty acid soaps present at the different locations of the intestinal tract, where feeding free fatty acids led to a higher presence of soaps in the distal small intestine. The reduced digestibility of divalent cations (Ca, Mg, Zn and Fe) in the presence of dietary fat is consistent with the formation of divalent cation-fatty acid soaps. The observed formation of fatty acid soaps in the small intestine

suggests that soap formation may be a mechanism for reduced fatty acid absorption, particularly that of saturated fatty acids.

CHAPTER SEVEN: General Discussion

7.1. Summary & Discussion

Obesity and its associated diseases caused more than 2.8 million deaths in 2011 (WHO 2011) and is a leading health issue for many countries worldwide. The overconsumption of dietary fat is one of the primary causes of obesity and, therefore, dietary interventions by which fat absorption can be reduced have received much attention in both the scientific community and in the food and health industries.

Dietary Ca has been associated with reduced serum lipids, reduced body weight and body fat, an effect which may be related to the ability of ingested Ca to bind to free fatty acids in the gastrointestinal tract (once released from the triglyceride molecule during digestion) and form insoluble indigestible Ca-fatty acid soaps. Few studies, however, have investigated the proposed mechanism for the latter phenomenon, largely because Ca-fatty acid soaps are difficult to determine analytically. While there are published methods for determining Ca-fatty acid soaps in biological material such as faeces, none appear to be ideal for the quantitative assessment of fatty acid-soap formation. Consequently, the objective of the first study reported here (Chapter Three) was to investigate aspects of the current methods described in the literature for isolating Ca-fatty acid soaps, and if necessary develop a new or revised method. A novel method for the indirect determination of fatty acid soaps in faeces and digesta was developed as described in Chapter Three and this method was applied to quantify fatty acid-soaps in faeces (Chapter Four) and in gastric chyme, intestinal digesta and faeces (Chapter Six).

The work described in Chapter Four of this dissertation investigated the effect of different dietary Ca concentrations on the presence of fatty acid-soaps in the faeces of growing pigs when the dietary fatty acids were given in the form of four different dietary fat sources, while for the work described in Chapter Six, the fatty acid-soaps were determined throughout the entire gastrointestinal tract of the growing pig to ascertain where in the gastrointestinal tract Ca-fatty acid soaps form. In addition to studying Ca in relation to the formation of fatty acid-soaps, other nutritionally important divalent cations were also examined to determine whether they also are able to form fatty acid-soaps. In that vein, an *in vitro* experiment investigating the precipitation of fatty acids in the presence of a range of nutritionally important divalent cations (Ca, Mg, Zn, Fe, Cu) was carried out (Chapter Five). Furthermore, the effect of the dietary fat source on the faecal excretion and faecal digestibility of divalent cations was also investigated as a secondary aim in the *in vivo* studies reported in Chapters Four and Six.

To investigate the formation of Ca-fatty acid soaps in the gastrointestinal tract, a method is required that can quantify soaps within the gastrointestinal contents (digesta and faeces). A small number of indirect methods for the quantification of insoluble fatty acid-soaps have been used in the past, but only two direct methods for extracting Ca-fatty acid soaps have been published. In most cases the indirect methods were not thoroughly validated. In particular, the determination of the recovery of soaps was not tested. The most promising published method (Sammons and Wiggs 1960) used diethyl ether to extract Ca-fatty acid soaps and this method was investigated as part of the present work. The recovery of Ca-stearic acid soap from faeces using the latter method was only 68% while the recovery of Ca-oleic acid soap was even lower (32%). A considerable proportion of the Ca-fatty acid

soaps (30 - 40%) remained in the aqueous-faecal phase. Additionally, approximately 20% of oleic acid (dissociated from its soap) was recovered in the diethyl ether phase, further suggesting that the latter method was not suitable for application in the presently described research. After several attempts to develop a direct method for the quantification of the Ca-fatty acid soap complex failed, an indirect method to determine the amount of insoluble fatty-acid soaps in digesta and faeces was developed. The indirect method aimed to extract all of the fatty acid containing compounds, (eg. tri-, di- and monoglycerides, free fatty acids, phospholipids and monovalent cation soaps, eg. Na-, K-fatty acid soaps) but leave behind the Ca-fatty acid soaps. This work described in Chapter Three focused on evaluating a range of solvents in terms of their ability to solubilise all of the different fatty acid-containing compounds present in faeces and digesta. Potential solvents (one for which Ca-fatty acid soaps were insoluble but other fatty acid-containing compounds were soluble) were identified. In the present work, the solvents chloroform and methanol were deemed to be unsuitable solvents for extracting non-soap fatty acids since synthetic Ca-fatty acid soaps were relatively soluble in the latter solvents. Interestingly, a chloroform-methanol (2:1) mixture has frequently been used by other workers to extract non-soap fatty acids as a means of determining Ca-fatty acid soaps from faeces. Based on the present work, however, the latter indirect soap assays would underestimate the amount of soaps present, due to a high solubility of Ca-fatty acid soaps in the solvent mixture used, particularly for soaps of unsaturated fatty acids. The Ca-fatty acid soaps would be co-extracted along with the non-soap fatty acids. The solvents that were found to be the most suitable for an indirect determination of Ca-fatty acid soaps were hexane, isopropanol and water, due to the low solubility of Ca-fatty acid soaps in the latter solvents but high solubility of other fatty acid containing compounds. The method developed here uses hexane to extract the non-polar

lipids (triglycerides and fatty acids), a hexane-isopropanol mixture to extract the polar lipids (phospholipids and monoglycerides) and water to extract the Na/K-fatty acid salts. To avoid solubilising Ca-fatty acid soaps containing unsaturated fatty acids, the extraction was performed at room temperature while for the Ca-fatty acid soaps containing saturated fatty acids, the extraction was performed at elevated temperatures (hexane incubation at 50°C, hexane-isopropanol incubation at 37°C, water incubation at 80°C). The newly developed method was found to be suitable for determining Ca-fatty acid soaps in digesta and faeces with the recovery of added Ca-fatty acid soaps being in excess of 90% across all of the soaps tested. Nevertheless, and although being valid under the conditions found in the presently discussed studies, the new method does have limitations. The new method still is an indirect one and does not measure the amount of Ca-fatty acid soaps *per se*. Moreover, the presence of other divalent cations could potentially interfere with the results. The method was developed for digesta and faeces of pigs receiving purified diets. Further method evaluation and development might be required for faeces obtained from humans consuming a mixed food diet where the lipid components are intrinsic parts of biological material.

A study was undertaken (Chapter Four) using the growing pig as an animal model for the adult human, that investigated the impact of dietary Ca concentration on faecal Ca, fatty acid and Ca-fatty acid soap excretion for several different fat sources containing quite different fatty acid compositions. The newly developed fatty acid-soap determination method was used to determine the amount of soaps in the faeces. The results of the latter study showed that faecal fatty acid-soap excretion increased with increasing dietary Ca concentration when the dietary fat source comprised predominantly (> 40%) saturated fatty acids. In contrast, no soaps were present in the faeces when pigs received diets containing oils comprising less

than 20% saturated fatty acids. The greatest amounts of Ca, fatty acids (particularly stearic acid) and fatty-acid soaps were excreted for the pigs receiving the tallow-based diets. Moreover, approximately 80% of the fatty acids excreted in the faeces were present in the form of fatty acid soaps. This would suggest that firstly the formation of Ca-fatty acid soaps is likely to be the mechanism by which dietary Ca impacts faecal fatty acid excretion, and secondly that the main reason fatty acids are not digested and absorbed is because they have been incorporated into fatty acid-soaps, and thirdly that stearic acid is likely to be the most affected fatty acid.

In terms of Ca-fatty acid soap formation being a mechanism by which dietary Ca affects body weight, pigs receiving the tallow-based diets in conjunction with 6 g kg^{-1} Ca excreted the greatest amount of total fatty acids (20 g kg^{-1} DMI) and had the lowest apparent faecal digestibility for the saturated fatty acids (83% and 66% for palmitic and stearic acid respectively). This reduction in digestibility would equate to an energy deficit of 566 kJ d^{-1} (or 135 kcal d^{-1}) compared to pigs receiving the unsupplemented Ca diet. When extrapolated to a dietary Ca intake commonly used in intervention studies conducted in humans (1.8 g d^{-1}), it is estimated that an additional 2.9 g kg^{-1} DMI of fat would be excreted in the faeces in comparison with an unsupplemented Ca diet (providing approx. 0.3 g Ca daily). That would correspond to an energy deficit of 109 kJ d^{-1} (26 kcal d^{-1}) in comparison to the unsupplemented Ca diet and a theoretical weight loss of 1 kg per year (based on the somewhat simplistic approximate assumption that an energy deficit of 3500 kcal leads to a body weight loss of 0.45 kg). A body weight reduction of 1 kg over a year although meaningful biologically, is small and would be difficult to detect experimentally, and may explain why many studies investigating the effect of dietary Ca on body weight fail to detect

a Ca effect on body weight. The results of the present study are, however, in agreement with those of a meta-analysis that included data from a number of randomised controlled trials investigating the effect of dietary Ca on faecal fat excretion (Christensen *et al.* 2009). The latter researchers calculated that subjects receiving the Ca-supplemented diets would excrete an additional 2.0 g fat (18 kcal) daily, which would equate to a loss of around 1 kg body weight over one year. The inclusion of higher levels of Ca in diets, particularly those containing high amounts of saturated fats, may be a useful strategy, when combined with other approaches to weight loss.

The formation of indigestible Ca-fatty acid soaps leading to greater faecal fat excretion is not the only mechanism that has been proposed to explain the effect that increasing dietary Ca has on body weight and body fat content. Indeed, two metabolic mechanisms based on the role of dietary Ca in the regulation of energy metabolism have been proposed in the literature (Shi and Zemel 2000; Xue *et al.* 2001; Sun and Zemel 2004b). One of these mechanisms involves shifting the fatty acid synthesis/fatty acid lipolysis balance via Ca-mediated hormonal regulation. A meta-analysis summarizing published studies investigating the effect of dietary Ca on fat oxidation concluded that a chronic high Ca intake increased fat oxidation by 11% (Gonzalez *et al.* 2012) equating to a loss of 3.7 kg body fat over one year. Comparing the potential body weight loss estimated based on either the effect of dietary Ca on faecal fat excretion (soap hypothesis) or on fat oxidation (metabolic hypothesis), it would appear that the metabolic mechanism may play a more prominent role in weight loss compared to that for the soap hypothesis. However, compared to the soap hypothesis, the results from studies investigating the metabolic hypothesis are more inconsistent and the metabolic effect remains uncertain.

An additional study was undertaken here to determine where in the gastrointestinal tract soaps actually form (Chapter Six). The results of this study showed that soap formation generally takes place in the small intestine with the concentration of soaps increasing throughout the small intestine. In addition, there was a strong correlation between the concentration of soaps at different locations of the small intestine and the pH at those locations suggesting, not unexpectedly, that pH is a key factor in soap formation. Interestingly, the correlation between saturated fatty acid soaps (comprising either palmitic or stearic acid) and pH was much stronger (r^2 = 0.7) than the correlation between the unsaturated fatty acid soaps (comprising oleic acid) and pH (r^2 = 0.2). *In vitro* and *in vivo* studies with synthetic Ca-fatty acid soaps have shown that soaps comprised of unsaturated fatty acids are more soluble compared to soaps comprised of saturated fatty acids of similar chain length (Gacs and Barltrop 1977; Graham and Sackman 1983). Therefore, other factors such as the solubility of fatty acid soaps or the presence of bile salts will likely impact fatty acid soap formation in the gastrointestinal tract.

Oleic acid soap formation throughout the gastrointestinal tract differed from palmitic and stearic acid soap formation in regards to the amount of soaps being formed (oleic acid-soap formation was lowest) and also the location in the gastrointestinal tract where soap formation peaked (oleic acid soap formation peaked in the distal jejunum and decreased in more distal regions of the small intestine, whereas palmitic and stearic acid soaps peaked in the terminal ileum). These results are in accordance with observations from our *in vitro* study (Chapter Five) where oleic acid precipitation with Ca was lower compared to the precipitation of palmitic and stearic acid in the presence of Ca. Furthermore, the observed lower presence of oleic acid soap in the terminal ileum compared to the distal jejunum is

most likely related to the solubility of Ca-oleic acid soap in the presence of bile salts. Observations from the *in vitro* kinetic experiments (see section 5.4.4) on soap formation reported in Chapter Five revealed a 30% lower precipitate of Ca-oleic acid soap in the reaction mixture (containing 10 mM bile salts and 1.45 mM phospholipids) after an incubation period of 2 h compared to an incubation period of 10 min. On the other hand, the precipitated amount of Ca-stearic acid soap was numerically higher after 2 h incubation than after 10 min, possibly indicating soap formation rather than soap dissociation. It would also appear that the small intestine rather than the large intestine is important for fatty acid soap formation. This is evidenced in that the concentration of fatty acid soaps comprising saturated fatty acids was greatest at the end of the small intestine (terminal ileum) and was not different between the digesta at the terminal ileum and in the faeces.

Fatty acid soap formation with Ca has been the main focus of the *in vivo* digestion studies conducted in the presently reported research. The dietary fat source (fat vs oil) as well as the amount of dietary fat (fat containing vs fat-free diet) had an effect on Ca flows. In particular, in the study that determined the concentration of soaps in the faeces of pigs fed different fat sources and dietary Ca concentrations (Chapter Four), the excretion of a high amount of fatty acid soaps as experienced for the tallow-based diet concomitantly led to increased Ca excretion in the faeces. Furthermore, in the mapping study (Chapter Six), the amount of Ca throughout the gut was significantly higher for pigs fed the fat containing diets (free fatty acid-based diet and tallow-based diet) which resulted in soap formation, compared to pigs receiving no fat in their diet (fat-free diet), where only insignificant amounts of soaps were formed.

The apparent Ca digestibility across the gastrointestinal tract was 20% lower in pigs receiving the fat containing diets compared to the fat-free diet in the mapping study. Although the interaction between diet and gastrointestinal location for Ca flow or apparent Ca digestibility were not statistically significantly different a numerical difference between pigs receiving the fat containing diets compared to the fat-free diet for different gastrointestinal locations was observed. For example, the mean apparent Ca digestibility of the duodenum was 40% for all three diets (39% - 43%) but from the distal jejunum to the rectum the apparent Ca digestibility was approximately 20% lower for pigs receiving the fat containing diets compared to pigs receiving the fat-free diet (apparent ileal Ca digestibility for pigs receiving diet A and B versus diet C; 63% vs 80%). Considering that the presence of fatty acid soaps increased from the distal jejunum onwards, it may be that the observed numerically lower Ca digestibility of 20% for the fat containing diets from the distal jejunum onwards is due to the effect of fatty acid soap formation with Ca, which makes Ca unavailable for absorption. This requires further investigation.

Soap formation at the jejunal pH (proximal jejunum pH 5.0, distal jejunum pH 6.0) as determined in the mapping study is in agreement with soap formation observed in our *in vitro* study, where the first precipitation of Ca-soaps of saturated fatty acids occurred at pH 5.5. *In vitro* soap formation of Ca-fatty acid soaps peaked at pH 6.5 and 7.5 with approximately 85% of the initial palmitic and stearic acid precipitated with Ca. Interestingly, *in vitro* Ca-fatty acid soap formation with unsaturated fatty acids did not occur at a pH below 7.5. Nonetheless, the highest fatty acid soap formation with oleic acid was observed for the distal jejunum (pH 6.0) when free fatty acids were fed to the pigs.

All of the work discussed above largely focussed on the formation of Ca-fatty acid soaps since Ca is quantitatively the most important divalent mineral in the diet. However, it was recognised that it is possible that other nutritionally important divalent cations may also form soaps. Consequently, a study using an *in vitro* simulation of gastrointestinal conditions was conducted to investigate the behaviours of Mg, Zn, Fe and Cu in relation to divalent cation-fatty acid soap formation in the gastrointestinal tract. The results of the latter study indicate that free fatty acids are able to complex with Mg, Zn, Fe and Cu. The extent to which Zn formed Zn-fatty acid soaps was marked, with Zn-fatty acid soap formation being greater than that for Ca when incubation occurred with lauric, oleic and linoleic acid at all of the intestinal pH's tested. Mg-stearic acid soap formation behaved similarly to Ca-stearic acid soap formation, however the precipitation of other saturated fatty acids tested occurred only at higher pH (e.g. palmitic acid precipitation at pH 6.5, myristic acid precipitation at pH 7.5), whereas no soap formation occurred with the unsaturated fatty acids oleic and linoleic acid. Fatty acid soap formation was lowest for Cu and Fe with less than 50% of the fatty acids initially present in the reaction mixture being precipitated in the presence of Cu or Fe. One reason that little fatty acid soap formation occurred with Cu and Fe may be that Cu and Fe hydroxide salts (NaOH was used to increase the pH of the reaction mixture) formed in preference to the Cu- or Fe-fatty acid soaps. Certainly, lowering the concentration of Cu in the reaction mixture below 1 mM, but keeping the concentration of stearic acid constant (13.2 mM), reduced the formation of $Cu(OH)_2$ and increased the amount of Cu that precipitated with stearic acid. As was the case with Ca, stearic acid appeared to form soaps with Mg and Cu most readily in comparison with shorter chain fatty acids or unsaturated fatty acids. Given that the concentration of divalent cations, other than Ca, is much lower in the gastrointestinal tract compared to Ca, the formation of Zn, Mg, Cu, or Fe soaps is

unlikely to significantly impact the digestibility of fatty acids. Soap formation may however significantly affect the absorption of Zn and Mg, and to a certain extent also Cu and Fe. These results have potential application to the nutrition and health of many people in developing countries, where micro-element deficiency is common.

The *in vivo* study examining the formation of fatty acid soaps throughout the gastrointestinal tract also examined the digestibility of selected divalent cations other than Ca. For example, Mg flow throughout the gastrointestinal tract of the pig in the mapping study, was higher for pigs receiving the free fatty acid diets and the tallow-based diets compared to pigs fed the fat free diet. Concomitantly the apparent Mg digestibility for the gastrointestinal tract was higher by approximately 45% for pigs receiving the fat free diet compared to pigs receiving diets containing fat. The *in vitro* experiments conducted with Mg in gastrointestinal simulated fluids showed that Mg-fatty acid soap precipitation occurred with the two saturated long chain fatty acids, palmitic and stearic acid at pH 6.5 and 7.5, pH values similar to the observed pH for the intestinal locations of the ileum and terminal ileum. Therefore, it is likely that the lower apparent Mg digestibility, as observed for the fat containing diets compared to the fat free diet, was due to Mg-fatty acid soap formation with palmitic and stearic acid, the major saturated fatty acids present in the experimental diets. Furthermore, the apparent Zn and Fe digestibility in pigs receiving fat containing diets was lower than for pigs fed the fat-free diet. The *in vitro* experiment conducted in the present work showed that both Zn and Fe were capable of precipitating fatty acids, therefore a reduced digestibility of the latter divalent cations might have occurred due to Zn- and Fe-fatty acid soap formation. Results for divalent cations (Zn, Fe, Cu) as presented in Chapter Four have shown a correlation between faecal fatty acid soaps and faecal Zn and Fe concentration when pigs

were fed the tallow- or the palmolein oil-based diets. No correlation between faecal fatty acids and faecal Zn or Fe concentration was observed for pigs receiving the olive oil or soya bean oil-based diets. These observations tie in nicely with the results from the *in vitro* experiments where oleic acid-soap and linoleic acid-soap precipitation in the presence of Zn or Fe was lower compared to saturated fatty acid-soap precipitation with Zn or Fe. Unsaturated fatty acids might not play a very important role in divalent cation-soap precipitation.

7.2. Conclusions & Future Research

Findings presented in this dissertation provide evidence that Ca can bind to fatty acids in the small intestine to form fatty acid soaps that are in turn responsible for reduced fat absorption from the gastrointestinal tract in the presence of dietary Ca. However, future research is necessary to provide evidence that the formation of fatty acid soaps is responsible for the reduction in body weight and body fat mass observed in humans as a result of increasing dietary Ca intake. To date, no human intervention study investigating the effect of dietary Ca on weight loss has looked into the amounts of fat and energy excreted with the faeces due to the formation of fatty acid-soaps. To quantify how much Ca-fatty acid soap formation contributes to body weight loss, an intervention study using human subjects receiving an energy-restricted diet and receiving either a high Ca intake or a low Ca intake needs to be conducted, where faecal fatty acid soaps, faecal fat and faecal energy are compared between the two intervention groups.

Based on the information from the experiments conducted within this research it has been determined that high concentrations of dietary Ca in combination with dietary fats high in

saturated fatty acids reduce fatty acid absorption, therefore it would be of interest to formulate a diet based on these principles and suitable for humans that would optimise the effect of reduced fatty acid absorption and determine the effect on daily energy uptake.

Within this research we have investigated different concentrations of dietary Ca as well as different fat sources. However, it would be of interest to further investigate if different sources of dietary ca (e.g. $CaPO_3$, Ca-gluconate, and dairy Ca, versus $CaCO_3$) have an effect on Ca-fatty acid soap formation. To date, intervention studies conducted in humans have used different forms of Ca supplementation to investigate the effect of dietary Ca on faecal fat excretion, but no study has undertaken a direct comparison of different sources of dietary Ca on the effect of faecal fat excretion, and Ca-fatty acid soap formation.

The findings presented in this thesis provide novel insight into the intestinal location where fatty acid soaps are formed and additionally show that fatty acid soap formation has the ability to reduce fat absorption at the major absorption site of fatty acids. Fatty acid soap formation occurred in more anterior locations of the small intestine and was higher when free fatty acids were fed to the pigs compared to feeding an intact triglyceride. However, it is triglycerides that are consumed in a human diet rather than free fatty acids, therefore the question arises, as to whether greater digestion of triglycerides in anterior locations of the gastrointestinal tract would reduce fat absorption via the mechanism of increased soap formation? Fat digestion in the stomach is relatively low (gastric lipase accounts for 10 to 30% of overall lipid digestion of ingested triglycerides in human adults), and gastric lipase preferably hydrolyses short and medium chain fatty acids. Increasing fat digestion in the stomach, thereby increasing the amount of free fatty acids entering the duodenum might potentially increase soap formation. For example, providing a food product (e.g. weight loss

shake) that contains a lipase which activates when ingested and cleaves fatty acids from the triglyceride molecule (particularly saturated long chain fatty acids) during digestion in the stomach would increase the amount of free fatty acids entering the small intestine. The fatty acids would be available to complex with dietary Ca and evade absorption in the form of insoluble Ca-fatty acid soaps. Future research would first need to establish if increased gastric lipid digestion due to an added dietary lipase has the potential to reduce fat absorption by increased soap formation. Preliminary studies using the pig as an animal model for the adult human would need to investigate the potential of this new found hypothesis.

Divalent cations such as Zn, Mg, Cu and Fe do form soaps *in vitro* and may have the potential to precipitate with fatty acids, particularly saturated fatty acids, *in vivo*, thereby contributing to a reduction in fat absorption. Considering that the dietary intake of these minerals is relatively low compared to Ca, the effect on fat absorption would be expected to be minor, but on the other hand, the formation of insoluble divalent cation-fatty acid soaps could potentially reduce the absorption of these nutritionally relevant minerals and have nutritional consequences. Further studies are required in this area and may include using isotopes of Mg, Zn, Fe and Cu in diets to determine whether the dietary minerals are absorbed or bound in fatty acid soaps.

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