Effect of calcium on bioaccessibility of milk fat during digestion of Cheddar-type cheeses

Thèse

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Résumé

Le fromage cheddar est reconnu comme une excellente source de calcium. Outre son intérêt nutritionnel intrinsèque, le calcium favorise la lipolyse lors de la digestion. Cet effet s'explique par la formation de savons de calcium avec les acides gras saturés à longue chaîne, ce qui entraîne l'exposition de nouveau substrat à l'interphase huile-eau des gouttelettes de gras laitier, permettant à la lipase de continuer son action. En contrepartie, les savons de calcium limitent l'absorption des acides gras impliqués. D'un point de vue technologique, le calcium joue un rôle clé dans la structure du fromage car il participe à la formation du gel de paracaséine. Ayant un effet sur la matrice fromagère et sur la digestion des lipides, le calcium peut alors modifier la biodisponibilité du gras laitier. L'objectif de ce projet était de mieux comprendre l'effet du calcium sur la biodisponibilité du gras laitier à partir de fromages de type cheddar avec le but éventuel de développer des aliments pouvant contrôler la digestion et l'absorption des lipides.

Dans un premier temps, des fromages de type cheddar enrichis en calcium par l'ajout de $CaCl_2$ ont été soumis à une digestion in vitro. L'analyse des chymes a permis de démontrer que les fromages enrichis se désintégraient plus lentement que leur contrôle sans calcium ajouté. D'une autre part, la libération d'acides gras des fromages enrichis progressait plus rapidement, mettant en évidence l'effet du calcium sur les mécanismes impliqués dans la lipolyse.

Dans un second temps, des fromages de type cheddar ont été fabriqués à partir de lait standardisé avec des huiles de beurre contrôle, oléine et stéarine et salés avec ou sans $CaCl_2$. Les fromages ont été digérés in vitro pour étudier l'effet du calcium sur la lipolyse et la formation de savons de calcium avec les huiles de beurre ayant différents profils d'acides gras. Les fromages préparés avec la fraction stéarine (avec le rapport le plus élevé d'acides gras saturés à longue chaîne) étaient plus résistants à la désintégration physique et présentaient une lipolyse plus lente que les autres fromages, en raison du point de fusion élevé de cette matière grasse. Les fromages enrichis en calcium présentaient des taux de lipolyse supérieurs aux fromages sans enrichissement. Cette lipolyse accrue a été expliquée par la

formation de savons de calcium avec des acides gras à longue chaîne. Ces composés insolubles pourraient toutefois réduire la biodisponibilité des acides gras impliqués en empêchant leur absorption.

Pour confirmer l'effet du calcium et du type de matière grasse sur la biodisponibilité des lipides, les fromages ont été utilisés par la suite pour une étude chez le rat. La lipémie postprandiale des animaux a été mesurée suite à l'ingestion du fromage. Les matières fécales ont été analysées pour quantifier les acides gras excrétés sous forme de savons de calcium. Les fromages ont eu des effets différents au niveau de la lipémie postprandiale. L'enrichissement en calcium a entraîné une augmentation de la lipémie avec les fromages à l'oléine, alors qu'un pic différé a été observé avec les fromages à stéarine. Ceci s'explique par la formation de savons de calcium avec des acides gras saturés à longue chaîne, favorisant indirectement une lipolyse plus rapide de ceux à courtes et à moyennes chaînes. Le retard du pic pour les fromages à base de stéarine s'expliquait par leur teneur plus élevée en acides gras saturés à longue chaîne, qui formaient des savons avec le calcium et se retrouvaient dans les fèces.

Les résultats confirment que le calcium affecte la digestion intestinale des lipides laitiers en augmentant le taux de lipolyse. Cependant, il limite également la bioaccessibilité des acides gras en produisant, au pH intestinal, des savons de calcium insolubles avec des acides gras saturés à longue chaîne. Ce projet démontre que la biodisponibilité des lipides peut être régulée par le calcium présent dans le fromage cheddar. Cette étude met en évidence l'interaction en cours de digestion du calcium et des lipides présents dans la matrice laitière et confirme sa répercussion physiologique. Ces effets sur la digestion et l'absorption des lipides sont d'intérêt pour la conception de matrices alimentaires pour la libération contrôlée de nutriments et bioactifs liposolubles. D'autres recherches dans ce domaine permettront de mieux comprendre le rôle joué par les aliments sur la santé humaine et d'habiliter le développement de produits laitiers pour contrôler la libération de nutriments afin de moduler les réponses métaboliques.

Mots clés : fromage, gras laitier, digestion, lipolyse, savons de calcium.

Abstract

Cheddar cheese is recognized as an excellent source of calcium. In addition to its intrinsic nutritional value, calcium promotes lipolysis during digestion. This lipolysis enhancing effect is explained by the formation of calcium soaps with saturated long-chain fatty acids, resulting in the exposure of new substrate to the oil-water interphase of the milk fat droplets, thus enabling lipase to continue its action. On the other hand, the formation of calcium soaps reduces the absorption of saturated long-chain fatty acids. From a technological point of view, calcium plays a key role in the cheese structure as it participates in the formation of the paracasein gel. By such effects on the cheese matrix and the digestion of lipids, calcium can modify the bioavailability of the dairy fat. The objective of this project was to better understand the effect of calcium on the bioavailability of dairy fat from Cheddar cheeses, in aim to developing food matrices for controlled digestion and absorption of lipids.

In a first step, Cheddar cheeses enriched with calcium by the addition of $CaCl_2$ were subjected to digestion in vitro. Chyme analysis showed that calcium-enriched cheeses disintegrated less rapidly than the non-enriched control but that their lipolysis progressed more rapidly, demonstrating the effect of calcium on the factors that influence lipolysis.

In a second step, Cheddar cheeses were made from standardized milk with control, olein and stearin butter oils and salted with or without $CaCl_2$. The cheeses were digested in vitro to study the effect of calcium on lipolysis and the formation of calcium soaps from butter oils with different fatty acid profiles. Cheeses prepared with the stearin fraction (with the highest ratio of saturated long-chain fatty acids) were more resistant to physical disintegration and presented slower lipolysis than the other cheeses because of the high melting point of this fat. Cheeses enriched with calcium had higher levels of lipolysis than cheeses without enrichment. This increased lipolysis was due to the formation of calcium soaps with saturated long-chain fatty acids. These insoluble compounds could reduce the bioavailability of the fatty acids involved by preventing their absorption.

To confirm the effect of calcium and type of fat on lipid bioavailability, the cheeses were subsequently used for an in vivo study. Postprandial lipemia of Wistar rats was monitored following ingestion of the cheese. The feces were analyzed to quantify the fatty acids excreted as calcium soaps. The cheeses had different effects in postprandial lipemia. Calcium enrichment led to a higher lipemic peak for the cheeses with olein, while a delayed peak was observed for cheeses with the stearin. This was explained by the increased affinity of calcium for saturated long-chain fatty acids, indirectly allowing faster lipolysis of other fatty acids, such as those with short- and medium-chains. The delay for stearin cheeses was due to their high content of saturated long-chain fatty acids, which formed soaps with calcium, thus reducing their absorption and ending up in feces.

The results confirm that calcium plays an important role in intestinal digestion of dairy lipids by increasing the rate of lipolysis. However, it also limits the bioaccessibility of fatty acids by producing insoluble calcium soaps with saturated long-chain fatty acids at intestinal pH conditions. This project demonstrates that the bioavailability of lipids can be regulated by calcium in Cheddar cheese. This study demonstrates the interaction of calcium and lipids present in the dairy matrix during digestion and confirms its physiological repercussion. These effects on digestion and lipid absorption are of interest for the design of food matrices for the controlled release of liposoluble nutrients or bioactive molecules. Further research in this area will provide a better understanding of the role of foods in human health and enable the development of dairy products to control the release of nutrients to modulate metabolic responses.

Keywords: Cheese, milk fat, digestion, lipolysis, calcium soaps

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List of Abbreviations

AMF	Anhydrous milk fat
ANOVA	Analysis of variance
BA	Bile acids
CCP	Colloidal calcium phosphate
CE	Cholesterol esters
CEL	Cholesterol-esterase lipase
CLSM	Confocal-laser scanning microscopy
CM	Chylomicron
CMP	Caseino-macropeptide
CS	Calcium soaps
CVD	Cardiovascular disease
DAG	Diglycerides (or diacylglycerides)
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
FA	Fatty acids
FABP	Fatty acid binding proteins
FCF	For coloring food
FID	Flame-ionization detector
GC	Gas chromatography
GI	Gastro-intestinal
HDL	High-density lipoproteins
HGL	Human gastric lipase
HL	Hepatic lipase
HPL	Human pancreatic lipase
iAUC	Incremental area under the curve
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
IDL	Intermediate density lipoproteins
LCFA	Long-chain fatty acids
LDL	Low-density lipoproteins
LRP	LDL-related peptide
LSD	Least square difference
MAG	Monoglycerides (or monoacylglycerides)
MCFA	Medium-chain fatty acids
MDI	Matrix degradation index
MFG	Milk fat globule
MFGM	Milk fat globule membrane
MW	Molecular weight
NEFA	Non-esterified fatty acids
OLTT	Oral lipid tolerance test
PL	Phospholipids
PLA2	Phospholipase A2
PES	Poly-ether sulfone
PUFA	Polyunsaturated fatty acids
PPL	Postprandial lipemia

SCFA	Short-chain fatty acids
SEM	Standard error of the mean
SPE	Solid-phase extraction
TAG	Triglycerides (or triacylglycerides)
TN	Total nitrogen
TPA	Texture profile analysis
TRL	Triglyceride-rich lipoproteins
TSC	Trisodium citrate
UFA	Unsaturated fatty acids
VLDL	Very low density lipoproteins
WSN	Water-soluble nitrogen

Dedication

Aire…

Cada respiro lleva tu nombre.

A mis abuelos, Laura, Elvia, Federico y Daniel, por mostrarme el horizonte,

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Foreword

This thesis is divided in 7 chapters, as follows:

Chapter 1 is an introduction to the nutritional importance of the food matrix. It presents different aspects of health relevance of dairy products, namely those concerning Cheddar cheese.

Chapter 2 is a review of literature on human digestion, with emphasis on lipid digestion and the chemical interaction of calcium with digestion products. The general characteristics of milk fat are presented and recent works on its health implications are discussed. Lastly, the manufacturing process of Cheddar cheese is presented, with emphasis on the factors influencing its texture and the functional role of calcium.

Chapter 3 presents the hypothesis and the general objectives of the project

Chapter 4, 5 and 6 are written as research papers presenting the experiments and results corresponding to the research objectives presented in chapter 3.

Chapter 7 provides a general discussion on the main findings of this project, and stresses the complementarity between in vitro and in vivo models.

A general conclusion highlights the main findings of this project and emphasizes on their significance towards the development of wholesome and healthy foods.

Chapter 1. Introduction

1.1 Cardiovascular disease risk and fat ingestion

From the estimated 58 million deaths in 2005, cardiovascular diseases (CVD) are the most important cause of mortality in the World (World Health Organization, 2007). In 2005, CVD accounted for about 17.5 million deaths, equivalent to more than 30% of all deaths causes (World Health Organization, 2007). In Canada, CVD are the second most important cause of mortality, being responsible for nearly 25% of the deaths in 2013, just after cancer (Statistics Canada, 2017).

One of the underlying pathologies of CVD is atherosclerosis, which develops over many years and is usually advanced by the time symptoms occur (World Health Organization, 2007). Research has shown that abdominal obesity and exaggerated postprandial lipemia are among the main risk factors[1] for CVD and mortality (Bohl et al., 2015) by contributing to the induction and progress of atherosclerosis (Wilhelm et al., 2003; Zilversmit, 1995). Both risk factors can be related to lifestyle, especially inappropriate dietary behavior (Bohl et al., 2015). For this reason, the association of fat with CVD risk factors has reflected on nutritional guidelines outlining ingestion limits and different nutritional impact of different fats (World Health Organization, 2007).

Nutritionally speaking, fat has been targeted since the early 1960s for its consequences on human health (Keys et al., 1957, 1965). However, the ingestion of saturated fat as a major risk factor for CVD has been revised and nuanced during the past two decades, especially in the case of fat from dairy products (Elwood et al., 2004a; Elwood et al., 2004b; German, 2008; German et al., 2009; Gibson et al., 2009). In sum, high-fat dairy products are not consistently associated with higher CVD risk and have even been found to have positive effects on overall health (German, 2008; German et al., 2009; Huth et al., 2012; Visioli et al., 2014). Given the wide array of structures and composition of dairy foods, further research is necessary to better understand their role on digestion, absorption and metabolism to predict their effect on CVD risk (Bohl et al., 2015).

[1] Factors like high blood pressure, diabetes, use of tobacco, low physical activity also come in play (Bohl et al., 2015).

1.2 Nutritional implications of the food matrix

Traditionally, foods have been formulated to meet special needs and market trends by replacement or elimination of specific components. Such compositional tailoring interprets the food as a simple addition of individual components, without accounting for the complex chemical and physical interactions that food components may have on nutrient release and absorption (Turgeon et al., 2011). Under the compositional approach, food structure has often been overseen and the implications on its health properties are far from being thoroughly explored (Argov et al., 2008). On the upside, food design is evolving towards food systems that promote health of the individual consumer. In this sense, parallel to the formulation, the role of the food matrix is being emphasized (Nehir El et al., 2012; Turgeon et al., 2011).

The food matrix is the continuous medium, of natural or technological origin, where nutrients can interact at different length scales with its components and structures (Parada et al., 2007). There is a need to understand the association between the food matrix and its role on nutrient release beyond simple food composition. Basic understanding of the physicochemical processes taking place during digestion is required to design food matrices that can modulate nutrient release and absorption (Norton et al., 2015; Parada et al., 2007). This will help identify food processing techniques and parameters that could modify the food matrix to enhance or hinder biological availability of certain nutrients (Hur et al., 2009; McClements et al., 2009a; McClements et al., 2009b).

1.3 Nutritional relevance of Cheddar cheese

Dairy products have widespread consumption and are of nutritional and economic importance. Cheddar cheese is a dairy product widely consumed in North America. On a technological basis, Cheddar cheese is a well-studied food, but its nutritional role is usually brought down to its composition, without considering the possible matrix effects during digestion and nutrient absorption. Although Cheddar cheese is recognized as a source of calcium, its reputation has been tarnished by the high amount of fat it contains. However, several studies have shown that cheese, as well as other dairy products, do not have the deleterious effects once associated with foods containing saturated fats and that some may even have a positive effect on health (Christensen et al., 2009; de Oliveira Otto et al., 2012; German et al., 2009; Tremblay et al., 2011).

These beneficial effects have been in part linked to the calcium content and to the structure of such products (Soerensen et al., 2014). Calcium largely determines the structural characteristics of cheeses like Cheddar by promoting association of casein micelles during renneting (Ong et al., 2013). Secondly, calcium plays a major role on lipid digestion by enhancing lipolysis, but simultaneously limiting the absorption rate of the fatty acids released by forming calcium soaps (Lorenzen et al., 2011; Welberg et al., 1994). These opposed effects are self-coordinated and have been identified as one of the main reasons for their positive health repercussions.

The main objective of this project was to better understand the role of calcium on the Cheddar cheese matrix, its microstructure and nutrient release, specifically lipids, during digestion. Hence, Cheddar-type cheeses were prepared with different calcium levels and milk fats with different fatty acid profiles to study the calcium-lipid interactions taking place under simulated digestive conditions. Successively, a rat model was used to validate in vitro data and assess the metabolic impact of the calcium-lipid interactions on lipid bioavailability.

In sum, this study describes the process by which Cheddar cheese releases its lipid nutrients, specifically fatty acids, during the digestion process and how such nutrients are absorbed. These findings could result in novel nutritional aspects that could be modulated in food systems and adapted for the food industry, which is the goal of the present project. Knowledge generated by this project will aid the food industry's capability of designing food matrices that enhance the consumers' health by modulating the physiological repercussions of foods, especially those concerning postprandial lipemia. Eventually, new strategies may be developed to produce food with regulated nutrient release and absorption or targeted delivery of bioactive molecules.

Chapter 2. Review of literature

2.1 Human digestion

2.1.1 Gastrointestinal tract

Digestion is the process by which complex nutrients in food are broken down into absorbable forms that can pass through the epithelial cells lining the mucosa of the gastrointestinal (GI) tract into the underlying blood and lymphatic vessels (Kong et al., 2008a; Versantvoort et al., 2004). Digestion starts as food enters the mouth and continues as it passes through the GI tract, which is the tubular organ that extends from the posterior oropharynx to the anus (Klein et al., 2006). The different parts of the GI tract and the main processes and physiological characteristics for each major segment are presented in Figure 1.

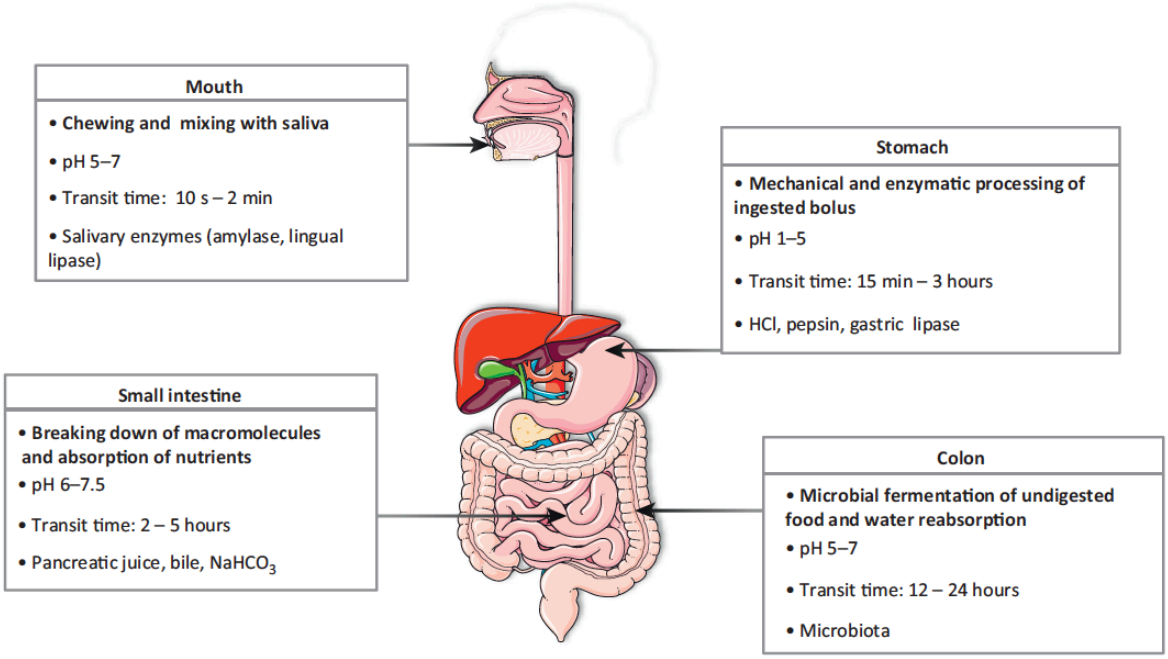

Figure 1. Gastrointestinal tract parts with the main processes and physiological characteristics for each major segment. Reproduced from Guerra et al. (2012).

Digestion and absorption are the two main processes taking place in the GI tract. During digestion, the food is broken down, physically and chemically, into absorbable forms. Then absorption takes place and the nutrient passes the GI mucosa to become available for metabolism. Although digestion and absorption are distinct processes, they are entwined throughout the different parts of the GI

tract. In each part, specific changes occur to the food constituents and the sites of absorption aim the uptake different nutrients.

2.1.1.1 Mouth and esophagus

The mouth has an important role in food digestion by activating the integrated response of the GI tract to a meal (Stenson, 2006). Formally, the mouth is not part of the GI tract but the mastication process allows deglutition and facilitates digestion (Klein et al., 2006). During its short time in the mouth, food pieces are reduced in size by mastication and are incorporated with saliva into a smooth bolus that can be swallowed (Klein et al., 2006; Kong et al., 2008a; Stenson, 2006). Saliva contains enzymes that immediately start nutrient breakdown. Amylases act on starch hydrolysis, although most of their action takes place in the stomach (Stenson, 2006). A lingual lipase is also secreted in saliva, but its lipolytic action is minimal when compared to lipases from the GI tract (Klein et al., 2006), and it has mainly been suggested to play a role in oral sensitivity to fat (Laugerette et al., 2006; Laugerette et al., 2005; Pepino et al., 2012). Residence time in the mouth goes from seconds to minutes (Oomen et al., 2002), until most particles in the bolus have a diameter of 3 mm or smaller (Hoebler et al., 2000; Jalabert-Malbos et al., 2007; Klein et al., 2006).

After being swallowed, the food passes into the esophagus and formally enters the gastrointestinal tract (Kong et al., 2008a). The adult esophagus is approximately 25 cm long and it transports the bolus from the mouth into the stomach. Besides some mucus to aid in transit, no digestive fluids are incorporated into the bolus during its passage through the esophagus. Contraction of the esophagus transports the food through the diaphragmatic hiatus and into the stomach (Klein et al., 2006).

2.1.1.2 Stomach

2.1.1.2.1 Physical and chemical disintegration of food

The stomach serves as a reservoir where food continues to be mechanically and chemically disintegrated (Kong et al., 2008b). As food is ingested, the fundus (i.e.

the upper part of the stomach) relaxes to contain arriving bolus (Stenson, 2006). Gastric fluids are secreted to achieve chemical breakdown. At the beginning of the fed state[2] the pH of the gastric medium is raised from around 2 up to 5 when buffered by food components (Klein et al., 2006). Gradually, the secretion of HCl lowers the pH back to 2 (Malagelada et al., 1976), which provides optimal conditions for pepsin activity (Minekus et al., 2014).

Besides the chemical disintegration, the antral musculature grinds food into smaller particles (Stenson, 2006). The repetitive contractions of the antral musculature squeeze the chyme towards the pyloric sphincter and the chyme squirts back into the body of the stomach (Stenson, 2006). This way, gastric motility grinds food particles and emulsifies fat into the chyme. An appreciable digestion of carbohydrates, proteins and lipids occurs in the stomach (Stenson, 2006). Some absorption (i.e. small hydrophilic molecules) takes place in the stomach, but most gastric contents are emptied into the small intestine.

2.1.1.2.2 Gastric emptying

Another important function of the stomach is regulating gastric emptying (i.e. the control of the chyme flow into the small intestine (Stenson, 2006)). Gastric emptying depends on the volume contained in the stomach and the degree of disintegration and chemical breakdown of the chyme. Gastric emptying delivers a relatively steady rate of 200 kcal/hour into the intestine, so the caloric density of the chyme also plays in regulating its emptying (Stenson, 2006). Other factors in response to food-related factors that modify the gastric emptying rate are osmolality, fat content, pH, and the mechanical food characteristics (e.g. liquid, viscous, solid) (Stenson, 2006).

The composition of the food impacts gastric emptying because not all nutrients are emptied at the same rate. For instance, fat floats over the aqueous medium, which

[2] Gastric conditions differ between the fed and the fasted state (i.e. with or without food, respectively) (Versantvoort et al., 2004). An important difference, being more acidic in the fasted state (i.e. $1 \leq pH \leq 2$ (Oomen et al., 2002)) than in the fed state (i.e. $5 \leq pH \leq 6$).

delays its emptying with respect to latter (Stenson, 2006). Furthermore, solid fats can bind to solid particles and delay their emptying. Liquids are emptied faster than solids because they do not require grinding (Stenson, 2006). Gastric emptying of solid food is also affected by the disintegration process, which is mainly affected by surface erosion and texture softening during digestion (Kong et al., 2008b). Smaller particles are more easily digested by enzymes due to more exposed surface (Stenson, 2006). In general food particles are reduced to a size of 1 to 2 mm before they pass to the small intestine (Kong et al., 2008a; Stenson, 2006). Since different food structures behave in a different manner during digestion, altering such structures may modify the emptying process (e.g. aiming for harder textures to delay physical breakdown) (Kong et al., 2008b).

2.1.1.3 Small intestine

The chyme arriving from the stomach contains partially digested nutrients and digestive breakdown is completed in the small intestine (Versantvoort et al., 2004). The small intestine is the organ with the largest absorption surface and it consists of three continuous segments: duodenum, jejunum and ileum (Table 1). As the chyme moves forward in the small intestine, the pH increases due to the secretion of carbonate and bile salts (Daugherty et al., 1999; Diem et al., 1970; Oomen et al., 2002; Versantvoort et al., 2004).

Table 1. Segments of the human small intestine and their approximate physical and biological parameters. Data adapted from Daugherty et al. (1999).

Segment	Surface area (m^2)	Length (m)	pH
Duodenum	1.9	0.35	4.0 - 5.5
Jejunum	184	2.8	5.5 - 7.0
Ileum	276	4.2	7.0 - 7.5

The duodenum is connected to the stomach through the pylorus and it is attached along to the pancreas, which secretes juices containing carbonate, bile salts and

the enzymes that finish food breakdown to enable absorption. The duodenum represents only a short segment of the small intestine, around 30 cm long, but it acts as a neutralizing and mixing chamber for the chyme exiting the stomach with the pancreatic secretions (Daugherty et al., 1999; Guerra et al., 2012). From the duodenum, chyme advances into the jejunum due to peristaltic movement (Klein et al., 2006). After the jejunum, the chyme reaches the ileum, the last segment of the small intestine, where absorption of released nutrients is completed. Most nutrients are absorbed at the jejunum and ileum, given their length and a longer residence time of the chyme, when compared to the duodenum (Daugherty et al., 1999; Oomen et al., 2002). The ileum also reabsorbs most of the bile salts (Jones et al., 2006). At the end of the ileum, the ileocecal valve marks the entry into the large intestine. About 90% of all nutrients are absorbed in the small intestine, with most of them being absorbed in the jejunum. The other 10% occurs in the stomach and large intestine (Versantvoort et al., 2004).

2.1.1.4 Large intestine

Any non-digested or non-absorbable components pass into the large intestine, where the water and mineral contents of the chyme will be recovered. Any bile salts remaining are also recovered and stored in the gallbladder for further use (Jones et al., 2006). At this point, absorption of macronutrients is complete and the intestinal flora consumes part of the non-digested or non-absorbed components. At the end, all residues are excreted as feces.

2.1.2 Nutrient bioaccessibility and bioavailability

The purpose of digestion is to enable nutrient absorption. However, not all ingested nutrients can be absorbed and used for normal body functions so the total amount of a nutrient in a food does not necessarily reflect the amount of such that can be absorbed (Oomen et al., 2002). Furthermore, not all nutrients in an absorbable form are necessarily bioavailable. Nutrient bioavailability (i.e. biological availability) is the fraction of an ingested component that eventually ends up in the systemic circulation (Versantvoort et al., 2004) and it is the key to nutrient effectiveness

(Watzke, 1998). Bioavailability depends on several physicochemical and biochemical processes, as depicted in Figure 2.

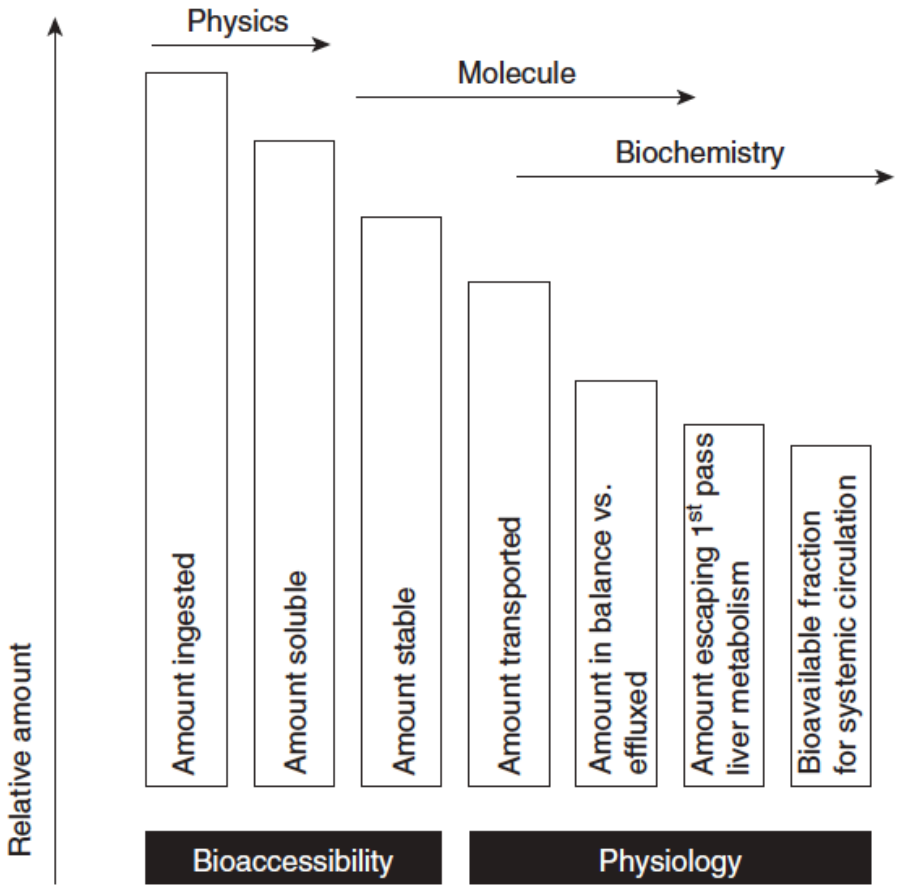

Figure 2. Ordering of factors in bioaccessibility and bioavailability. Reproduced from Duchateau et al. (2009).

In simplified terms, bioavailability (F) can be defined by 3 factors as F = Fb * Ft * Fm (McClements et al., 2009a; Oomen et al., 2003). Bioaccessibility (Fb) represents the fraction of an ingested component that is released from the food matrix into the juices of the gastrointestinal tract (Versantvoort et al., 2004). Processes affecting bioaccessibility of components include the release from the food matrix, their solubility and their stability at gastrointestinal conditions (Duchateau et al., 2009). Transport (Ft) represents the fraction of the released component that is transported across the intestinal epithelium (Versantvoort et al., 2004). Such transport is determined by the gut wall permeability to the specific

component (Duchateau et al., 2009), including active and passive transport mechanisms. Finally, metabolism (Fm) represents the fraction of the component that can reach its site of action (McClements et al., 2009a; Versantvoort et al., 2004). Such fraction differs if nutrients are transported into systemic circulation through the lymph or the portal vein (Duchateau et al., 2009). For the latter, the fraction is highly dependent on the liver first-pass effect, which implies major remodeling of nutrients that are absorbed through the portal vein (Duchateau et al., 2009). The transport pathway depends on the physicochemical properties of the compound. Hydrophilic compounds are preferentially transported though the portal vein, whereas lipophilic compounds are transported through the lymph (Duchateau et al., 2009). Altogether, bioavailability is the outcome of a complex physicochemical, biochemical and physiological stepwise reduction processes (Duchateau et al., 2009), which complicates its study by in vitro approaches.

The term bioaccessibility is commonly used when using in vitro approaches to digestion because it specifically designates the fraction of a substance that is freed from the food matrix and that is available for absorption from the gastrointestinal environment (Failla et al., 2005; Ruby et al., 1999; Versantvoort et al., 2004). In other words, bioaccessibility gives information on the digestibility of a food component or a nutrient (Failla et al., 2005). In vitro approaches to digestion are useful to study digestion processes, even if they lack the complex physiology that takes place in living organisms (van Aken, 2010). Nevertheless, in vitro models can be used to predict bioavailability and to screen and target experimental conditions before proceeding with in vivo models or clinical studies (Failla et al., 2005).

2.2 Digestive handling of nutrients

Different food components follow specific digestion processes, which differ according to the chemical nature of each nutrient. Macronutrients (i.e. carbohydrates, proteins and lipids) follow a specific breakdown pathway and are absorbed at different sites in the gastrointestinal tract. Absorption of micronutrients (e.g. vitamins and minerals) is influenced by macronutrient breakdown, which releases them from the food matrix. Once released, some nutrients follow similar uptake pathways as the macronutrients (Borel, 2003).

Salivary amylase is secreted in the mouth, where it begins to break down starches into smaller carbohydrates and continues its hydrolytic action in the stomach while the buffering effect of food lasts. Salivary amylase is inactivated eventually at low pH in the stomach (Keim et al., 2006; Versantvoort et al., 2004). The pancreas also secretes other amylases (Keim et al., 2006). Starches are further hydrolyzed by pancreatic amylase into disaccharides, trisaccharides and α-dextrins (i.e. 5 to 10 glucose units) (Versantvoort et al., 2004). Finally, enzymes at the brush border complete the digestion to monosaccharides, which are then absorbed (Versantvoort et al., 2004). Most simple sugars present or produced by amylases and other intestinal enzymes are water-soluble and are transported from the small intestine to the liver via the portal vein (Keim et al., 2006). Non-hydrolyzed carbohydrates (e.g. dietary fiber), pass to the large intestine (Keim et al., 2006).

Under normal conditions, most dietary and endogenous protein is absorbed (Mackie et al., 2010; Matthews, 2006). Digestion of proteins begins in the stomach by pepsin, when activated from pepsinogen by the acidic conditions given by HCl secreted in gastric juice (Klein et al., 2006). Most of proteolysis occurs in the small intestine (Klein et al., 2006), where protein digestion is continued by trypsin and chymotrypsin, which are present in the pancreatic fluids secreted into the duodenum (Dupont et al., 2010; Matthews, 2006; Versantvoort et al., 2004). Other enzymes in pancreatic juice (carboxypeptidase and elastase) and in the brush border (aminopeptidase and dipeptidase), produce dipeptides, tripeptides and

amino acids (Mackie et al., 2010; Norton et al., 2015; Versantvoort et al., 2004). Such proteolysis products are absorbed by active transport through the enterocytes, where hydrolysis of peptides is completed by cytosolic peptidases (Klein et al., 2006). Amino acids pass through portal circulation to the liver ultimately regulates their metabolism and flow into systemic circulation (Matthews, 2006).

2.2.1 Digestion of lipids

Due to the hydrophobic nature of lipids, different mechanisms than those for proteins and carbohydrates take place for digestion and absorption (Klein et al., 2006). Besides lipolysis (i.e. hydrolysis of ester bonds in triglycerides), a transport system through the intestinal medium enables absorption. These processes will be discussed in more detail in this section. Appendix B presents a short review on structure and nomenclature of glyceride-related lipids.

Triacylglycerols (TAG) represent around 95% of dietary lipids (Favé et al., 2007; Jones et al., 2006). The other lipids are a heterogeneous mix of more or less hydrophobic molecules like phospholipids, diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids, free cholesterol, cholesterol esters, lipid soluble pigments, lipid-soluble vitamins and other minor components (Favé et al., 2004; Jones et al., 2006). In the first place, lipids must be released from the food matrix by mechanical or enzymatic action so they can be accessible to lipases, which act on the surface of lipid droplets (Borel, 2003; Golding et al., 2010). Fat-soluble vitamins and minor lipophilic molecules also congregate in the fat droplets and follow the absorption paths of major dietary lipids (Borel, 2003; Machlin, 1984; Norman et al., 1984; Olson, 1984; Suttie, 1984).

2.2.1.1 Gastric digestion

Lipid components are dispersed as oil-in-water emulsified lipid droplets with diameters between 10 and 100 μm in the aqueous gastric medium (Mekki et al., 2002). Lipolysis starts in the stomach by human gastric lipase (HGL), secreted by

chief cells in the gastric mucosa (Klein et al., 2006). The enzyme is resistant to pepsin and it is not inactivated under the acidic conditions in the stomach (Klein et al., 2006). HGL is stable up to pH ≈ 2 and shows optimal activity between 4.5 and 6 (Favé et al., 2007; Sams et al., 2016; Stenson, 2006). Gastric lipase does not require a cofactor (cfr. human pancreatic lipase requires colipase to be effective) (Bodmer et al., 1987).

HGL acts preferentially on sn_3-linked, and generally leads to hydrolysis of 10 to 30% of esterified fatty acids (Carriere et al., 1993; Favé et al., 2004; Favé et al., 2007). Hydrolysis extent by HGL from 5 to 40% has also been reported (Armand, 2007). Fatty acids (FA) are cleaved from the glycerol backbone according to their carbon-chain length. Short-chain fatty acids (SCFA) are preferentially hydrolyzed from TAG than longer ones (German, 2008). As lipolysis advances, protonated long-chain FA inhibit gastric lipolysis when they accumulate around the fat droplets and form vesicles that entrap the lipase and physically isolate it from its substrate (Favé et al., 2004). Small droplets (i.e. with a high interface area) delay such inhibition (Favé et al., 2004). HGL is resistant to pepsin, but sensitive to trypsin and chymotrypsin (Favé et al., 2007).

The release of amphiphilic lipid digestion products, combined to shear forces in the stomach and gastric emptying, facilitates the formation of an emulsified chyme that empties into the duodenum (Versantvoort et al., 2004). For this reason, lipid digestion in the stomach is a crucial step because it facilitates subsequent TAG hydrolysis by providing fatty acids that promote fat emulsification and increase the available lipid-water interface for pancreatic lipases (Borel et al., 1994b; Favé et al., 2004; Klein et al., 2006).

Gastric lipolysis is essential for newborns and individuals with pathologies that limit the secretion of lipases after the gastric passage (e.g. pancreatic insufficiency, cystic fibrosis, reduced hepato-biliary functions), individuals with special needs (e.g. pregnant and lactating women, elderly people), who could benefit from an enhanced lipid bioavailability (Armand, 2007; Armand et al., 1999; Borel et al., 1994b; Favé et al., 2004; Favé et al., 2007).

2.2.1.2 Intestinal digestion

Fat entering the duodenum consists of 70% TAG and a mixture of partially digested hydrolysis products (Jones et al., 2006). Bile salts are delivered to the duodenal lumen, where they aid in emulsifying fat (Jones et al., 2006). Hydrolysis continues in the duodenum, through the synergistic action of HGL, colipase-dependent human pancreatic lipase (HPL) and bile (Favé et al., 2004). Adsorption of the enzyme to the interface causes a conformational rearrangement, exposing the catalytic site of the enzyme (Golding et al., 2010). Bile salts inhibit HPL activity by desorbing it from the substrate interface, but colipase, an interfacial coenzyme, acts as an anchor for lipase at the lipid droplets so it can exert its lipolytic activity (Bauer et al., 2005; Borgström et al., 1982; Klein et al., 2006). To optimize lipolysis, colipase is required in a 1:1 molar ratio to lipase (Bezzine et al., 1999). Under normal digestion conditions, HPL is extremely rapid and lipolysis occurs faster than the depletion of reaction products from the water-fat interphase (Jones et al., 2006).

The main interfacial processes occurring during pancreatic lipolysis (Figure 3) are as follow:

- Pancreatic lipase binds to co-lipase to access the interface of emulsified fat (Figure 3, i and ii) (Golding et al., 2010).
- The interfacial structure of some surfactants can inhibit the adsorption of the co-lipase/pancreatic lipase complex (Figure 3, iii) (Golding et al., 2010).
- Bile salts can remove such inhibitory surfactants from the interface via an orogenic displacement mechanism (Figure 3, iv) (Golding et al., 2010).
- As lipolysis proceeds, there is a buildup of 2-monoglycerides and free fatty acids on the surface of the fat droplet, which can limit lipase adsorption. Bile salts have water soluble and lipid soluble portions, which assemble with the digested lipid products to remove them from the fat-water interface (Figure 3, v) (Golding et al., 2010; Klein et al., 2006). The formed structures are

called mixed micelles and they transport digestion products to the enterocyte border for absorption (Klein et al., 2006).

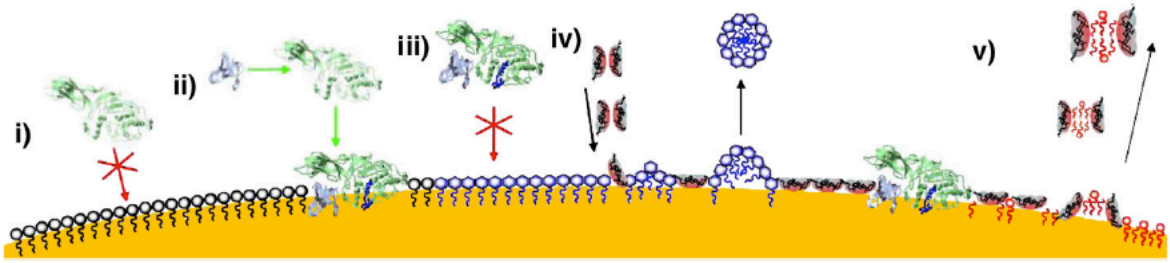

Figure 3. Interfacial processes occurring during pancreatic lipolysis at oil-water interface of fat droplets. Reproduced from Golding et al. (2010).

Hydrolysis of TAG by digestive lipases occurs on sn_1 and sn_3 fatty acids, yielding progressively sn_1,sn_2-DAG or sn_2,sn_3-DAG and finally FA and sn_2-MAG (Carey et al., 1983) (Figure 4). When lipolysis is completed, each TAG yields 2 FA and 1 MAG (Berry et al., 2005). In the end, less than 5% of fat remains in the form of DAG and TAG (Favé et al., 2004). Fat digestion is very effective, and most ingested TAG are hydrolyzed to sn_2-MAG and free FA within the first 100 cm of the jejunum (Klein et al., 2006).

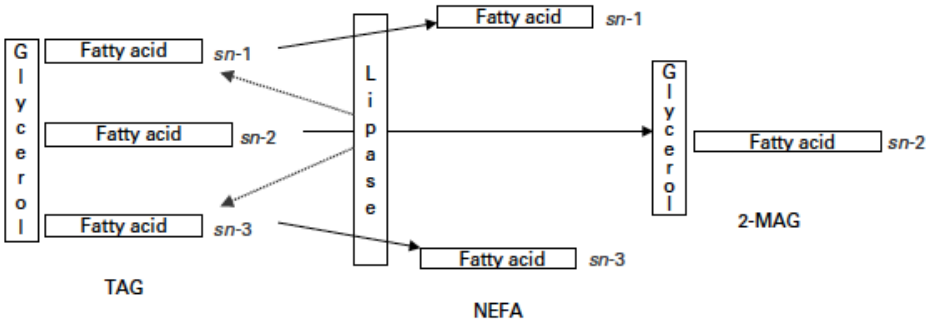

Figure 4. Lipolysis of triacylglycerol (TAG) by digestive lipases, producing non-esterified fatty acids (NEFA) and sn-2 monoacylglycerols (2-MAG). Reproduced from Berry et al. (2005).

Phospholipids, most of which reach the gastrointestinal tract via biliary secretion (i.e. only small amounts are from dietary sources (Versantvoort et al., 2004)), are hydrolyzed by phospholipase A2 (PLA2) at the sn_2 position into lysophospholipids and free FA (Favé et al., 2004). Alkaline sphingomyelinase hydrolyzes sphingomyeline (Duan, 2006; Duan et al., 2009). Cholesteryl esters are hydrolyzed

by pancreatic cholesterol-esterase lipase[3] (CEL) (Favé et al., 2004). Esterified vitamins are also hydrolyzed by CEL to enable uptake (Borel, 2003). Some hydrolysis of sn_2-MAG by CEL and PLA2 has also been reported (Sethi et al., 1993).

2.2.2 Absorption of lipids

Lipid absorption follows an intricate strategy to transport hydrophobic molecules through an aqueous medium and to transfer them to the absorption sites across an unstirred water layer at the surface of the intestinal microvillus membrane (Bauer et al., 2005; Klein et al., 2006). Daily intake of fat usually ranges from 50 to 150 g (Jones et al., 2006).

Overall, assimilation of lipid nutrients depends on the efficiency of lipolysis, with the activity of lipases being governed by the physicochemical properties of emulsions. In this sense, droplets of different size and composition are digested differently and, thus, are absorbed and metabolized differently (Borel et al., 1994a). Nonetheless, absorption effectiveness of TAG is higher than 95% (Sethi et al., 1993). Vitamins and other minor components have different absorption rates, but they are generally high and positively correlated to TAG absorption, since their uptake follows similar mechanisms (Borel, 2003).

There are two major macromolecular aggregates involved in lipid solubilization for absorption within the intestinal lumen (Armand, 1997; Favé et al., 2004; Woollett et al., 2006): The first are unilamellar vesicles (i.e. with only one phospholipid bilayer), spanning several hundred angstroms, containing phospholipids, long-chain fatty acids, monoglycerides, cholesterol and some bile acids (Klein et al., 2006). Unilamellar vesicles are an early product of lipolysis. They are mainly present in the duodenum, where they supply mixed micelles with lipolytic products to ensure their saturation, ensuring an optimal environment for lipid absorption (Christophe et

[3] CEL (i.e. Cholesterol-esterase lipase) is also referred to under the following names: Carboxyl-esterase lipase, cholesterol lipase and bile-salt activated lipase (Bauer et al., 2005; Carey et al., 1983; Klein et al., 2006).

al., 2000). The second and most important type of aggregates are mixed micelles, which are less than 100 angstroms (Klein et al., 2006).

2.2.2.1 Mixed micelles

Mixed micelles contain FA, MAG, bile acids, cholesterol and liposoluble vitamins (Klein et al., 2006). The amphiphilic components are essential to create the mixed micellar system and their production is triggered by the action of lipases on TAG (Duchateau et al., 2009). These structures must pass through a 40 µm unstirred water layer located at the surface of the intestinal epithelium to deliver their contents to the apical portion of the enterocytes (Klein et al., 2006). A schematic representation of the transport of lipolysis products by mixed micelles is presented in Figure 5.

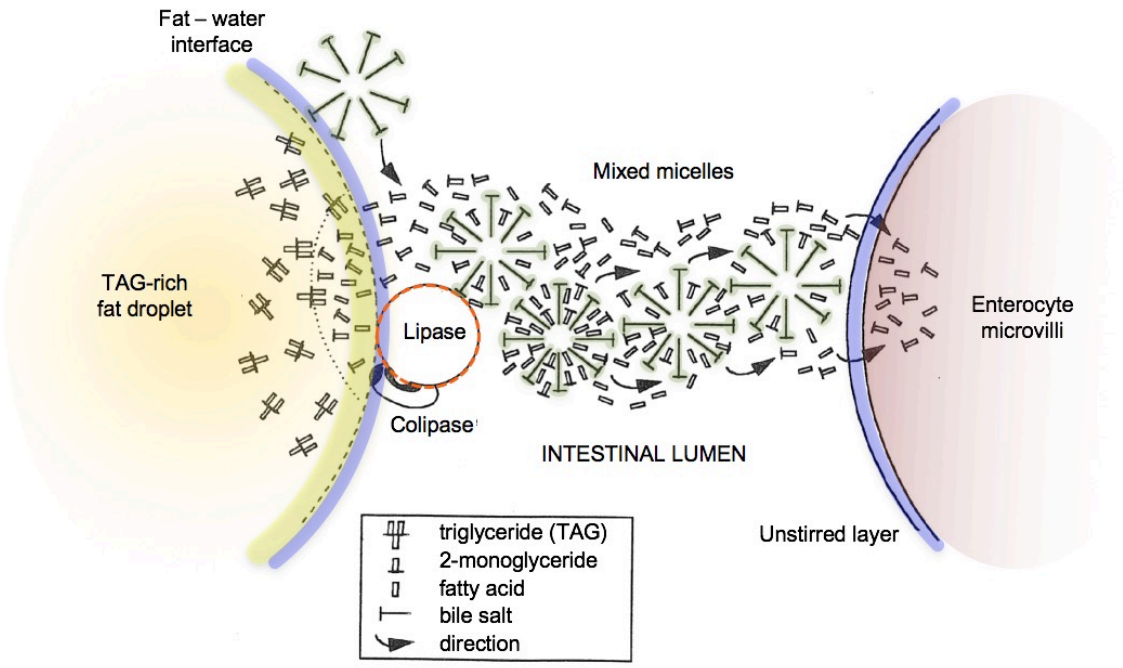

Figure 5. Schematic representation of transport of lipolysis products assembled in mixed micelles from the lipid droplet to the enterocyte. Modified from Jones et al. (2006).

Bile salts are required to achieve optimal absorption of lipids (Bauer et al., 2005) because they play an important role on lipid digestion by promoting emulsification, transferring lipase to new lipolysis sites and solubilizing lipolysis products into

mixed micelles (Jensen, 1971). This implies removing lipolysis products from the oil-water interface of the fat droplets (Figure 5).

2.2.2.2 Paths for lipid absorption

Absorption of dietary lipids mainly takes place in the jejunum, but continues with an increasing ratio of endogenous fat throughout the ileum (Klein et al., 2006). Absorption of FA may require or not the assembly of mixed micelles, according to their hydrophilicity (Bernard et al., 1991). The absorption strategy will also dictate the subsequent handling of the lipids through the portal vein or the lymphatic system (Duchateau et al., 2009).

2.2.2.2.1 Absorption without mixed micelles

Some lipids with relatively high hydrophilicity can pass directly to the portal vein and be transported to the liver. Such lipids do not require assembly into mixed micelles to be absorbed. Short-chain fatty acids (SCFA), being relatively hydrophilic, are not incorporated into mixed micelles and they are mainly absorbed by direct passage to the enterocyte (Bernard et al., 1991). Medium-chain fatty acids (MCFA) can be absorbed through mixed micelles or in their free form. FA that are transferred through the portal vein are subject to the liver first pass, where they can be directly oxidized as an immediate source of energy (Bach et al., 1982). When different milk fat fractions were administered to rats, no SCFA or MCFA were detected in their chylomicrons (Lai et al., 1998).

The differentiation between the portal vein or the lymphatic route for FA is at about carbon 12 (i.e. lauric acid), with longer chains being transported through the lymph (Duchateau et al., 2009).

2.2.2.2.2 Absorption mediated by mixed micelles

Long-chain fatty acids (LCFA) are mainly absorbed through mixed micelles assembled in the lumen (Jones et al., 2006). Unsaturated fatty acids (UFA) are also absorbed this way, and they are more rapidly absorbed than saturated FA

(Graham et al., 1983). The lymphatic handling makes FA less prone to catabolism and more easily stocked in adipose tissue, as opposed to those FA passing directly to the liver (Legrand, 2010).

In addition to LCFA, other lipids such as sn_2-MAG produced by lipolysis and highly lipophilic compounds require assembly into mixed micelles for absorption (Borel, 2003; Carey et al., 1983; Jones et al., 2006). MAG are absorbed without further lipolysis, so fatty acids remain bound to the glycerol backbone (Carey et al., 1983). This is the case for most dietary polyunsaturated fatty acids (PUFA), since they are often located at the sn_2 position (Sethi et al., 1993). Other highly lipophilic micro-constituents like cholesterol and fat-soluble vitamins must be incorporated by co-solubilization within FA and MAG (Patton et al., 1985) into mixed micelles in a non-esterified form to be absorbed (Borel, 2003; Machlin, 1984; Norman et al., 1984; Olson, 1984; Suttie, 1984). Absorption of carotenoids is similar to that of other non-polar lipids (Castenmiller et al., 1998).

Mixed micelles are not absorbed intact (Jones et al., 2006). Conversely, they empty their contents when presented to the microvilli of the enterocyte by providing a concentration gradient (higher in the lumen than inside the enterocytes) for absorption of monomeric lipids (Hussain, 2014; Versantvoort et al., 2004). The gradient, combined with the low pH conditions in the vicinity of the absorptive sites that cause the dissociation of the monomeric lipids, enable their entrance into the enterocyte cytoplasm (Niot et al., 2009; Versantvoort et al., 2004). Lipid molecules are absorbed either by passive diffusion (through the phospholipid bilayer of the enterocytes plasma membrane) or by active transport (involving specific proteins in the enterocytes brush border membrane) (Favé et al., 2004; Niot et al., 2009). The main processes involved in the intestinal absorption of LCFA are shown in Figure 6 and include the following:

- Micellar dissociation mediated by the acidic microclimate at the brush border membrane of enterocytes, causing protonation of fatty acids ($FA^- + H^+ \rightarrow FAH$) and enabling cellular uptake through passive and active

transport. Main active transporters are plasma membrane fatty acid binding protein (FABPpm), fatty acid transport protein 4 (FATP4) and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) (Niot et al., 2009; Pepino et al., 2014).

- Fatty acids lose their proton as they reach the cytoplasm, where they are handled by soluble fatty acid binding proteins (FABP) for intracellular trafficking. Acyl-CoA-synthases (ACS) produce FA-CoA, which can be used as an energy source or be directed into the endoplasmic reticulum (ER) for TAG re-synthesis (Niot et al., 2009). Through the microsomal triacylglycerol transfer protein (MTP), the reformed TAG are assembled with cholesterol esters (CE) and phospholipids (PL) into triacylglycerol-rich lipoproteins (TRL) (Niot et al., 2009).
- The TRL produced during the postprandial period are chylomicrons (CM), which are emptied by exocytosis into the lymph (Niot et al., 2009). Small amounts of very low-density lipoproteins (VLDL) are produced by the enterocyte, but only during the fasting state (Mason, 1998). Some nascent high-density lipoproteins are also produced by the enterocyte (Hussain, 2014).

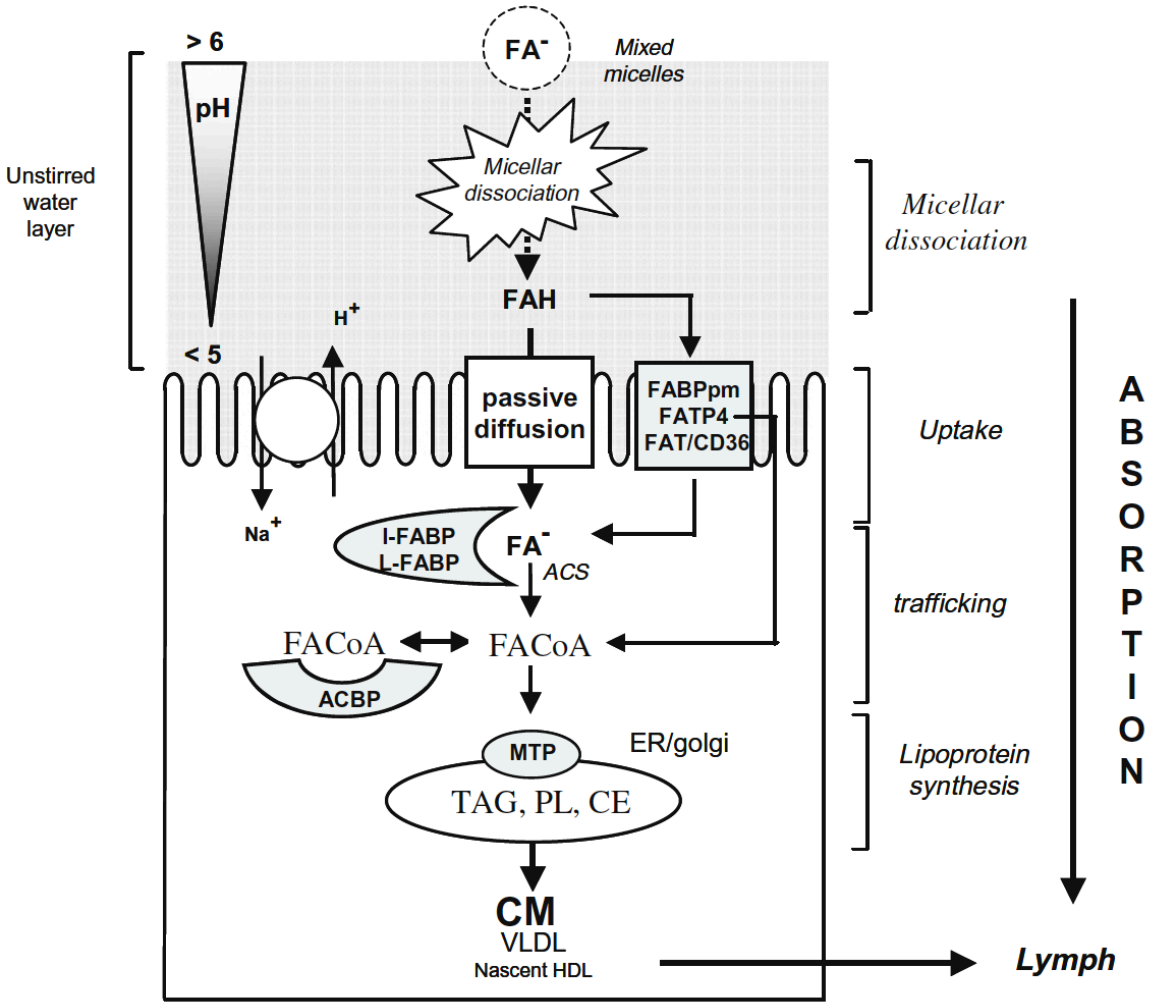

Figure 6. Main processes involved in intestinal absorption of long-chain fatty acids (LCFA). Adapted from Niot et al. (2009).

2.2.3 Calcium and lipid bioaccessibility

As previously defined, bioaccessibility is the fraction of an ingested component that is released from the food matrix into the juices of the gastrointestinal tract (Versantvoort et al., 2004). Calcium plays a complex role in the lipid digestion process and bioaccessibility. On one side, it increases lipolysis rate of TAG, which results in faster FA release. On the other side, calcium ions precipitate with LCFA as calcium soaps under intestinal conditions, reducing their absorption.

2.2.3.1 Calcium soaps

The interaction between calcium and FA has been documented for over a century (Givens, 1917), and it is still a current research topic on fat digestion (Guéguen et al., 2008). Calcium soaps are calcium salts of fatty acids, where one divalent calcium cation is bound to two monovalent fatty acid anions (Patton et al., 1985). Calcium soaps form highly hydrophobic complexes and precipitate in aqueous media (Figure 7) (Pereira et al., 2012).

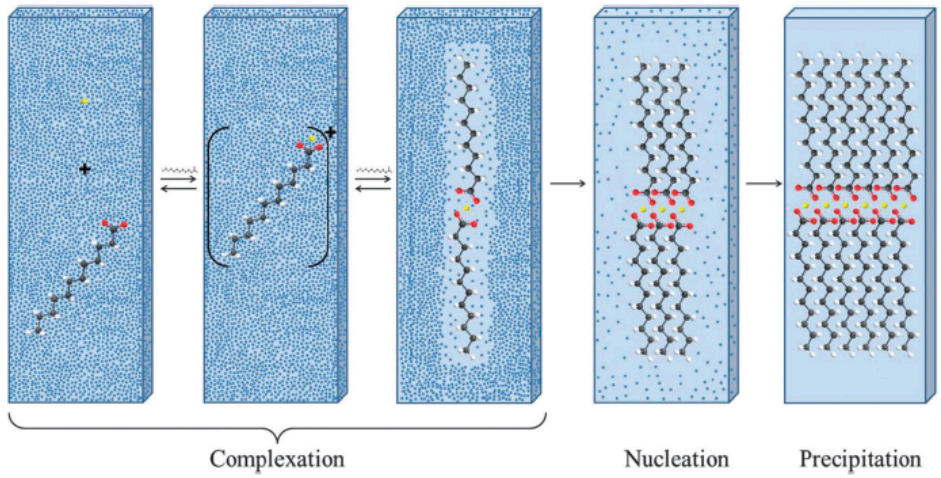

Figure 7. Schematic representation of complexation between calcium and saturated fatty acids (lauric acid depicted) in aqueous media. Reproduced from Pereira et al. (2012).

During the gastric phase of digestion, dietary calcium exposed to the acidic medium of the stomach dissolves. Calcium soap formation is limited at acidic pH because FA are mainly protonated and cannot react with calcium. It is assumed that calcium must be in its soluble form to be absorbed in the small intestine. The major site of absorption is the duodenum, where highly efficient active transporters are present (Goss et al., 2007). Under physiological conditions, calcium soap formation is limited because of the low free calcium concentration and the acidity of the chyme entering the duodenum (Patton et al., 1985). On the other hand, in the fed state, there is an increase in Ca^{2+} levels to ≈ 15 mM in the small intestine because of biliary secretions, which decreases to 3 to 4 mM after 4 hours of digestion (Golding et al., 2010). As chyme advances through the small intestine,

the remaining calcium is exposed to neutral to slightly alkaline conditions given by the secretion of bicarbonate (Goss et al., 2007). Also, such pH conditions cause free FA to lose their proton to be ionized and can react with calcium (Patton et al., 1985).

2.2.3.2 Effect of calcium soap formation on lipolysis

As lipolysis takes place, amphiphilic reaction products, namely LCFA, accumulate at the water-lipid interface of the emulsified lipids. LCFA accumulation on the droplet surface prevents access of lipolytic enzymes to their substrate, eventually inhibiting lipolysis (Favé et al., 2004). Calcium ions increase the rate and extent of lipid hydrolysis by reacting and forming insoluble soaps with such accumulated FA (Armand et al., 1992; Favé et al., 2004) (Figure 8). This removes the LCFA from the lipid surface of fat droplets, which renews the lipids at the interface with TAG and enables lipase access to them (Patton et al., 1979; Patton et al., 1985). Calcium soaps are poorly solubilized by bile salts (Patton et al., 1985), so intestinal absorption of the precipitates formed is much less effective than that of the respective free FA, leading to a net reduction in their bioaccessibility (Karupaiah et al., 2007; Lorenzen et al., 2007).

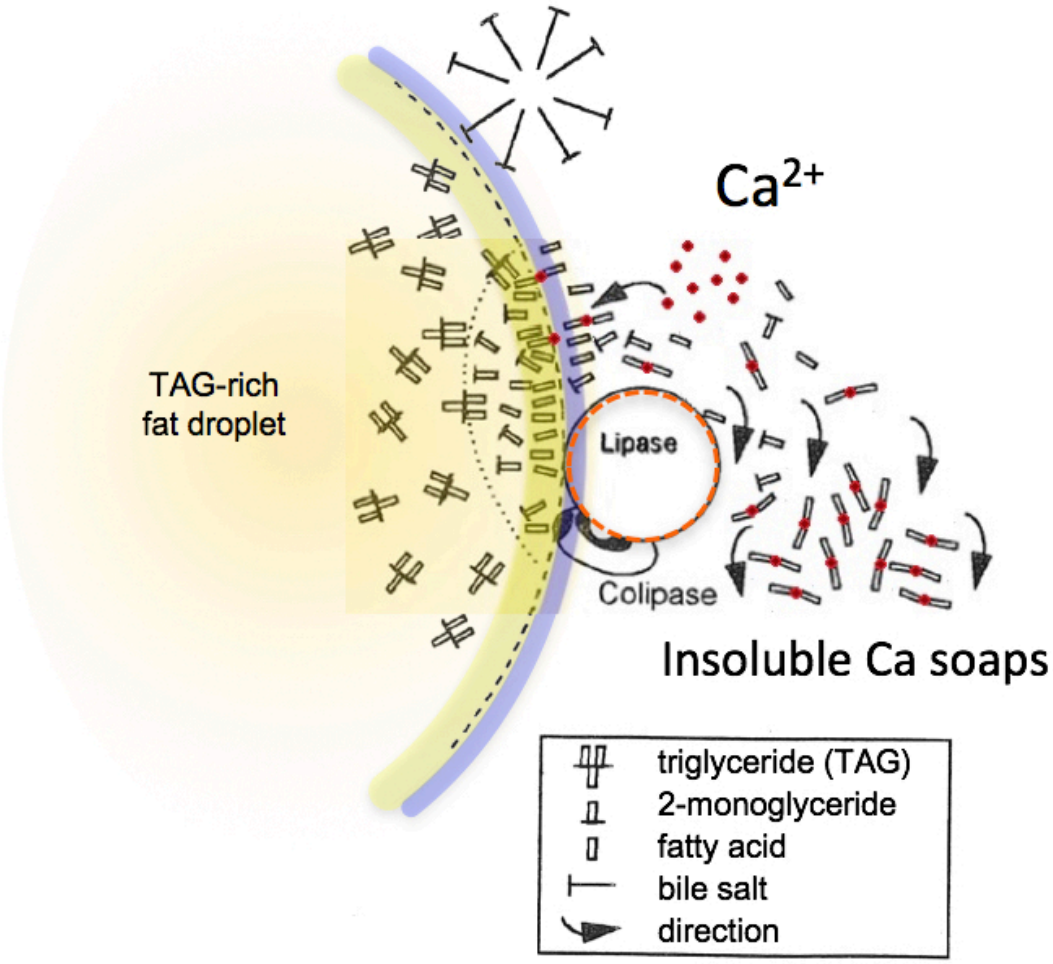

Figure 8. Schematic representation of the formation of calcium soaps at the lipid-water interphase with FA produced by lipolysis. Based on the figure by Jones et al. (2006).

As the chyme advances towards the ileum, pH increases and gives the necessary conditions to form calcium salts with very low solubility (Goss et al., 2007), such as phosphates, carbonates and soaps (Bronner et al., 1999). At pH 7.5, the solubility of calcium soaps in aqueous media, is extremely low (Harrison, 1924), so any ionized calcium may precipitate with fatty acids in the intestine before absorption occurs. For this reason, calcium soaps are mainly present in the terminal ileum (Owen et al., 1995), where the pH conditions are neutral or slightly alkaline and induce their precipitation. This also explains why calcium soap precipitation occurs even after most calcium has been absorbed in the duodenum (Guéguen et al., 2008).

The solubility of calcium soaps of different fatty acids differs according to the length and saturation degree of their carbon chain (Graham et al., 1983). Saturated FA react more extensively with cations than unsaturated FA in the digestive system. Among saturated fatty acids, increasing chain length leads to higher affinity for calcium and lower solubility of the soaps (Graham et al., 1983; Jenkins et al., 1982). Therefore, saturated LCFA account for a significantly higher proportion of calcium soaps (Jenkins et al., 1982). In sum, calcium soap solubility decreases as the pH of the medium increases, FA carbon chain length increases, and saturation level of the FA increases (Table 2). The solubility products of main calcium soaps are presented in appendix C.

Table 2. Solubility of calcium soaps in water at 50°C (Pohle, 1941).

Calcium soap of	Solubility (g/L)
Caprylic acid	3.10
Lauric acid	0.13
Myristic acid	0.09
Palmitic acid	0.07
Stearic acid	0.03
Oleic acid	0.32

Therefore, calcium has mainly an effect on bioavailability reduction of LCFA, with decreasing impact on PUFA, MCFA and SCFA (Fox, 1989; Owen et al., 1995). For example, calcium palmitate and stearate are the most abundant soaps after milk fat ingestion in a high calcium diet (Owen et al., 1995). Also, due to the stereospecificity of digestive lipases, LCFA in sn_1 and sn_3 positions are susceptible to produce calcium soaps, leading to lower absorption rates (Lorenzen et al., 2007).

2.2.3.3 Bioavailability of calcium soaps

The physiological role of calcium on lipid digestion and metabolism has been intensively studied (Zemel, 2009). The bioavailability of calcium soaps is generally recognized to be lower than that of the respective fatty acids involved.

Supplemental calcium has been reported to cause an increase of fecal lipids in various studies with healthy male and female subjects, with varied diets (Bendsen et al., 2008; Lorenzen et al., 2007; Major et al., 2007; Major et al., 2009; Shahkhalili et al., 2001; Welberg et al., 1994). Furthermore, some research points to calcium from dairy sources as more effective than single calcium salts to reduce lipid absorption (Astrup et al., 2010). Recent meta-analyses of various intervention studies on calcium and energy balance indicated that increasing dairy calcium intake by 1200 mg/day resulted in increased fecal fat by an average 5.2 g/day (confidence internal reported from 1.6 to 8.8) (Astrup et al., 2010; Christensen et al., 2009).

Calcium supplementation of high-fat diets does not result in a stoichiometric equivalent reduction in fatty acid absorption (Guéguen et al., 2008). This is because not all dietary calcium reaches the neutral pH regions of the small intestine and also because some calcium soaps can be absorbed by co-solubilization within FA in mixed micelles (Jandacek, 1991). This allows some soaps to reach the brush border along with FA and MAG. Absorption, which requires dissociation of the soaps, is achieved by the low pH microclimate associated with the absorptive site of enterocytes (Hogben et al., 1959; Niot et al., 2009; Versantvoort et al., 2004) (Figure 6). Although this parallel path for absorption exists, calcium soaps reduce LCFA bioavailability by delaying their absorption (Astrup et al., 2010; Golding et al.).

2.3 Postprandial lipids

2.3.1 Fatty acid handling within the enterocyte

The fate of the FA within the enterocyte depends on their chain length. Lipid transport through the cell cytoplasm occurs with the aid of specific cytosolic proteins (e.g. FABP) (Favé et al., 2004; Klein et al., 2006). After crossing the enterocyte membrane, free FA are bound to specific FABP with a high affinity for LCFA (Sethi et al., 1993) and low affinity for FA containing less than 10-12 carbon atoms (Klein et al., 2006).

SCFA and MCFA pass into the portal vein directly without re-esterification into TAG and, bound to albumin, they are transported as non-esterified fatty acids to the liver and other tissues (Bernard et al., 1991; German, 2008; Sethi et al., 1993). Hence, FA passing through portal circulation into the liver (4:0, 6:0, 8:0, 10:0 and a portion of 12:0) are not incorporated into chylomicrons and have no serum cholesterol-raising effect (Parodi, 2016). Once in the liver, SCFA and MCFA are rapidly oxidized and serve as an immediate source of energy, although some MCFA can be assembled into TAG by the liver (German, 2008). Free glycerol from lipid hydrolysis is also transported by portal blood.

MAG and FA with more than 12 carbons are re-esterified to TAG and free cholesterol into cholesteryl esters within the enterocytes (Hussain, 2014; Sethi et al., 1993). The assembly of lipid molecules takes place in the endoplasmic reticulum of the enterocyte through a complex mechanism leading to the formation of lipoproteins, mainly chylomicrons (Hussain, 2014), which are mainly composed of re-esterified lipids packed within a monolayer of phospholipids and apolipoproteins (Figure 9) (Favé et al., 2004; Sethi et al., 1993).

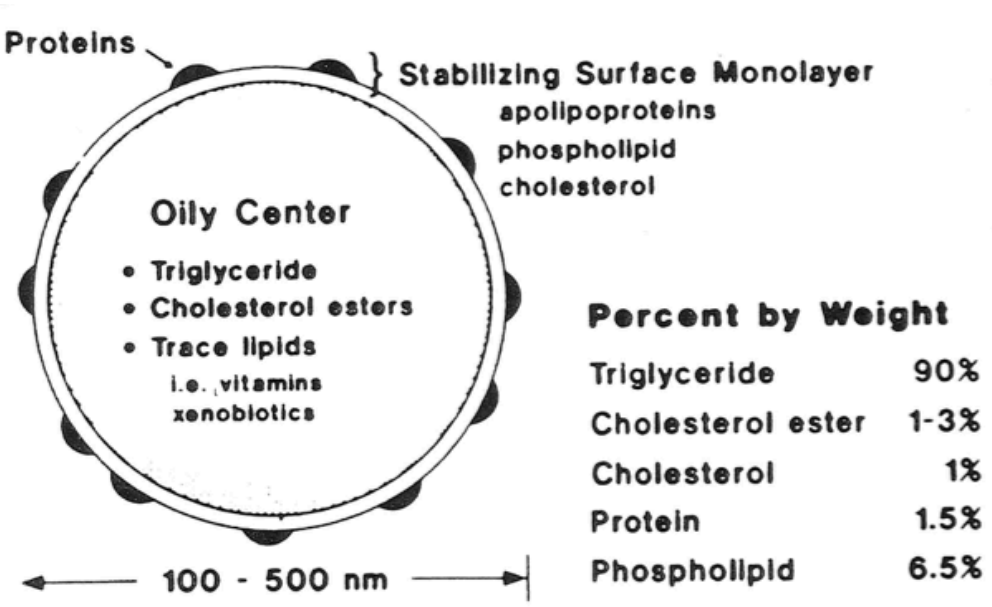

Figure 9. Schematic structure and composition of chylomicrons, the main lipoproteins secreted by the enterocytes into the lymphatic system (Klein et al., 2006).

2.3.2 Postprandial lipemia

Postprandial lipemia[4] (PPL) is the physiological response to fat ingestion (Lorenzen et al., 2007) and it refers to the status of lipids and lipoproteins (Figure 10) in blood following a fat load or a meal (Ooi et al., 2011). The main lipoproteins are presented in Figure 10. Triglyceride-rich lipoproteins (TRL) include chylomicrons, very low-density lipoproteins (VLDL) and intermediate-density lipoproteins (IDL) (Ooi et al., 2011). Cholesterol-rich lipoproteins include low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Ooi et al., 2011).

[4] The terms “lipidemia” and “lipaemia” are also used.

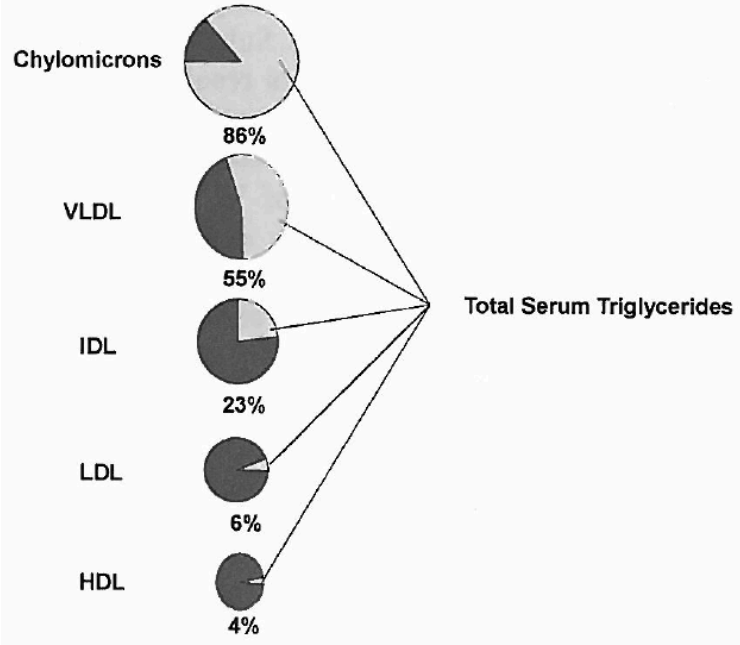

Figure 10. Main lipoproteins and their approximate dry-weight mass ratio triglyceride content (%) in each fraction, all contributing to total serum triglyceride concentration. Reproduced from Ooi et al. (2011).

In the postprandial phase, chylomicrons are poured into the intestinal lymph, pass through the thoracic duct and enter the bloodstream through the subclavian vein (Sethi et al., 1993). PPL is a dynamic process (Figure 11) where lipids are depleted from chylomicrons under the effect of lipoprotein lipase (LPL) (Berry, 2009). During the later stages of the postprandial response, hepatically-derived lipoproteins also come into play (Berry, 2009) (Figure 11). PPL is characterized by the rise in circulating lipids due to the accumulation and competition for clearance between hepatically- and intestinally-derived TRL[5] (Lairon, 1996; Ooi et al., 2011). Chylomicrons begin to appear in the plasma within 60 minutes of ingestion of the fat (Sethi et al., 1993), triggering a pattern of rapid rise and fall in TRL (Lorenzen et al., 2007). Usually a single peak occurs within 120 to 300 minutes (Lorenzen et al., 2007; Michalski et al., 2006), although biphasic and triphasic responses after up to 10 hours after ingestion of the meal are not rare (Sethi et al., 1993). Generally, the

[5] Main intestinal- and hepatic-derived TRL during the postprandial phase include Chylomicrons and VLDL, respectively.

major part of the TAG components is usually removed from circulation within 6-8 h after meal ingestion (Sethi et al., 1993). This implies that humans eating three or more times a day spend most of their day in the postprandial state (Berry, 2009; Lairon et al., 2007).

PPL reflects the ability of the organism to process the fat of a meal from its secretion as chylomicrons to its clearance from circulating lipoproteins by transferring lipids into adipose tissue and clearing remnant particles (Lairon et al., 2007). The amplitude and time course of the occurrence of the postprandial triglyceride response is highly influenced by the quantity of dietary lipids (mainly TAG) and their FA profile and structure (Berry et al., 2007; Berry et al., 2005; Lairon, 1996; Mekki et al., 2002).

A schematic representation of the circulation and metabolic fate of postprandial lipids in the body is presented in Figure 11. The hydrolysis of dietary and endogenous lipids (bile acids (BA), phospholipids (PL), cholesterol esters (CE), triglycerides (TAG)) is followed by their assembly into TRL, mainly as chylomicrons (CM) (Niot et al., 2009). TRL are then transported through the lymph and blood. TAG in CM are hydrolyzed by LPL, and the resulting LCFA are used as energy source in myocytes or reassembled into TAG for storage in the adipocytes. LDL-related peptide (LRP) and hepatic lipase (HL) act on CM remnants, which are reassembled into VLDL in the hepatocytes and secreted into the bloodstream, which also influences PPL (Niot et al., 2009).

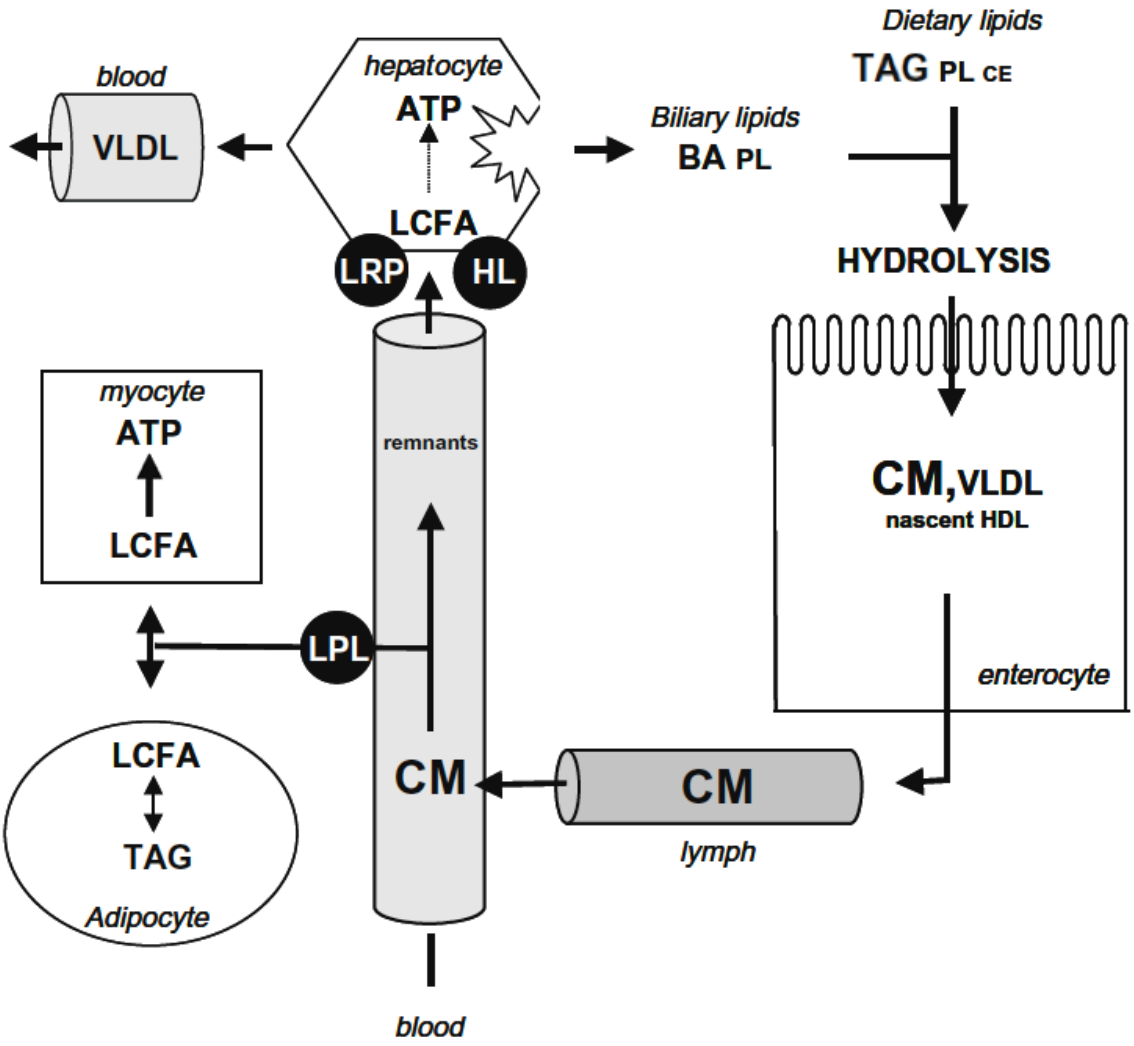

Figure 11. Representation of the circulation and metabolic fate of postprandial lipids in the body. Modified from Niot et al. (2009).

2.3.2.1 Postprandial lipemia and cardiovascular disease

Exacerbated PPL, namely a high and rapid rise in blood TAG with a delay in postprandial TAG elimination (Michalski, 2008) has been repeatedly associated with cardiovascular risk (Lairon et al., 2007). The magnitude and duration are linked to CVD risk by their effect on endothelial dysfunction, activation of factor VII[6], elevated circulating chylomicron remnants, generation of small dense LDL

[6] Factor VII is a clotting factor.

particles and impaired insulin sensitivity (Berry, 2009). In fact, non-fasting TAG levels in plasma have been demonstrated to be useful as a cardiovascular disease (CVD) risk predictor (Su et al., 2009) due to their contribution to both thrombotic and atherogenic processes (Berry et al., 2005).

Furthermore, a postprandial increase of intestine-derived chylomicrons over a significant part of the day may also be related to CVD risk associated with liver-derived VLDL (Su et al., 2009). Research has demonstrated that TRL and their remnants have a direct role with atherosclerosis (Kolovou et al., 2011; Sethi et al., 1993). Delayed clearance of postprandial TRL increases the delivery of TAG and cholesteryl esters to the arterial wall and can accelerate the progression of atherosclerosis (Sethi et al., 1993; Su et al., 2009). This is explained by the large TRL particles that can induce the assembly and function of coagulation complexes, leading to the activation of factor VII, linking increased postprandial triglyceridemia with increased CVD risk (Berry et al., 2007; Berry et al., 2005; Lefevre et al., 2004).

For this reason, exaggerated intake of lipids, especially associated with a reduced metabolic capacity, can induce an accumulation of TRL remnants, with a subsequent and, exaggerated cellular uptake of atherogenic particles (Lairon, 1996). Conversely, moderate lipid intake, associated with efficient TAG handling may reduce the buildup of atheroma plaque (Lairon, 1996).

2.3.2.2 Postprandial lipemia and dietary fat

Abundant research related to PPL has focused on the quantity and composition of fat, and more recently on the structure of such fat (Berry, 2009; Grundy et al., 2002; Lairon, 1996). The structure of dietary fats, ranging from the molecular to the macroscopic level within the food matrix, affects FA bioavailability and metabolism, as extensively reviewed by Michalski et al. (Michalski et al., 2013). During the past decades, it has been shown that TAG with similar fatty acid composition but different structure (i.e. sn- position on the glycerol backbone of their fatty acids) differ in their physiological effects due to their different biochemical and physical properties (Berry et al., 2005; Kubow, 1996). For example, during digestion, fats

that are crystalline solids at body temperature have lower lipolysis rates and form micelles less readily slowing their absorption (Berry, 2009; Berry et al., 2007). Hence, stereospecific-induced properties translate into different behaviors during digestion, absorption and PPL (Berry et al., 2005; Mekki et al., 2002). Beyond the molecular structure of TAG, the food matrix containing the fat may also influence PPL.

2.3.2.3 Postprandial lipemia and dairy products

Milk fat present in dairy products with different food structures is not digested in the same way, which results in different postprandial responses (Clemente et al., 2003; Michalski et al., 2006). Furthermore, calcium has been pointed as a component in dairy products associated with the reduction of CVD risk (Lorenzen et al., 2011), mainly by its effect on reducing PPL (Astrup et al., 2010; Lorenzen et al., 2007).

Dairy foods represent an excellent model to better understand the underlying mechanisms by which the food matrix may have an impact on PPL. From a technological viewpoint, they include a spectrum of foods offering a wide array of textures, with adjustable composition and structure. Nutritionally speaking, dairy foods are highly digestible and can serve as carriers for other nutrients. Given their variety and generalized consumption, they also represent an opportunity to provide the population with healthier foods. Indeed, if foods can be designed to modify PPL by controlling the way they are digested, then there may be potential health benefits, especially on a long-term basis.

2.4 Milk fat

2.4.1 Composition and structure

Milk fat is secreted as a complex structure called the milk fat globule (MFG). The native milk fat globules consist of a hydrophobic core containing the vast majority of the TAG, esterified cholesterol and fat-soluble vitamins, surrounded by a protein and phospholipid multilayer surface called the milk fat globule membrane (MFGM) (Christie, 1995; Keenan et al., 1995a; Smith et al., 2007) (Table 3). Several minor components of physiological and sensory importance present in milk fat include glycerolipids, sterols and steroidal hormones, hydrocarbons, lipid-soluble vitamins, prostaglandins, carnitine and acylcarnitines, ceramides, glycosphingolipids and other sensory-relevant aliphatic compounds like ketones, alkyl ketones, lactones, aldehydes and short-chain fatty acids (Christie, 1995).

Table 3. Usual composition of major lipid classes in bovine milk (Christie, 1995).

Lipid class	Mass fraction (%)
Triacylglycerols	97.5
Diacylglycerols	0.36
Monoacylglycerols	0.027
Cholesteryl esters	Trace
Cholesterol	0.31
Free fatty acids	0.027
Phospholipids	0.6

Composition of milk fat extracted by centrifugation differs from that of the native milk fat globules due to the removal of the MFGM during processing, which drastically reduces the amount of phospholipids, mono and diglycerides, cholesterol and other sterols, carotenoids, vitamin A and other minor components. Anhydrous milk fat (AMF) or anhydrous butter oil is defined as containing 99.8% milk fat and 0.1% water, whereas butter oil (not anhydrous) is composed of 99.3%

milk fat and 0.5% water (Kaylegian et al., 1995). Milk fat is composed of 98% TAG and only traces of MFGM remain (Kaylegian et al., 1995).

Over 400 FA have been identified in milk fat, with only less than 20 accounting for more than 95% of the total FA (Kaylegian et al., 1995) (Table 4). FA are not homogeneously distributed among the TAG, due to the intrinsic physiology of the assembly and secretion by the mammary gland (Christie, 1995). Conversely, the FA distribution profiles in milk TAG are stable (Table 5) and certain generalizations on their stereospecific position on the glycerol backbone (Kaylegian et al., 1995) include the following:

- C4 and C6 are in sn_3 position.
- C8 is in sn_2 and sn_3 positions.
- C10 and C12 are located preferentially in sn_2 position.
- C14 is in sn_2 position.
- C16 and C18:0 are almost equally distributed in sn_1 and sn_2 positions.
- C18:1 is predominantly in sn_1 or sn_3 positions.

Table 4. Composition (weight % and mole %) of the major fatty acids in butterfat (Sichien et al., 2009).

Fatty acid	Common name	Mass fraction (%)	Mole fraction (%)
C4:0	butyric	3.7	9.6
C6:0	caproic	2.5	4.9
C8:0	caprylic	1.6	2.2
C10:0	capric	2.9	3.9
C12:0	lauric	3.2	3.7
C14:0	myristic	11.2	11.4
C14:1	myristoleic	1.5	1.1
C15:0	pentadecylic	1.3	1.4
C16:0	palmitic	27.4	24.8
C16:1	palmitoleic	1.7	1.5
C18:0	stearic	13.9	11.4
C18:1	oleic	28.0	23.0
C18:2	linoleic	2.0	1.7
C18:3	linolenic	1.0	0.8
C20:0	arachidic	0.2	0.1

Table 5. Relative abundance of main fatty acids esterified in cow's milk triacylglycerols (Christie, 1995; Jensen et al., 1995).

Fatty acid	Name	Mole fraction within each sn position (%)			Total mole fraction (%)
		sn_1-	sn_2-	sn_3-	
C4:0	butyric			35.4	11.8
C6:0	caproic		0.9	12.9	4.6
C8:0	caprylic	1.4	0.7	3.6	1.9
C10:0	capric	1.9	3.0	6.2	3.7
C12:0	lauric	4.9	6.2	0.6	3.9
C14:0	myristic	9.7	17.5	6.4	11.2
C16:0	palmitic	34.0	32.3	5.4	23.9
C16:1	palmitoleic	2.8	3.6	1.4	2.6
C18:0	stearic	10.3	9.5	1.2	7
C18:1	oleic	30.0	18.9	23.1	24
C18:2	linoleic	1.7	3.5	2.3	2.5
TOTAL	∑=	96.7	96.1	98.5	97.1

2.4.2 Technological and nutritional functionality of milk fat

2.4.2.1 Fractions of milk fat

Milk fat with different fatty acid profiles is commercially available. Some specialty applications of milk fat require it to be refined into different fractions, like those with higher melting points for baked products or with lower melting points for low-temperature spreadability (Makhlouf et al., 1987). There are various methods to fractionate milk fat, yielding final products that differ in their melting characteristics, which are determined by the FA profiles of their TAG (Kaylegian et al., 1995; Sichien et al., 2009). Fractionation by crystallization, or dry fractionation, is the most common method to separate milk fat into stearin and olein, respectively for higher and lower melting-point fractions (Sichien et al., 2009). Hence, the olein fractions are enriched in FA leading to low melting points whereas stearin fractions are enriched with FA leading to high melting points (deMan et al., 1980; Sichien et al., 2009). The specific FA composition of olein and stearin fractions differs depending on the cut-off temperatures used to fractionate the original AMF but, in general, olein fractions are enriched in SCFA and UFA, whereas stearin fractions are enriched in saturated LCFA (deMan et al., 1980; Kaylegian et al., 1995) (Figure 12).

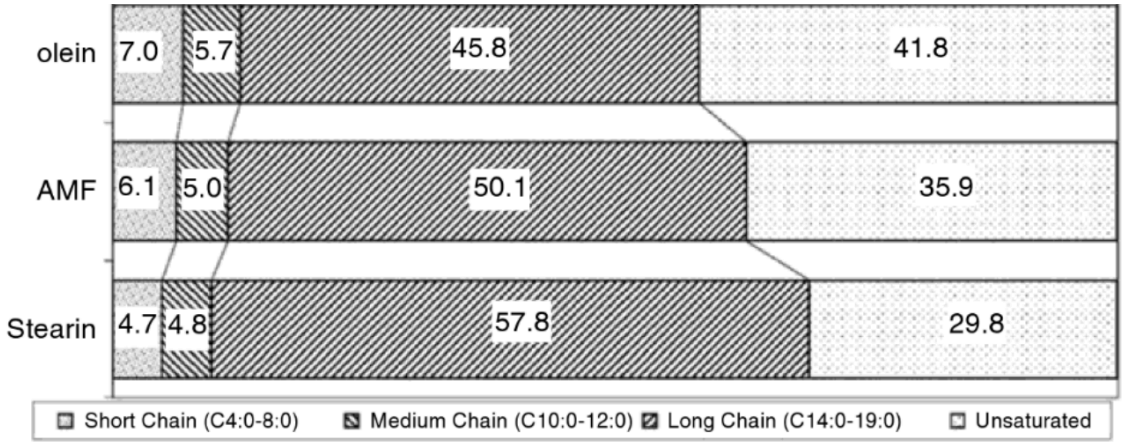

Figure 12. Distribution of different fatty acid groups in AMF and its fractions obtained by crystallization from the melt. Values indicate mass fraction (%) of each FA group. Modified from Augustin et al. (2006).

The different melting profiles of milk fat fractions can be seen by the displacement of the three main endothermic events during melting on differential scanning calorimetry (DSC) thermograms (Figure 13). In the thermograms presented, the three endothermic events include triglyceride groups melting independently and identified as the low-melting fraction (LMF) which melts below 10°C, a middle-melting fraction (MMF) which melts from 10°C to 19°C, and a broad high-melting fraction (HMF) which melts above 20°C (Sichien et al., 2009).

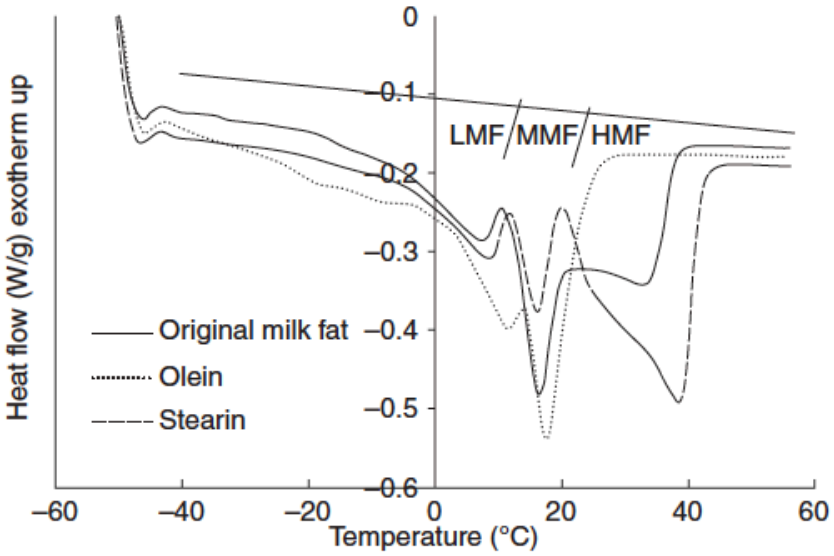

Figure 13. Melting profile of milk fat, stearin and olein determined by differential scanning calorimetry (DSC) with a heating rate of 5°C/min. Reproduced from Sichien et al. (2009).

2.4.2.2 Homogenization of milk fat

Milk fat is mainly consumed through foods where it is dispersed in the matrix within the other components. In non-homogenized milk, the MFGM serves as an amphipathic layer that stabilizes the droplets in an aqueous environment. When milk is homogenized, the native MFG is disrupted and smaller droplets are formed (typically from about 4 μm to below 1 μm), increasing their surface area about 6 to 10 times (Argov et al., 2008; Jensen et al., 1995). The original MFGM cannot cover the increase of the fat-water interface, so it is mainly compensated, in non-pasteurized milk, by casein adsorption, resulting in a protein-phospholipid surface

(Jensen et al., 1995; Michalski et al., 2002). Higher homogenization pressures increase the amount of protein adsorbed per gram of fat because smaller droplets, hence more surface, are produced (Mulder et al., 1974). When bulk fat, such as AMF, is homogenized in skim milk, the surface layer of the fat droplet is composed of adsorbed casein and some serum proteins, stabilizing the emulsion (Walstra, 1995). Changing the surface composition of the milk fat droplets modifies their functionality, both from a technological and from a nutritional point of view (Argov et al., 2008).

2.4.2.3 Technological properties of milk fat

Technologically, the adsorption of casein implicates the milk fat droplet surface as a structure promoter in acid milk gels (Michalski et al., 2002), but reduces casein functionality in rennet induced gels by promoting interactions of the milk fat droplet with the casein matrix (Michalski et al., 2007). The presence of casein in homogenized droplets can form permanent cross links with the casein network (Michalski et al., 2002). Defects linked to such interactions include weaker rennet curd, curd shattering and improper curd matting[7] (Metzger et al., 1994, 1995), but an improvement of the body and texture of products like Cheddar cheese has also been reported (Metzger et al., 1995). In cheese, casein-stabilized milk fat droplets embedded in the coagulum would be less prone to coalesce during cheese production than native MFG (Michalski et al., 2005; Tunick et al., 1993).

2.4.2.4 Nutritional properties of milk fat

2.4.2.4.1 Size of the milk fat droplets

From a nutritional perspective, MFG modified milk fat droplets are digested and metabolized differently. Under normal physiological conditions, lipases are in excess relative to substrate availability so increasing the substrate exposure allows the binding of more lipase molecules, resulting in a much higher degree of lipolysis

[7] Matting refers to the fusion of curd pieces to form a continuous rubbery slab (Sivasankar, 2002).

of the TAG (Favé et al., 2004). There seems to be a consensus in that, at least for emulsions stabilized with the same surfactant, small droplets facilitate lipolysis because more surface is available for lipase action than in large droplets (Armand et al., 1999; Favé et al., 2004). For example, an in vitro study with pancreatic lipase showed that lipolysis of small native globules (1.8 μm) was faster than that of large globules (6.7 μm) (Berton et al., 2012). The increase in the lipolysis rate was explained by the increase (3.6-fold) of available surface (Berton et al., 2012). A clinical trial showed that emulsified milk fat in skim milk resulted in an earlier and sharper lipemia peak than non-emulsified milk fat consumed as a spread (Vors et al., 2013). The responses were related to the emulsified or non-emulsified state of the milk fat (Vors et al., 2013).

2.4.2.4.2 Emulsifying agent of the milk fat droplets

Emulsifiers can change lipolysis rates by competition with the enzyme adsorption to the surface of the fat droplets (McClements et al., 2009a). An in vitro study found that, despite a 25-fold surface increase, homogenized milk fat droplets had only a 2-fold lipolysis increase, when compared to native MFG (Berton et al., 2012). Such results were explained by the milk proteins providing a less favorable interface for lipolysis (Berton et al., 2012). In a rat-model study comparing different emulsifiers, fine milk fat droplets coated with casein were metabolized slower than large droplets coated with phospholipids and non-emulsified fat (Michalski et al., 2005). The large droplets and non-emulsified fat caused a fast and high lipemic peak with a sharp fall, whereas lipemia caused by the fine droplets was more gradual raise and recovery (Michalski et al., 2005). Other works showed that pasteurization of human milk reduced the lipolysis rates (de Oliveira et al., 2016b) and gastrointestinal bioaccessibility of some fatty acids (de Oliveira et al., 2016a) using a preterm newborn in vitro digestion system, when compared to raw human milk. In both cases, such effects were in part explained by heat-induced protein aggregation around the MFMG in pasteurized human milk (de Oliveira et al., 2016a; de Oliveira et al., 2016b).

2.4.2.4.3 Milk fat distribution within the food matrix

Milk fat distribution can vary considerably from one dairy product to another (Tamime, 2007). For example, milk fat distribution can range from finely emulsified droplets in homogenized milk to a mass of intact and coalesced MFG aggregates in butter (Martini et al., 2007). Several studies have shown that dairy products with different fat distribution lead to different metabolic responses (Visioli et al., 2014).

In rats, cream caused a faster lymphatic fat rise than cream cheese and butter (Fruekilde et al., 2004). Another study with rats compared two high-fat (20%) diets of identical composition, but with milk fat from cheese powder or a butter-casein mixture (Higurashi et al., 2016). After 9 weeks, the cheese-based diet reduced the hepatic accumulation of triglycerides and cholesterol and reduced the plasmatic non-HDL levels (Higurashi et al., 2016). Such differences were explained by differences on their digestion and absorption kinetics.

In humans, various intervention studies have compared the effects of consuming milk fat in a structured matrix (cheese) as compared to a relatively unstructured one (butter).

- Meals containing milk fat from butter caused a delayed PPL when compared to milk fat from milk and mozzarella cheese. According to such study, free fat (i.e. butter) may require more time to emulsify than small droplets (i.e. milk) and fat mixed with proteins (i.e. cheese) (Clemente et al., 2003). The same study concluded that the physical structure of milk fat-rich foods did not have a major effect on PPL, but that the timing of the peak could be delayed for butter and cheese, when compared to milk.
- When 40 g of milk fat was consumed as butter or matured Cheddar cheese by mildly hypercholesterolemic participants for 4 weeks, the total and LDL cholesterol increased significantly for the butter group as compared with the cheese group (Nestel et al., 2005).

- When 13% of total daily calories were replaced with cheese or butter (143 g or 47 g, respectively with equivalent milk fat content) for 6 weeks in a randomized crossover trial with 49 healthy participants, cheese did not increase LDL cholesterol concentrations, when compared with the run-in period. Furthermore, when compared with butter, cheese induced a significantly lower increase in total (5.7%) and LDL (6.9%) cholesterol (Hjerpsted et al., 2011).
- At equal amounts of milk fat (42 g) in an isoenergetic diet for three weeks, cheese induced a lower increase in cholesterol concentrations than butter in healthy adults. The calcium content of the cheese was proposed as explanation of such effect (Biong et al., 2004).

The works cited show the influence that the food matrix can have on its metabolic consequences. Furthermore, they also support the choice of a cheese matrix as a pertinent model to study the digestive and absorptive processes leading to different metabolic responses.

In the end, milk fat absorption from the lumen is generally quite efficient, and only about 4% of the ingested fat escapes into the feces (Carey et al., 1983). Nevertheless, the physiological outcome changes for foods with different structures and compositions because they are digested and metabolized differently (Michalski et al., 2005). By harnessing such processes, it could be possible to modulate the metabolic handling of lipids and, for example, favor their beta oxidation rather than their storage (Vors et al., 2016).

2.5 Cheese

2.5.1 Nutritional relevance of cheese

Cheese is the main form of solid and hard dairy products, as opposed to fluid or semi-solid products such as beverages and yogurt. Nutritionally speaking, cheese is recognized as nutrient-dense food, while, on a technological viewpoint, it is highly customizable matrix. Among dairy foods, cheese is a product that may contain appreciable amounts of fat, which has tarnished its nutritional value. Nevertheless, recent intervention studies (Biong et al., 2004; Nestel et al., 2005; Tholstrup et al., 2004) suggest that cheese has the lowest LDL-cholesterol raising effect out of full-fat dairy products. As a matter of fact, a reassessment on the impact of milk fat and dairy foods on CVD during the past years seems to converge towards excluding dairy products, including cheese, from CVD risk factors (German et al., 2009; Gibson et al., 2009; Jahreis et al., 2010; Kratz et al., 2013; Steijns, 2008; Visioli et al., 2014). Interestingly, even epidemiological studies that have reported dairy products as a factor increasing CVD risk have found the lowest and even negative correlations for cheese with respect to other dairy products (Artaud-Wild et al., 1993; Moss et al., 2003; Renaud et al., 1989).

2.5.2 Studies on cheese digestion

To better understand the beneficial effects of cheese, an increasing number of in vitro and in vivo works have followed, studying different cheese matrices, their structure and the interactions of their components. It has been shown that cheeses with different textures have different disintegration and nutrient release rates during in vitro digestion (Fang et al., 2016b), which can result in different peptide release rates (Fang et al., 2016a). In vivo studies with mini-pigs have shown that acid and rennet gels have different protein breakdown during digestion, which led to different bioavailability of amino-acids (Barbe et al., 2014).

Different cheese matrices were shown to have different lipolysis rates during in vitro digestion, leading to different FA bioaccessibility levels (Lamothe et al., 2012;

Lamothe et al., 2017). Both in vivo and clinical studies have shown beneficial effects of cheese on health, like preventing hepatic fat accumulation and improving serum lipid parameters in rats (Higurashi et al., 2016) or improving serum lipid parameters and increasing fat excretion in humans (Soerensen et al., 2014). Also, studies have shown that the composition and the structure of ripened cheeses influence the digestion and metabolic fate of the milk fat they contain (Lopez et al., 2010). Although a significant progress on how the cheese matrix influences the digestion of its components has been achieved, it is far from being fully understood.

2.5.3 Cheddar cheese

2.5.3.1 Definition

Cheddar cheese is the most widely consumed cheese in Canada (Agriculture and Agri-Food Canada, 2017) and one of the most consumed in the world (Canadian Dairy Commission, 2015). Cheddar is mainly produced with pasteurized cow's milk (Canadian Dairy Commission, 2015), which is coagulated and subjected to the cheddaring process (or any other process[8] that yields a cheese with the same characteristics) (Minister of Justice of Canada, 2016).

According to Canadian standards of identity for Cheddar cheese, its moisture content cannot exceed 39% and its fat content may not be less than 31% (Minister of Justice of Canada, 2016). The typical composition of Cheddar cheese is presented in Table 6. Saturated FA represent about two thirds of Cheddar cheese fat, with the rest being unsaturated FA (Health Canada, 2010a), according to the intrinsic composition of milk fat. Total calcium in Cheddar cheese ranges from 550 to 675 mmol per kilogram of solids in a non-fat basis (Lawrence et al., 2004). Such

[8] Cheddar cheese can also be made by the stirred curd process, which is easier and faster than the traditional process. After whey drainage, curds are constantly stirred, preventing them to mat, until the desired acidity is reached. The curds are then salted, pressed and aged. Cheddar cheese for further processing is often prepared by this method (Canadian Dairy Commission, 2015).

calcium content is equivalent to approximately 350 mg in a standard 50 g portion[9] (Health Canada, 2010c). Typical pH range of Cheddar cheese extends from 4.9 to 5.3. These calcium and pH conditions determine the textural and sensory characteristics of Cheddar cheese (Lawrence et al., 2004).

Table 6. Typical composition of Cheddar cheese (Health Canada, 2010a).

Component	Mass fraction %
moisture	37.1
ash	3.7
protein	24.0
lipids	33.8
carbohydrate	1.3

2.5.3.2 Manufacturing process

Cheddar cheese manufacture involves several interrelated operations. A generic process showing the main operations for Cheddar cheese manufacture is presented in Figure 14, and it is explained in the following sections.

[9] The adequate daily intake of calcium suggested in Canada is 1300 mg (Health Canada, 2010b).

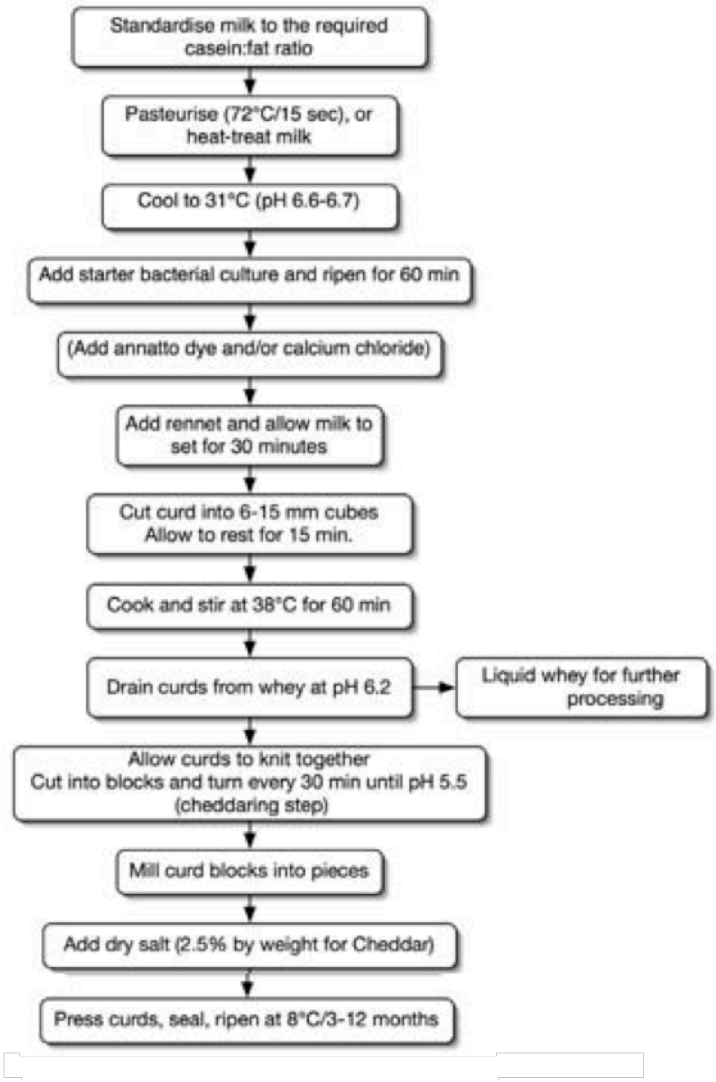

Figure 14. Generic process for Cheddar cheese manufacture. Adapted from Everett (2007).

2.5.3.2.1 Conditioning of milk

First, milk is standardized to the required fat content (and fat-to-protein ratio) (Everett, 2007). Milk in not homogenized prior to Cheddar cheese production. The milk is then pasteurized with low heat treatments. The cheese-making capacity of milk is reduced if heat treatments exceed 15 to 20 seconds at 74°C by considerably increasing calcium and inorganic phosphate insolubilization, as well as whey protein denaturation and aggregation (Gaucheron et al., 2004; Mietton et al., 2004).

Once milk is pasteurized, it is cooled down to around 32°C so the starter culture can be added (Walstra et al., 2005). About one hour after the cultures have been added and the acidification has begun, food additives, like color (if required) and calcium chloride, are added to the vat.

Calcium chloride is permitted as a firming agent in amounts not to exceed 0.02% of the milk (Minister of Justice of Canada, 2016). Calcium chloride is added before the renneting of pasteurized[10] milk in an attempt to re-establish calcium ion activity for an optimal formation of the curd (Horne, 1998) (about 1 mM equivalent (Walstra et al., 2005)). A secondary effect of increasing the ionized calcium is a slight pH drop (Mietton et al., 2004). The added calcium compensates for any losses due to precipitation of calcium phosphates during the heating of milk (Horne, 1998). Simultaneously, lower amounts of rennet are required to form the gel (Walstra et al., 2005).

2.5.3.2.2 Clotting of milk

When the rennet is added, agitation is halted to allow the formation of a coagulum. Clotting takes place under the effect of renneting enzymes, mainly chymosin (EC 3.4.23.4) and smaller amounts of pepsin (EC 3.4.23.1) (Walstra et al., 2005). Around pH 6.7, chymosin rapidly destabilizes casein micelles by cleaving the Phe105-Met106 bond of the κ-casein, with the para-κ-casein remaining in the micelle and the hydrophilic strand of caseino-macropeptide (CMP) dissolving in the aqueous medium (Walstra et al., 2005). The loss of CMP destabilizes the colloidal suspension because steric and electrostatic repulsion is diminished. Aggregation of the micelles is originally driven by Van der Waals attraction between CMP-depleted patches of the paracasein (Walstra et al., 2005). When CMP loss approaches 70%, the altered casein micelles aggregate under the high Ca^{2+} activity in milk serum (Walstra et al., 2005).

[10] A consequence of pasteurization is a decrease in the solubility of calcium phosphate. This implies withdrawal of calcium bound to casein and a consequent increase in the protein charge, weakening the bonds, measurable as a decrease in gel strength (Horne, 1998).

Calcium ions intervene in clotting by neutralizing negative charges on the micelles (more effective than H^+ ions at pH conditions during renneting), reducing electrostatic repulsion and promoting association (Walstra et al., 2005). Nevertheless, the presence of phosphate in the colloidal structures is still required for the coagulation process (Famelart, 2004). The elimination of colloidal calcium phosphate (e.g. during excessive acidification of the curd) would increase electrostatic repulsion within the micelles, resulting in weaker structures (Famelart, 2004).

Up to a certain point[11], adding $CaCl_2$ to milk increases the concentration of ionized calcium, leading to the association of some calcium ions with phosphate and citrate in the aqueous phase and in the micellar structures (Gaucheron et al., 2004). Paracasein micelles are locked together by calcium bridges, as positively-charged colloidal calcium phosphate between the negative sites of the phosphoserine on the micelle surface (Famelart, 2004; Walstra et al., 2005). Hence, the increase of the colloidal calcium phosphate contributes to the aggregation rate of casein, accelerates the clotting process for formation of the curd and yields a firmer gel (Mietton et al., 2004).

As aggregation progresses, a particle gel is formed (Figure 15), with particles and micelles aggregating into strands and the latter into a network where the junctions become stronger (Walstra et al., 2005). Eventually, this leads to syneresis as the network rearranges and more junctions between the gel structures are formed (Walstra et al., 2005). The resulting gel is a protein network, mainly composed of casein where calcium acts as a structuring agent (Horne, 1998). The network entraps milk fat globules (Mistry et al., 1993), while allowing whey to drain out (Canadian Dairy Commission, 2015). In the case of Cheddar cheese, the gel is allowed to set until it is firm enough to withstand the cutting step without disaggregating, but not too firm to cause shattering of the curd (Walstra et al., 2005). Another advantage of adding $CaCl_2$ during milk conditioning is that fines

[11] Added calcium levels over 10 to 15 mM have been reported to have adverse effects on the clotting process (Famelart, 2004).

(i.e. small particles of shattered curd) are reduced so the yield is improved (Mietton et al., 2004).

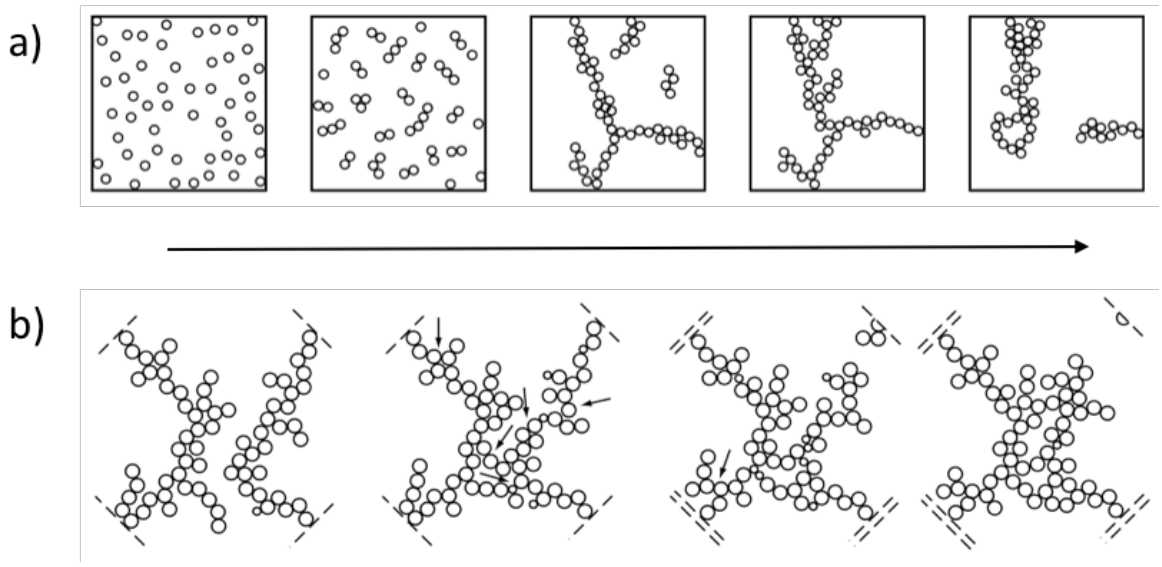

Figure 15. Schematic representation of a) paracasein aggregation during the gel formation and b) the subsequent rearrangement events leading to local shrinkage and whey expulsion from the gel (syneresis). Adapted from Walstra et al. (2005).

2.5.3.2.3 Cooking of the curd

Once the coagulum is cut, the cooking process takes place. The cooking process of the curd consists in raising the temperature of the vat up to 38°C under gentle agitation to increase whey expulsion from the curd grains (Everett, 2007). The temperature rise must be done slowly to prevent an extreme syneresis of the outer layer of the curd grains which would lock in moisture and cause problems during ripening (Walstra et al., 2005). The cooking process is continued until the required pH, around 6.2, is reached. At that moment, the whey is drained and the curds are left in the vat for the cheddaring step.

2.5.3.2.4 Cheddaring

Cheddaring involves leaving the warm curds in the vat to allow further expulsion of whey while the pH drop continues (Everett, 2007). Cheddaring leads to the matting (i.e. fusing) of the curd pieces into a continuous mass (Walstra et al., 2005). During

cheddaring, the curd is cut and turned several times to aid the release of whey while the curd further acidifies up to pH 5.5 (Everett, 2007). This contributes to the formation of a rubbery mass with a fibrous texture when torn apart (Fox et al., 2000; Lawrence et al., 2004). This fibrous texture, also called “chicken breast texture” marks a visual endpoint for the traditional cheddaring process (Fox et al., 2000). At this point, the curd is ready to be milled into pieces or strips and dry-salted.

2.5.3.2.5 Salting and forming

In dry salting, salt crystals are mixed with the milled curd pieces (Lawrence et al., 2004). This leads to the diffusion of salt into the curd, but simultaneously causes a moisture counterflow, creating a concentrated brine on the surface of the milled curds (Walstra et al., 2005). At this stage, the outward migration of water is osmotically driven and causes the curd to shrink (Walstra et al., 2005). This is in contrast with early syneresis during clotting, where the shrinking of the curd causes the expulsion of water.

After the salting and mixing, the curd is molded and pressed. Up to 50% of the salt is lost in the press whey (Walstra et al., 2005). The remaining salt continues to diffuse, and residual moisture equilibrates within the cheese (Walstra et al., 2005). Salt distribution does not reach complete equilibrium within the timespan of a fresh Cheddar cheese, and variations in salt concentration persist even after 9 weeks (Walstra et al., 2005).

When finished, fresh Cheddar cheese is a firm-to-hard cheese with a color ranging from white to pale yellow (except when authorized colors are added) (Canadian Dairy Commission, 2015). Cheddar cheese can be consumed fresh or allowed to age from a few months to several years. Depending on the ripening duration, Cheddar cheese ranges from a mild to sharp flavor (Canadian Dairy Commission, 2015).

2.5.3.3 Texture and structure of the Cheddar cheese matrix

Texture is an important functional and sensory attribute of Cheddar cheese that evolves through the manufacture and ripening processes. The rheology of cheese is a function of combined effect of various factors (Lucey et al., 2003), including:

- The composition of the cheese and the intrinsic effect of each component (Mistry et al., 1993).
- The spatial arrangement of its components, such as the distribution of fat globules within the protein network (Michalski et al., 2002).
- The strength of the attractions between the structural elements of its microstructure, such as the degree of casein association (Horne, 1998).
- The hydrolysis extent of the structural proteins, namely caseins (Fox, 1989).
- The physicochemical state of its components, like calcium solubility and milk fat melting properties (Guinee et al., 2004b).

2.5.3.4 Role of proteolysis in Cheddar cheese texture

The structural frame of Cheddar cheese is the protein network composed of interconnected casein particles, mainly α_{s1}-casein and β-casein. Such protein network greatly determines its rheological properties (Lucey et al., 2003). During the ripening process, the texture of Cheddar cheese evolves within days from a firm rubbery and fibrous texture to a smooth and softer mass as the structural α_{s1}-casein molecules are cleaved at Phe23-Phe24 by residual chymosin activity (Lawrence et al., 2004). Other endogenous milk and bacterial enzymes may also contribute to proteolysis (Fox, 1989; Tunick et al., 1993). Major textural changes occur during ripening, especially during the first 21 days after manufacture (O'Mahony et al., 2005). During this time, the strain of fracture increases as curd pieces continue to fuse, but begins to decrease within days as colloidal calcium

phosphate[12] (CCP) solubilizes (O'Mahony et al., 2005) and proteolysis continues (Guinee et al., 2000a; Lawrence et al., 2004). Such two processes are responsible for the softening of the cheese during the first 2 to 4 weeks. As ripening takes place, the melting capacity increases and the stretch capacity is reduced (Lucey et al., 2003). Finally, the crumbly and dry texture of Cheddar cheese that has been aged for over a year is also explained by proteolysis occurring during ripening (Creamer et al., 1982). Such proteolysis implies the chemical consumption of water in the formation of new ionic group and also leads to an increased competition for remaining water (Creamer et al., 1982).

2.5.3.5 Role of calcium in Cheddar cheese texture

Parallel to proteolysis, calcium, namely as CCP, influences cheese texture due to its bonding role on protein association during clotting and to acidity variations during curd evolution (O'Mahony et al., 2006). Acidity changes during manufacture and ripening mainly influence the texture by changing the soluble fractions of CCP, which has an impact on texture. As pH decreases during cheesemaking and ripening, more CCP solubilizes, leading to a weakening of the protein network (Lucey et al., 1993). This is associated with proteolysis to the softening of Cheddar cheese (Lucey et al., 2003). For this reason, the functionality of calcium and CCP in dairy products has been extensively studied, with a particular aim on its role in bonding caseins within the micelle (Figure 16) and on the formation of micellar gels like cheese curd (Horne, 1998). In the casein micelle, a dual bonding of the casein is achieved by the bonding of hydrophobic regions and by linkage of hydrophilic regions containing phosphoserine clusters to colloidal calcium phosphate clusters (CCP) (Figure 16) (Horne, 1998). Molecules of κ-casein limit further growth of the micelle (Horne, 1998). Under the action of rennet, the hydrophilic strand of κ-casein is released as CMP. This leads to micelle association by hydrophobic interaction, which causes the formation of the micellar gel (Horne, 1998).

[12] Colloidal calcium phosphate is the insoluble part of calcium and phosphate attached to the protein matrix in its colloidal form that are of importance for cheese structure, texture and functionality. CCP is a major structural element of the casein micelle and it impacts the texture of cheese (O'Mahony et al., 2005).

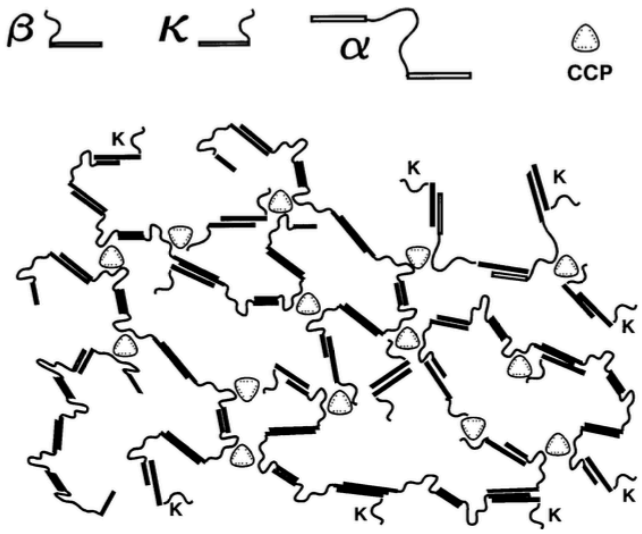

Figure 16. Dual bonding model of structure of casein micelle, with β, κ and α-casein and CCP depicted as indicated and hydrophobic and hydrophilic bonding regions represented by parallel bars and CCP clusters, respectively. Reproduced from Horne (1998).

In Cheddar cheese, varying calcium levels during the manufacture process, whether by addition or by draining the whey at a specific pH level, results in different textures and water contents. Increasing calcium abundance prior to renneting modifies the microstructure of the curd, as shown in Figure 17 for gel samples, cooked curd, cheddared curd, and final cheese. Higher $CaCl_2$ concentration (300 mg/L) produced a less porous gel during clotting and a denser protein network after the cooking process than that obtained with low (50 mg/L) or no calcium addition (Ong et al., 2013).

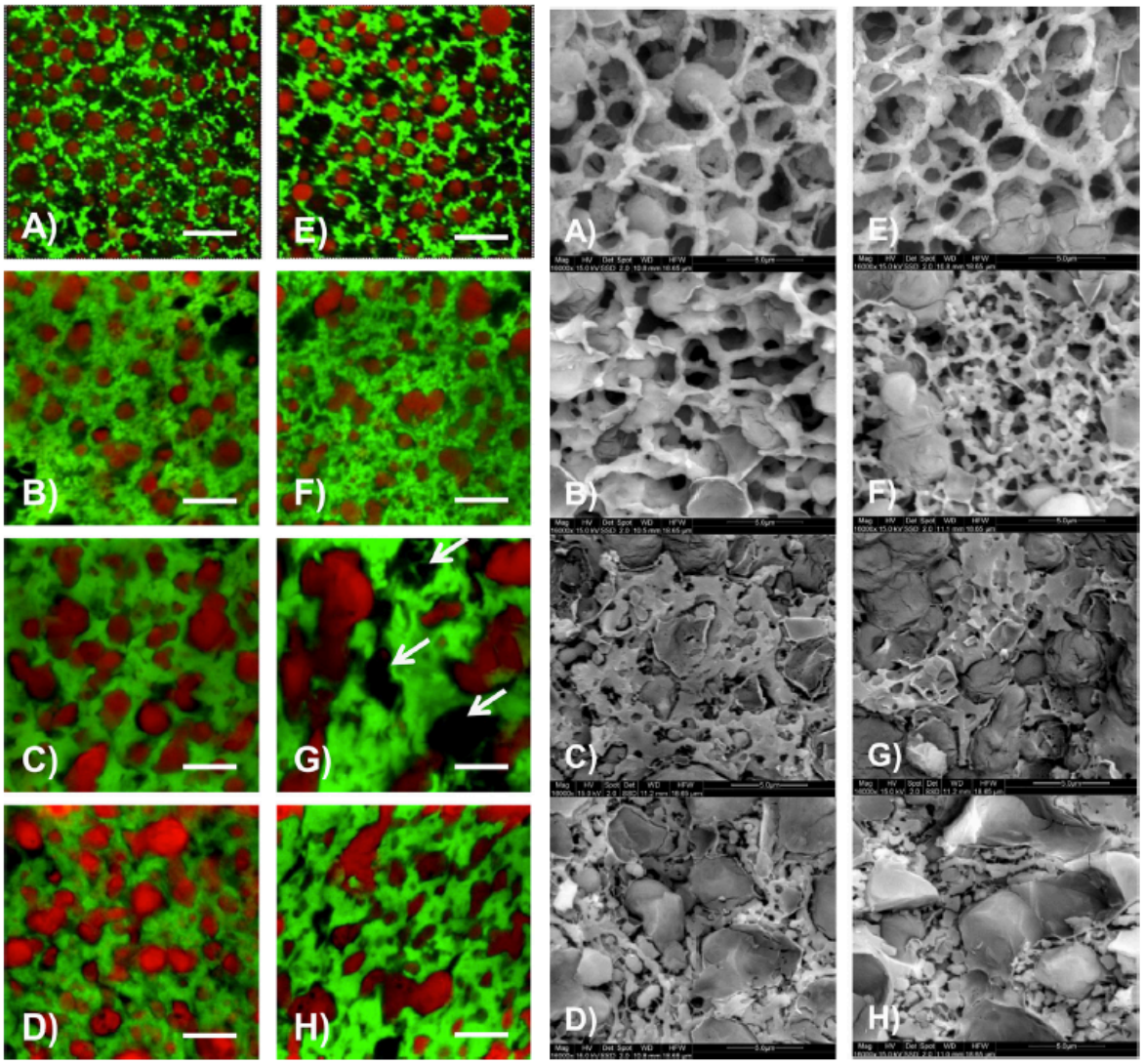

Figure 17. Confocal laser scanning microscopy (CLSM) (left, bar = 10 μm) and Cryo-scanning electron micrographs (right, bar = 5 μm) images of samples with the addition of 50 (left) or 300 mg/L $CaCl_2$ (right), respectively for A, E: gel; B, F: cooked curd; C, G: cheddared curd and D, H: cheese. For CLSM, red and green channels represent protein and fat, respectively. Arrows indicate micro-pores. Adapted from Ong et al. (2013).

The effect of calcium on Cheddar cheese texture has been studied by including a calcium salt ($CaCl_2$) or calcium chelator (trisodium citrate – TSC) during the salting step (Brickley et al., 2009). Figure 18 shows that the structuring effect of calcium reaches beyond the clotting and cheddaring steps. Such modifications affected the final texture of the Cheddar-type cheeses, where higher calcium levels (obtained

by salting with $CaCl_2$) led to harder cheeses, while TSC led to reduced hardness but more elastic cheeses at high temperatures (Brickley et al., 2009).

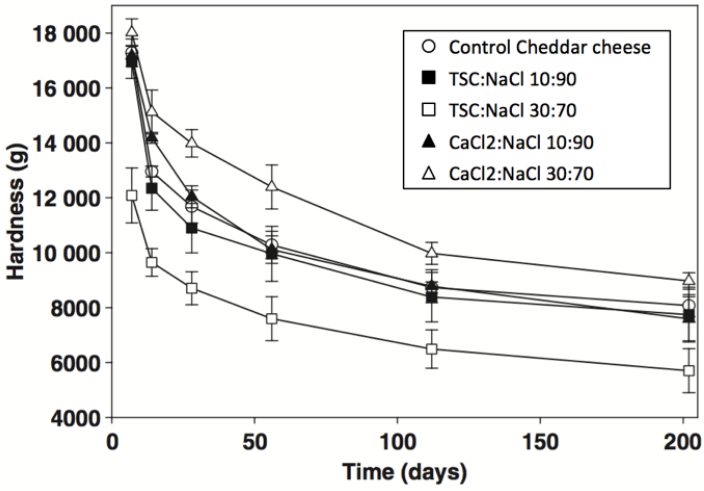

Figure 18. Hardness values as determined by texture profile analysis for a control Cheddar-type cheese and cheeses salted with different amounts of NaCl and $CaCl_2$ or trisodium citrate (TSC). Ratios indicate the contribution of each salt to the ionic strength. Adapted from Brickley et al. (2009).

2.5.3.6 Role of fat in Cheddar cheese texture

The other major element of the Cheddar cheese matrix is the fat embedded within the protein gel (Bryant et al., 1995; Mistry et al., 1993). Fat can be present in various structures of different shapes and sizes (Guinee et al., 2000a) (Figure 19). There is a persistence of the linearized structural orientation of the paracasein matrix developed during the cheddaring and pressing processes (Kalab et al., 1978; Lowrie et al., 1982). While some fat is still present as discrete globules of various sizes, most fat is in the form of clumps and coalesced fat reservoirs (Guinee et al., 2000a; Guinee et al., 2000b; Mistry et al., 1993). The clumping of MFG starts by shearing of the fat globule during processing, followed by the shrinkage of the paracasein matrix that forces MFG into closer contact during the early stages of curd formation (Guinee et al., 2000b). This causes disruption of individual milk fat globules, followed by coalescence during cheddaring while the curd is still warm (Guinee et al., 2000a). This allows lipids to fill any voids in the protein matrix (Michalski et al., 2007). Also, some coalescence of fat can occur

during ageing of Cheddar cheeses, mainly because of structure softening due to proteolysis (Mistry et al., 1993).

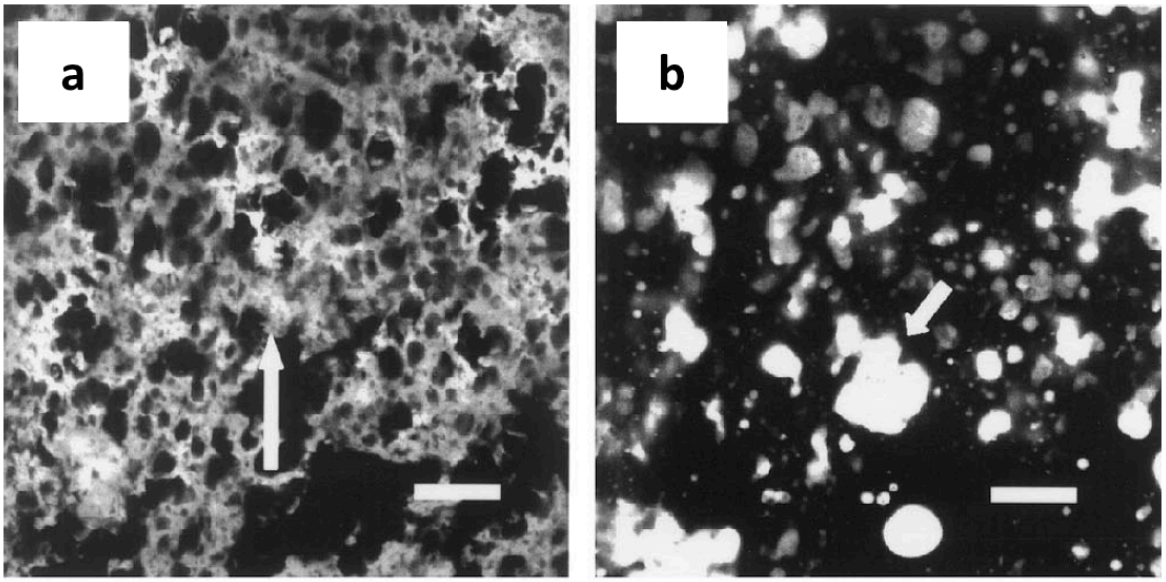

Figure 19. Confocal laser scanning micrographs of the protein (a) and fat (b) in full fat Cheddar cheeses. The protein (long arrow) and fat (short arrow) are shown as light areas against a dark background. The micrographs were taken at 1 d. Bar = 25 μm. Adapted from Guinee et al. (2000a).

It is noteworthy to mention that, in general, milk used for Cheddar cheese is standardized and pasteurized, but not homogenized. This is to avoid interaction of fat globules with the protein matrix during gelation (Metzger et al., 1994; Michalski et al., 2007). However, in experimental cheeses prepared with homogenized milk, fat reservoirs were greatly reduced (Metzger et al., 1995) because casein adsorbs to the fat globule surface during homogenization, which improves emulsification and decreases free fat (Metzger et al., 1995). The typical fat distribution of Cheddar cheese can be altered if homogenized fat (as a fine dispersion) is used instead of a native cream. The use of homogenized milk reduces clumping and coalescence during cheese manufacture, which in turn modifies the rheological behavior of the cheese (Guinee et al., 2000b).

In sum, Cheddar cheese is a complex food matrix. Calcium plays a structural role in promoting the clotting process and holding the protein network together. Fat is

distributed within the protein network in globular and bulk forms. Such distribution is modified depending on the dispersion characteristics of the milk fat prior to the cheese-making process.

In this project, Cheddar cheese was selected as a model because it is a solid food matrix that naturally contains high levels of calcium and milk fat, and because its processing can be adjusted to modify its structure and composition. From a nutritional point of view, the high levels of calcium in Cheddar cheese should play an important role on milk fat digestion and absorption. Calcium is expected to accelerate or increase lipolysis, and such increase is explained by the production of calcium soaps. Therefore, by modifying the structure and composition of Cheddar-type cheeses, it may be possible to change the way its nutrients are digested and absorbed, with potential repercussions on postprandial lipemia. Such a study could be conducted by using both in vitro and in vivo to measure the effect of structure and composition on digestion and absorption.

2.6 Digestion models

2.6.1 In vitro digestion models

An in vitro digestion model is a research tool to study the behavior of food under digestive conditions. The main drawback of such models is their limited physiological relevance. This is due to their limited capacity to accurately recreate the spatial, temporal and physiological integration of the digestion, secretion and absorption processes (Minekus et al., 2014). Nevertheless, in vitro models present several advantages with respect to human nutritional studies because they are less expensive and have no ethical restrictions (Minekus et al., 2014).

Several types of in vitro digestion systems exist, with complexity levels ranging from a simple enzyme solution to very elaborate dynamic models that can mimic peristaltic movements and uptake of some nutrients. Unfortunately, such complex dynamic in vitro systems, are very labor-intensive and are not adapted to handle screening protocols with numerous samples (Failla et al., 2005). Conversely, simple static in vitro digestion models are useful for screening purposes and to study specific substrates or meals under defined digestive conditions (Minekus et al., 2014). This makes them especially useful for bioaccessibility studies (Versantvoort et al., 2004). Although complex dynamic models can simulate more aspects of human physiology, simple static models are easy to perform, have high reproducibility and allow simultaneous handling of large numbers of samples (Egger et al., 2016; Minekus et al., 2014; Oomen et al., 2002).

2.6.1.1 Versantvoort in vitro digestion model

The Versantvoort in vitro digestion model simulates the pregastric, gastric and small intestine phases, respectively using simulated saliva, gastric and intestinal fluids (Versantvoort et al., 2005; Versantvoort et al., 2004). The simulated intestinal fluid includes duodenal (pancreatin) and hepato-biliary (bile and $NaHCO_3$)

solutions (Versantvoort et al., 2004). The fluids[13] can be adapted to simulate the fasted or the fed state. The food samples are digested in tubes by successive addition of the fluids and mixed by head-over-heels agitation (Versantvoort et al., 2004). Recently, this model was used to digest cheese samples, obtaining satisfactory results (Lamothe et al., 2012) and it has been adapted in further works to handle cheese samples (Ayala-Bribiesca et al., 2016). The main modifications to the original model are the inclusion of glass beads to enhance mechanical abrasion during mixing and the adjustment of pH-regulating solutions to overcome the buffering effect of cheese (Ayala-Bribiesca et al., 2016). The duration of the in vitro digestion stages can easily be adapted to attain a complete disintegration of the food sample.

[13] Detailed composition of the simulated fluids is presented in appendix A. Gastric lipase is not commercially available, so in vitro models do not include it in simulated fluids, or include a fungal or microbial analog (Bodmer et al., 1987; Diakidou et al., 2009).

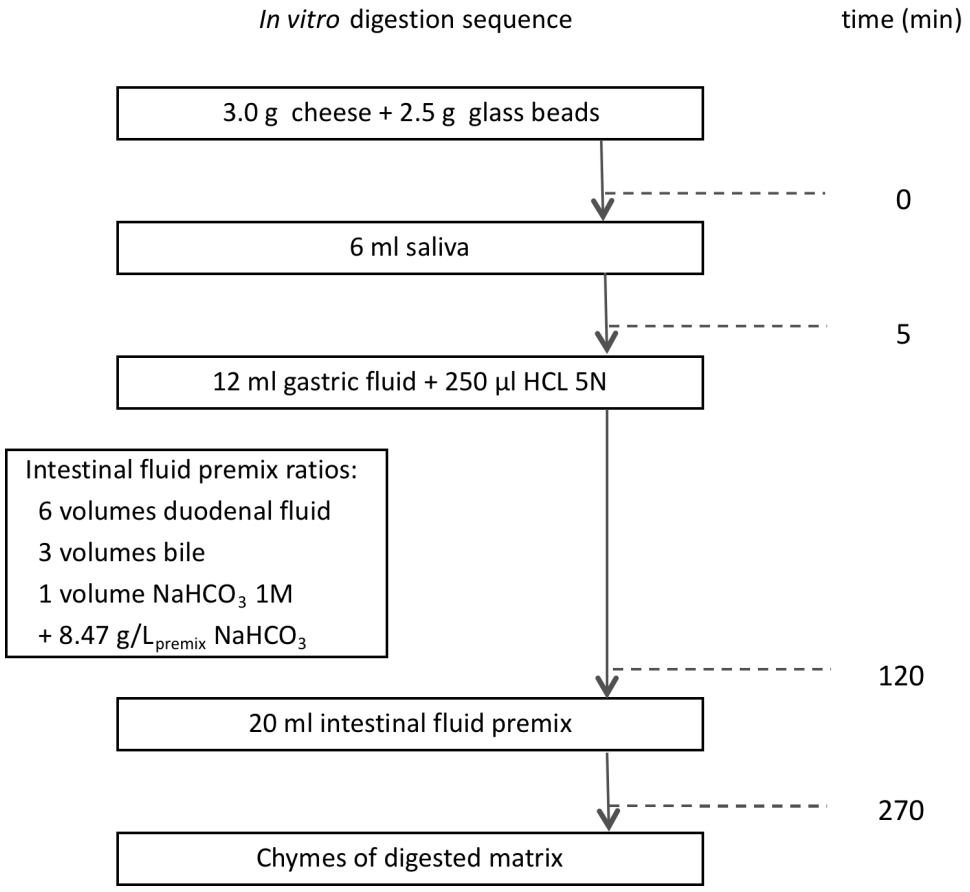

Figure 20. In vitro digestion sequence for cheese digestion, based on the model proposed by Versantvoort et al. (2004) and adapted to Cheddar cheese (Ayala-Bribiesca et al., 2016).

2.6.2 In vivo digestion models

Overall, bioavailability studies require in vivo approaches since a metabolic feedback is required to verify absorption and to confirm that the molecules of interest reach their metabolic target. Often, bioavailability is estimated by balance studies, which compare the ingested and the excreted amount of a nutrient (Jandacek et al., 2004), but the complex physicochemical, biochemical and physiological processes involved in between are often treated as a black box. Due to practical and ethical restrictions to perform clinical studies, in vivo models are a tool of choice to further elucidate the processes intervening in nutrient effectiveness. Furthermore, in vivo models may complement the understanding of the metabolic effects of foods and validate in vitro models (Hunt et al., 2003).

Animal models, such as the mini pig and the rat, have been used to study the behavior of food matrices under digestive conditions (Barbe et al., 2013; Michalski et al., 2006). The rat model is commonly used to study metabolic responses to the diet. Wistar rats are easily available, easily contained, can be trained and the similarity between their physiology with that of humans makes them a relevant model for dietary interventions regarding fat digestion (Armand et al., 1999; Lai et al., 1995; Michalski et al., 2006; Panzoldo et al., 2011). As with other in vivo models, conclusions cannot be fully extrapolated to humans without confirmation through clinical studies. Nevertheless, the information obtained is pertinent and, due to protocol limitations regarding human subjects, would not be available otherwise.

Chapter 3. Hypothesis and objectives

3.1 Hypothesis

Digestion of milk fat in Cheddar cheese is modulated by calcium and the milk fat fatty acid composition through their effect on the cheese matrix and the formation of insoluble soaps with long-chain fatty acids, which activates lipolysis but reduces their bioaccessibility.

3.2 Objectives

1. To assess in vitro bioaccessibility of lipids, namely fatty acids, from cheese matrices enriched with calcium.

2. To assess the impact of calcium on fatty acid bioaccessibility from cheeses prepared with different milk fats under simulated digestive conditions.

3. To modulate bioavailability of cheese lipids by modifying the composition and structure of their matrices.

Each objective has been addressed the following three chapters, respectively.

Chapter 4. Effect of calcium enrichment of Cheddar cheese on its structure, in vitro digestion and lipolysis

The present chapter is a research paper reporting a study where calcium chloride is added to model Cheddar cheeses during the salting step and subjected to different ripening times. The effect of both parameters on cheese composition, microstructure and in vitro digestion, with emphasis on fatty-acid bioaccessibility, is presented. The paper has been published in the International Dairy Journal in 2016. The role of the coauthors is as follows: Erik Ayala Bribiesca is the main author and did the experimental design, laboratory experiments, statistical analysis of the data, and writing of the paper. Martine Lussier participated in the in vitro digestion experiments. Denise Chabot led the microscopy imaging procedures and Sylvie Turgeon and Michel Britten supervised the project and assisted in proofing the manuscript. All coauthors authorized the inclusion of the paper in this thesis.

4.1 Résumé

Cette étude visait à établir un lien entre les caractéristiques des fromages de type Cheddar avec différents niveaux de calcium et leur impact sur la digestion in vitro des lipides. Le caillé a été salé avec du $CaCl_2$ pour produire des fromages avec trois niveaux de calcium. La composition, la texture et la structure des fromages ont été caractérisées. La désintégration physique et la lipolyse ont été suivies lors de leur digestion in vitro. Les résultats ont montré que la dureté du fromage augmente avec l'ajout de $CaCl_2$ lors du salage, ce qui a mené à une désagrégation plus lente pendant la digestion. Malgré une désintégration plus rapide, le fromage témoin présentait la lipolyse la plus lente. La lipolyse dépend de la teneur en calcium et de la structure de la matrice fromagère, qui agiraient en modulant l'accès des enzymes à leurs substrats. L'étude plus approfondie de l'interaction calcium-matrice devrait permettre de mieux comprendre les facteurs qui affectent la bioaccessibilité des lipides.

4.2 Abstract

The purpose of this study was to establish a link between the characteristics of Cheddar-type cheeses with different calcium levels and their impact on cheese in vitro digestion. Curds were enriched with $CaCl_2$ during the salting step to produce control, high-calcium, and very high-calcium cheeses. Cheese composition, texture and structure were characterized, while physical disintegration and lipolysis were monitored during their in vitro digestion. Cheese hardness increased with higher calcium content. This resulted in a slower disintegration during in vitro digestion. Despite showing faster disintegration, the control cheese had the slowest lipolysis progression. The results suggest that lipolysis depends on calcium content and the structure of the matrix modulating the access of enzymes to their substrates. Further studies should provide a better understanding of the calcium-matrix interaction affecting lipid bioaccessibility.

4.3 Introduction

When food enters the human body, digestion takes place to release the nutrients from the matrix so that they can be absorbed. The mechanical characteristics and intrinsic composition of the matrix influence the entire digestion process by controlling the matrices' disintegration and biochemical behavior under gastrointestinal conditions (Hur et al., 2011; McClements et al., 2009a). Hence, the food matrix acts as a nutrient-release regulator as it disintegrates during the digestion process (Turgeon et al., 2011), thus determining nutrient bioaccessibility, which is defined as the fraction of a substance that is soluble in the gastrointestinal environment and is available for absorption (Ruby et al., 1999).

Among nutrients, lipids demand a more complex digestion and absorption strategy than water-soluble substances do (Klein et al., 2006). Dietary lipids are composed mainly of TAG. After being released from the matrix, the TAG-rich fat droplets are exposed to lipases, which cleave the ester bonds on the glycerol backbone, releasing FA. Lipolysis products and other fat-soluble components must be incorporated into mixed micelles and transported to the enterocyte brush border for absorption (Jones et al., 2006). Therefore, lipid bioaccessibility may be estimated from the amount of lipids transferred into the aqueous micellar fraction (Failla et al., 2005). Several studies have shown that the emulsion characteristics may affect lipase activity, an effect that is explained mainly by the accessibility to the TAG (Armand et al., 1999; Clemente et al., 2003; Favé et al., 2004; McClements et al., 2009a; Michalski et al., 2005). Extensive studies on emulsion engineering have provided a fundamental understanding of digestion mechanisms (McClements et al., 2009a) (Singh et al., 2009), but further research on actual food is needed.

In dairy products, a wide array of matrices can be found, and an increasing number of studies report that the physical structure of milk fat-rich foods modifies the way lipids are digested and absorbed (Clemente et al., 2003; Lopez et al., 2008). Besides lipids, a key nutrient associated with dairy foods is calcium. Calcium is known to interact with milk components, influencing the structural properties of

dairy matrices such as cheese (Lucey & Fox, 1993). Furthermore, calcium has been reported to enhance lipolysis during digestion by precipitating free FA in the intestinal medium, a process that in turn may reduce their bioaccessibility (Lopez et al., 2008; Lorenzen et al., 2007; McClements et al., 2009a). This reduction occurs because FA, as lipolysis products, accumulate at the lipid–water interface and limit the access of lipase to its substrates (Favé et al., 2004). Calcium enhances lipolysis by removing such FA from the interface in the form of calcium soaps, which are not water-soluble under intestinal conditions (Hu et al., 2010). Insolubility limits FA bioaccessibility and translates into reduced absorption (Lorenzen et al., 2007).

Cheese is a complex matrix of milk proteins (mainly casein), fat, minerals, and water, where casein and calcium form the major structural network and entraps the fat (Mistry et al., 1993). Roughly speaking, protein and calcium contribute to hardness, and fat and water contribute to smoothness (Metzger et al., 1994, 1995). The protein network determines the rheological properties of cheese, mainly due to calcium-casein interactions and proteolysis, which contribute significantly to its textural properties (Lucey et al., 2003). In cheeses such as Cheddar (pH between 4.9 and 5.4), higher mineralization levels lead to a harder texture (Lucey et al., 1993; Metzger et al., 1994, 1995). The rheological properties of cheese also depend on the size distribution and membrane composition of fat globules (Michalski et al., 2002). In cheese, coalesced fat globules may lead to the formation of fat pools, which are non-globular inclusions of milk fat that result from the disruption of individual milk-fat globules, so that lipids can fill voids in the protein matrix (Michalski et al., 2007). Such fat pools occur mainly during the manufacturing process, when the warm curd is cheddared, and they influence the texture properties of the cheese (Guinee et al., 2000a).

Cheddar cheese is suitable for studying the impact of the food matrix on lipid bioaccessibility. Modifying the microstructure of the matrix could alter the way the matrix itself is digested. For example, it is reasonable to postulate that a harder matrix will resist breakdown and delay nutrient release. In Cheddar cheese,

increasing the amount of calcium strengthens the protein matrix and could lead to a delayed disintegration in the digestive system. The dissolution of food matrices will depend on water absorption and diffusion of acid and enzymes (Van Wey et al., 2014). The rate fat is released during digestion may result in different bioaccessibility profiles, as previously observed for different types of cheese (Lamothe et al., 2012).

The evolution of the cheese matrix during the first weeks of ripening could impact the way the matrix behaves during digestion. During ripening, cheese structure evolves mainly because of proteolysis and colloidal calcium solubilization (Johnson et al., 2006; O'Mahony et al., 2005). Proteolysis is one of the factors that may modify the texture of young (or mild) Cheddar cheese by softening the para-casein network (Guinee et al., 2000a; Lawrence et al., 2004; O'Mahony et al., 2005), but the most important factor that modifies Cheddar cheese texture during the first weeks of ripening is the level of calcium, namely in the form of colloidal calcium phosphate (CCP). CCP is the insoluble part of calcium and phosphate attached to the protein matrix in its colloidal form (Lucey et al., 1993; O'Mahony et al., 2006). By strengthening the para-casein matrix, CCP acts as a structuring element (Hassan et al., 2004). In Cheddar cheese, CCP greatly decreases during the first 21 days of ripening and is highly correlated with texture softening during the same period (O'Mahony et al., 2005). In sum, physicochemical events that modify the texture of cheese may have an impact on the resistance of the cheese matrix to digestion.

It has been shown that the characteristics of the food matrix influence the kinetics of digestion and nutrients bioaccessibility (Ellis et al., 2004; Hornero-Méndez et al., 2007; Lopez et al., 2008), but such processes are still poorly understood. Modifying cheese microstructure and composition through technological processing could modulate the bioaccessibility of lipids. The purpose of this study was to examine the effect of calcium enrichment and short-term ripening on Cheddar cheese structure and to assess the impact of that structure on lipolysis.

4.4 Materials and methods

4.4.1 Preparation of Cheddar-type cheeses

One standardized batch of Cheddar curd was produced in a pilot plant (Research and Development Centre, Saint-Hyacinthe, Canada) from whole pasteurized milk (Laiterie Chalifoux, Sorel, QC, Canada). The milk was warmed to 32°C. A commercial starter (CH-FRS-102 culture; Chr. Hansen, Hørsholm, Denmark) and 0.26 ml/kg of 45% w/v $CaCl_2$ solution (Cal-Sol; Fromagex, Rimouski, QC, Canada) were added. After 1 h, 0.085 ml/kg of microbial chymosin solution (ChyMax; Chr. Hansen, Hørsholm, Denmark) was added. Once set, the gel was cut and then cooked by increasing the temperature to 38°C at a rate of 0.2°C/min. The whey was drained when the pH reached 6.0. The curd was cheddared for 1 h and milled when the pH reached 5.1. The milled curds were separated into three batches and salted with NaCl and $CaCl_2$•$2H_2O$[14] (Sigma-Aldrich) in the amounts shown in Table 7 to obtain the different Cheddar-type cheeses.

Table 7. Experimental conditions used in the salting step for Cheddar-type cheeses.

Cheese calcium level	NaCl (mass %)	$CaCl_2 \cdot 2H_2O$ (mass %)	Ca added * (g/kg curd)
Control	1.80	0	0
High-calcium	1.80	1.47	4
Very high-calcium	1.80	4.40	12

* Ca added represents the amount of elemental calcium added through the $CaCl_2$•$2H_2O$

The salted curds were then packed into 13 kg-capacity stainless steel molds lined with synthetic cheesecloth and pressed at room temperature for 1 h at 40 psi (≈ 275 kPa). The cheeses were removed from the molds, vacuum-packed, and kept at 4°C. After 1 week, one third of each cheese was cut into 300-g blocks, which were then individually vacuum-packed and frozen at −20°C until required for

[14] Further references to $CaCl_2$•$2H_2O$ used for the salting step is noted simply as $CaCl_2$.

the experiments. The same was done with another third after 2 weeks and the last third after 4 weeks. The cheeses were thawed at 4°C for 4 d before the experiments to allow the matrix to stabilize and to limit the crumbly texture that has been reported in Cheddar cheeses after a freeze–thaw cycle (Kasprzak et al., 1994).

4.4.2 Cheese composition

All reagents were obtained from Fisher Scientific (Ottawa, ON, Canada) unless otherwise specified. Moisture was quantified by difference from total solids, obtained by oven-drying 1 g of cheese in an aluminum cup at 100°C for 16 h. Protein (N × 6.38) was quantified by the Kjeldahl method (AOAC, 1995a). Fat was quantified by the Mojonnier method (AOAC, 1995b). Dry samples were incinerated in a muffle furnace at 550°C for 16 h, and the residues were weighed to determine ashes. Cheese pH was measured in a slurry prepared with 10 g of cheese and 10 g of distilled water.

The cheese ashes were dissolved in 0.23 M HNO_3 and used to quantify calcium by inductively coupled plasma–optical emission spectroscopy (ICP-OES) with a Teledyne Leeman Prism spectrometer (010-00084-1; Hudson, NH, USA). Commercial standards (Fisher Scientific, Ottawa, ON, Canada) were diluted in the same solution. Detection was done in radial mode on the argon plasma torch. Colloidal calcium, or CCP, was determined by acid-base titration (pH 7 to 3) and back titration (pH 3 to 7) of a cheese slurry, in accordance with a previous study (Remillard et al., 2011). The results were reported as milligrams of calcium per gram of protein (mg/g).

To monitor proteolysis during the ripening period, water-soluble nitrogen in the cheeses was determined (Christensen et al., 1991). Cheese slurries were prepared in distilled water (2:1 water-to-cheese ratio). The slurries were kept at 40°C for 1 h and then centrifuged for 30 min at 3010 × *g* and 4°C. The aqueous phase was filtered, and the solids were extracted once again. The nitrogen in the pooled aqueous phases was quantified by the Kjeldahl method. The results were reported

as a percentage of water-soluble nitrogen (WSN) with respect to total nitrogen (% WSN/TN).

4.4.3 Texture profile analysis

A texture profile analysis (TPA) was performed to evaluate the impact of calcium supplementation on cheese texture. Cylindrical samples (r = 5 mm; h = 10 mm) were cut at 4°C and stored for 30 min at 22°C. A double compression cycle to a 30% strain with a Plexiglas probe moving at a rate of 0.4 mm/s rate was done using a TA-XT2 texture analyzer equipped with a 5-kg load cell (Stable Micro Systems, Surrey, UK) controlled with Exponent software (version 6.1.4.0; Stable Micro Systems). Hardness, springiness, and cohesiveness were computed from the TPA data (Tunick, 2000). The TPA was done in triplicate, and each sample was composed of 10 cheese cylinders. Outlier cylinders within samples, with more than two standard deviations from the average maximum force during the first compression, were omitted from the data analysis.

4.4.4 Scanning electron microscopy

The cheeses were cut into sticks measuring 3 × 3 × 7 mm with a razor blade. The sticks were immediately covered with a protein-fixation buffer (pH 7.2) containing 2% glutaraldehyde (18426; Ted Pella, Redding, CA, USA) in 0.1 M sodium cacodylate (Sigma-Aldrich) and then fixed for 2 h at 21°C under gentle agitation. The protein-fixation buffer was removed, and the sticks were dehydrated in a graded series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%, and twice in 100%), each for 15 min under gentle agitation. The samples were then defatted three times with hexane under the same agitation conditions. The samples were freeze-fractured in liquid nitrogen, dried in carbon dioxide with a critical point dryer (Biodynamics Research Corp., Rockville, MD, USA), and mounted on aluminum stubs. The mounted samples were covered with a 10-nm layer of gold using an Emitech K550X sputter coater (Quorum Technologies, Kent, UK). Scanning electron micrographs were obtained with an ESEM XL-30 microscope (Philips, Eindhoven, the Netherlands) operating under high vacuum at 5 kV, with a

secondary electron detector with a HD filter, a spot size of 3, and a working distance of 7 to 10 mm. At least 15 fields of each cheese were observed, and typical fields were imaged. Fields showing curd junctions were discarded.

4.4.5 Confocal laser microscopy

The cheeses were cut on a refrigerated surface into 3 mm cubes and frozen in an isopentane bath cooled in liquid nitrogen. Slices (20 µm thick) were cut using a microtome (Reichert Jung, Wetzlar, Germany) at −27°C. Each slice was mounted on a microscope slide with an adhesive marker (Fro-Tissuer, 22302, Ted Pella, Redding, CA, USA) allowed to dry for 10 min at −18°C, and stained with a drop of 0.01% aqueous solution of Nile Blue A (Sigma-Aldrich) for 15 min over ice. The use of Nile Blue A allows simultaneous staining of fat and protein (Auty et al., 2001). With the microscope slide still being on ice, each sample was rinsed, the excess water was removed, and the sample was mounted with Fluoromount G and observed under a Zeiss Meta-510 confocal microscope equipped with a Plan Apochromat 40× objective with a numerical aperture of 1.4 (Carl Zeiss GmbH, Jena, Germany). Fat and protein were detected, respectively, with an Argon/2 laser, line 488 nm for excitation and band-pass (BP) 530-to-600 nm emission, and with a HeNe laser, line 633 nm for excitation and long-pass (LP) 650 nm emission. The slides were kept over a refrigerated surface during observation to reduce fat mobility. The images were processed with Zen software (version 2009; Carl Zeiss GmbH). The pseudo-colours chosen were green for fat and red for protein, and both images were superimposed to show the relative distribution of fat and protein. At least five samples of each cheese were observed, and typical images were captured. Images showing curd junctions were discarded.

4.4.6 In vitro digestion of Cheddar-type cheeses

4.4.6.1 In vitro digestion model

The in vitro digestion model and fluid composition simulating the physicochemical conditions of human digestion during the fed state (Versantvoort et al., 2005) were

adapted for the digestion of cheese. Briefly, the oral, gastric, and small-intestine stages of digestion were simulated with digestive fluids added sequentially to the sample in a conical 50-ml tube and mixed by head-over-heels agitation at 50 rpm. All reagents and extracts for in vitro digestion were obtained from Sigma-Aldrich. Bovine serum albumin (A7906), commercial porcine α amylase (A3176-1MU), mucin (M1778), pepsin (P7000), pancreatin (P7545), pancreatic lipase (L3126), and bile (B8631) extracts were used to recreate the composition of the digestive fluids (Versantvoort et al., 2005).

The cheeses were cut to a standardized surface-to-volume ratio of 20 cm^{-1} (i.e. 3 mm cubes) (Lamothe et al., 2012). Each digestion tube contained 4.5 g of cheese and 2.5 g of 4 mm glass beads to ensure thorough mixing of the samples. Oral (6 ml), gastric (12 ml), and intestinal (20 ml) fluids were added after 0, 5, and 120 min, respectively. The fluids for the intestinal phase (6:3:1 ratio of duodenal, bile, and $NaHCO_3$ 1 M solutions, respectively) were premixed for 5 min before being added to the tubes. To compensate for the buffering effect of the cheese, 250 µl of HCl 5 N was added to each tube at the beginning of the gastric phase to maintain acidic conditions, and 6.776 g of $NaHCO_3$ was added to 700 ml of the intestinal fluid premix. The duration of the intestinal phase was set to 180 min, for a total digestion time of 300 min. Samples for analyses were taken after 5, 60, 120, 150, 180, 240, and 300 min. One digestion tube was taken at every sampling time, analyzed, and then discarded. Digestions were performed in triplicate.

4.4.6.2 Matrix degradation index

Cheese disintegration was quantified using the matrix degradation index (MDI), obtained from the undigested cheese fraction retained by a metallic sieve (1.5 × 1.5 mm), as previously described (Lamothe et al., 2012). The solids were washed twice with 5 ml of unused digestive fluids (saliva, gastric, or intestinal fluid, depending on the moment of sampling) at 37°C. The sieve with the retained solids was drained and blotted on a filter paper cone for 10 min to remove any excess fluid. The solids were transferred to a pre-weighed aluminum dish and dried in a forced-air oven at 100°C for 12 h. The dry solids were weighed, the mass of the

glass beads was deducted, and the remaining mass of solids was used to obtain the MDI, using Equation 1.

Equation 1. Matrix degradation index.

$$\text{MDI}\ (\%) = 100 \times \frac{CS_0 - CS_t}{CS_0}$$

where CS_0 is the mass of cheese solids originally present in the digestion tube, and CS_t is the mass of cheese solids remaining at time t.

4.4.6.3 Lipolysis

The extent of lipolysis was measured using a non-esterified fatty acid (NEFA) enzymatic kit (Roche Diagnostics, Indianapolis, IN, USA). The drained liquid from the MDI samples was diluted 100-fold with a solution of ethanol and Triton X-100 (Sigma Aldrich) (6 ml and 5.7 g, respectively, completed to 100 ml with distilled water) to solubilize the fatty acids and halt lipolysis (Lamothe et al., 2012). The assay was performed according to the instructions provided with the kit. Absorbance was measured at 546 nm with a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). Oleic acid was used as the calibration standard. The NEFA were expressed as milligrams per gram (mg/g) of milk fat present in the cheese sample, assuming an average molecular weight of 247 g/mol for milk fatty acids (Lamothe et al., 2012). Data used to calculate the average FA molecular weight is presented in appendix D. Lipolysis was expressed as the percentage of fatty acids released from the theoretical maximum of 66% [i.e. pancreatic lipase cleaves only sn_1- and sn_3-fatty acids from the triglyceride molecule (Jones et al., 2006)].

4.4.7 Statistical analysis

Cheese composition analyses were repeated three times. Data were analyzed for statistical differences by a two-way analysis of variance (ANOVA) in a split-plot array with the calcium level nested in the main plot and ripening in the sub-plot. Multiple comparisons were done using the least square difference (LSD) with a

significance level of $P \leq 0.01$. For the in vitro digestion experiments, data were analyzed using a three-way ANOVA in a split-split-plot with the digestion time in the sub-sub-plot. All statistical analyses were performed with SAS-Server Interface (version 2.0.4; SAS Institute Inc., Cary, NC, USA).

4.5 Results and discussion

4.5.1 Cheese appearance and composition

All freshly made cheeses were similar in appearance to commercial fresh Cheddar cheese. During the salting step, the temperature of the curds salted with the highest level of $CaCl_2$ increased 6 ± 2°C relative to the control because of the heat released during $CaCl_2$ solubilization. The temperature change could be avoided by using a concentrated solution of $CaCl_2$ instead of using the dry salt. After pressing, the curds had begun to bond and all the cheese blocks could be easily handled. The very high-calcium cheese was slightly oily to the touch, possibly because of the warming of the curd during the salting step. After 1 week, all the cheeses could be cut easily with a wire cutter to divide them into separate portions for the different ripening periods. No differences were observed after thawing for any of the experimental cheeses except the very high-calcium one ripened for 1 week, which was found to have weakened milled-curd junctions when handled.

The composition of the experimental cheeses is presented in Table 8. The moisture level decreased as the calcium level increased ($P < 0.0001$), owing mainly to the osmotic pressure caused by the amount of $CaCl_2$ added. This effect was noticeable during the pressing of the cheeses, when more whey was expelled from the molds of the calcium-enriched cheeses. The lower moisture levels led to a higher level of protein in the calcium-enriched cheeses ($P < 0.0001$), but the protein-to-fat ratio was similar for all the cheeses (0.76 ± 0.03). As expected, ash residue was lowest for the control and increased ($P = 0.0002$) with the amount of $CaCl_2$ used during the salting step. Differences for fat content were not statistically significant ($P > 0.05$). Finally, the pH of the cheeses decreased with $CaCl_2$ enrichment ($P = 0.001$) owing to H^+ ions released by the interaction of calcium with phosphate and citrate (Philippe et al., 2003). No composition changes were observed during ripening.

Table 8. Composition of a typical fresh Cheddar cheese and the experimental Cheddar cheeses.

Cheese	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
Typical [d]	37	25	33	4	5.10
Control	38.6 [c]	23.8 [a]	32.8 [a]	3.30 [a]	5.15 [c]
High-calcium	35.4 [b]	25.8 [b]	32.6 [a]	3.92 [b]	5.10 [b]
Very high-calcium	32.2 [a]	25.9 [b]	33.9 [a]	4.93 [c]	4.99 [a]
SEM [e]	0.1	0.1	0.6	0.12	0.01

[a,b,c] Different letters within columns denote significantly different means. Only the experimental cheeses were compared. [d] Data obtained from the Canadian Dairy Commission (2015). [e] SEM, standard error of the mean.

Total calcium and colloidal calcium concentration in experimental cheeses are presented in Table 9. As expected, cheese calcium concentration increased as the calcium level during the salting process increased ($P = 0.0005$). The proportion of colloidal calcium in the control cheese after 1 week of ripening was 62%, with respect to the total calcium, which is within the range for a normal Cheddar cheese (Hassan et al., 2004). The addition of $CaCl_2$ during the salting step increased the colloidal calcium concentration by as much as twofold relative to the control cheese. Ripening had a slight effect ($P = 0.0499$) on CCP, but the effect was limited to the control cheese, where CCP solubilization during ripening was observed after 4 weeks. The proportion of CCP did not vary during the ripening of the calcium-enriched cheeses, because of calcium oversaturation, preventing CCP solubilization. Based on those results, a more tightly structured protein matrix could be expected as the calcium enrichment level increased (Johnson et al., 2006; Lucey et al., 2003).

Table 9. Total and colloidal calcium (mg/g protein) of the experimental cheeses after different ripening periods.

Cheese	Total calcium [d]	Colloidal calcium after ripening (weeks)		
		1	2	4
Control	25.0 [a]	15.5 [a,2]	12.0 [a,1,2]	8.6 [a,1]
High-calcium	29.6 [b]	28.0 [b,1]	26.8 [b,1]	26.7 [b,1]
Very high-calcium	46.4 [c]	32.8 [b,1]	29.3 [b,1]	31.9 [b,1]

[a,b,c,1,2] Different letters denote different means within columns, whereas different numbers denote differences within rows; the overall standard error of the mean was 0.06 for total calcium and 0.15 for colloidal calcium. [d] Quantified by ICP-OES.

WSN evolved differently during the ripening process depending on the calcium level in the cheese, as confirmed by the calcium × ripening interaction (P = 0.0022) observed. As expected, WSN increased in the control cheese during ripening (O'Mahony et al., 2005), but the increase was slower in the high-calcium cheese, and no increase was observed in the very high-calcium cheese (Table 10). These differences were probably due to higher ionic strength and lower water activity, which reduced the proteolytic activity in the cheeses to which higher amounts of $CaCl_2$ had been added during the salting step (Upreti et al., 2006). Proteolysis progression in Cheddar cheeses has been reported to be higher with increased moisture in the non-fat substance (Guinee et al., 2000a). In this study, such ratios were 0.565, 0.525, and 0.487, respectively for the control, high-calcium, and very high-calcium cheeses, so the trend in their proteolytic activity (Table 10) would be in agreement with their moisture in the non-fat substance ratios.

Table 10. Water-soluble nitrogen (WSN) to total nitrogen (TN) ratio (as % WSN/TN) in the experimental cheeses after different ripening periods.

Cheese	% WSN/TN after ripening (weeks)		
	1	2	4
Control	8.5 ^{c,1}	9.8 ^{c,2}	12.2 ^{c,3}
High-calcium	5.8 ^{b,1}	5.6 ^{b,1}	7.9 ^{b,2}
Very high-calcium	4.2 ^{a,1}	4.8 ^{a,1}	4.6 ^{a,1}

^{a,b,c,1,2,3} Different letters denote different means within columns, whereas different numbers denote differences within rows; the overall SEM was 0.23.

4.5.2 Cheese texture

Cheese texture was analyzed using TPA data from a two-compression cycle. The results for TPA properties are presented in Table 11. Cheese hardness was influenced by added $CaCl_2$ and, for the very high-calcium cheese, by ripening duration ($P = 0.0029$). Hardness increased as the level of calcium in the cheese increased, mainly because of the loss of moisture during the pressing step, which resulted in a drier matrix that exerted a higher resistance to compression (Creamer et al., 1982), as well as because of the higher CCP content, which increased the strength of the protein matrix (Lucey et al., 2003). The effect of ripening on hardness was not statistically significant for the control and high-calcium cheeses but it was for the very high-calcium cheese after 4 weeks. Normally, Cheddar cheese is expected to soften during the first 4 weeks after production, mainly because of CCP solubilization and, to a lesser extent, proteolysis (Creamer et al., 1982; Guinee et al., 2000a; O'Mahony et al., 2005). However, no such effect was observed in the experimental cheeses: hardness remained stable in all the cheeses except the very high-calcium one, which was markedly harder after 4 weeks of ripening. This greater hardness observed after four weeks may result from the gradual fusion of milled curds during ripening. High concentration of calcium has been suggested to interfere with the fusion of milled curds during pressing (Ong et al., 2013) by increasing curd surface dehydration and rigidity. The

effect of calcium on curd bonding is, however, expected to decrease during ripening due to the gradual elimination of moisture and salt gradients.

Springiness, which is the height recovery ratio between the two compressions (Bourne, 1978), was also dependent on the calcium level in the cheeses ($P = 0.0002$). In general, sample-height recovery was almost total, although it was slightly lower in the control than in the high-calcium and very high-calcium cheeses (Table 11). A small but significant difference between the two calcium-enriched cheeses was also detected. It seems that calcium, mainly as CCP, had a structuring effect that yielded a matrix that was more capable of recovery after compression. Perhaps a higher strain would have ended in an abrupt fracture from which the samples would not have recovered, but this was not the case under the test parameters used.

Finally, cohesiveness, which represents the applied-work ratio between the second and first compressions (Bourne, 1978), was statistically similar for all the cheeses, meaning that the strength of the internal bonds of the food was reduced proportionally after the first compression for all the cheeses. Nevertheless, a trend towards a more cohesive matrix with higher calcium levels was observed.

Table 11. Texture properties of cheeses with different calcium levels.

TPA [d] property	Hardness (kPa)			Springiness [e]			Cohesiveness [e]		
Ripening (weeks)	1	2	4	1	2	4	1	2	4
Calcium level									
Control	36.9 [a,1]	34.7 [a,1]	37.0 [a,1]	0.805 [a]	0.806 [a]	0.795 [a]	0.644	0.637	0.637
High calcium	64.6 [b,1]	65.9 [b,1]	61.0 [b,1]	0.875 [b]	0.878 [b]	0.873 [b]	0.669	0.660	0.639
Very high calcium	77.4 [c,1]	78.4 [c,1]	85.9 [c,2]	0.893 [c]	0.901 [c]	0.896 [c]	0.676	0.673	0.679
SEM [f]	1.3			0.008			0.010		

[a,b,c,1,2] Different letters denote differences within columns (i.e. calcium level), whereas different numbers denote differences within rows (i.e. ripening duration); no significant differences were detected for cohesiveness. [d] Texture profile analysis. [e] Properties expressed without units are dimensionless. [f] Standard error of the mean.

4.5.3 Cheese microstructure

Scanning electron micrographs of the cheeses after 4 weeks of ripening are shown in Figure 21 The images show the protein matrix and the voids previously occupied by milk fat, which was removed during sample preparation.

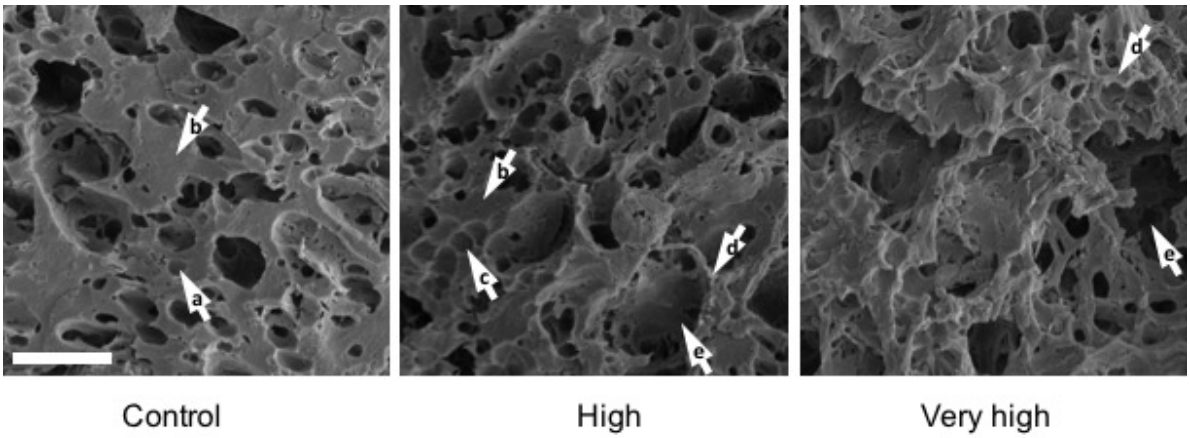

Figure 21. Scanning electron micrographs of control, high-calcium, and very high-calcium cheeses. Arrows indicate examples of a) a space occupied by an individual milk-fat globule, b) an intact protein matrix, c) a space occupied by partially coalesced milk-fat globules, d) a dehydrated protein matrix, and e) a space occupied by large fat reservoirs. Bar = 10 μm.

The control cheese had a continuous protein matrix containing individual and coalesced MFG, in keeping with previous observations for a young Cheddar cheese of similar composition (Lamothe et al., 2012; Mistry et al., 1993). The distribution of the fat globules changed when higher levels of $CaCl_2$ were added at the salting step. As the calcium level increased, the coalescence of the milk-fat globules seemed to increase, producing larger fat pools within the protein matrix (Figure 21). Also, as the calcium level increased, the protein matrix appeared stringier than it was in the control. In Cheddar cheese, protein filaments align in stringy patterns during cheddaring and, without losing their oriented nature, swell with the available moisture, slowly evolving into a uniform compact mass during ripening (Everett, 2007; Kalab et al., 1978). Hence, the greater moisture loss when higher amounts of $CaCl_2$ were added limited the amount of water available for the hydration of protein filaments. The more compact protein matrix resulting from such

water loss allowed milk fat to coalesce further into larger and more continuous reservoirs, as seen in the very high-calcium cheese (Figure 21).

As with the scanning electron micrographs, the CLSM images were obtained from the cheeses ripened for 4 weeks. The superimposed images of protein (represented in red) and fat (represented in green) show the fat-embedded protein matrix (Figure 22).

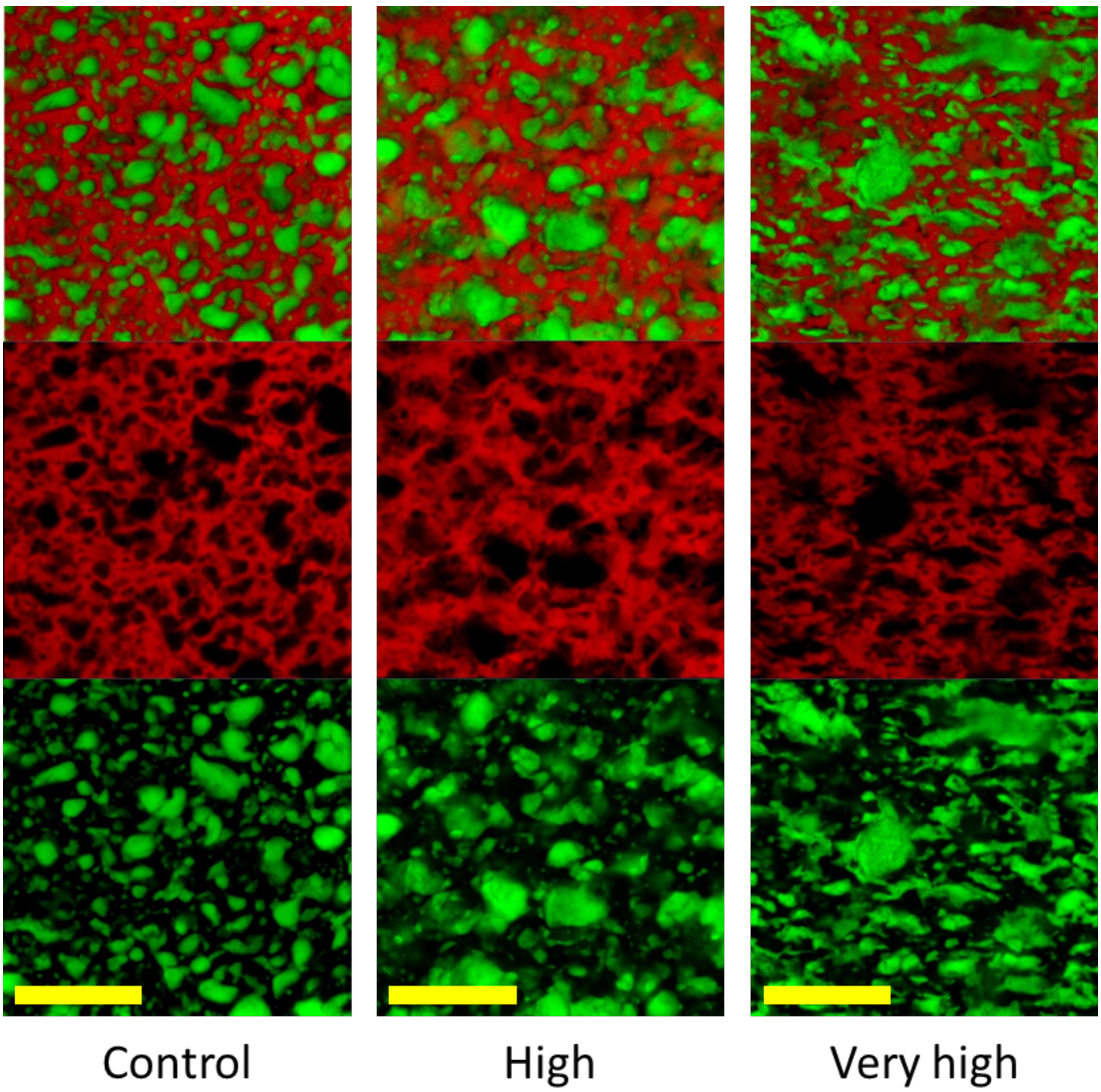

Figure 22. Confocal laser micrographs of control, high-calcium, and very high-calcium cheeses. Green and red channels represent fat and protein, respectively. Overlapping channels (top images) show the distribution of fat and protein within the cheese matrix. Bar = 40 μm.

The control cheese had a more continuous protein matrix containing smaller fat reservoirs than the calcium enriched cheeses. As the calcium level increased, the uniformity and continuity of the para-casein matrix with respect to fat dispersion seemed to decrease. In the control cheese, fat was more uniformly dispersed in individual globules or in small pools, whereas larger fat pools were visible in the high-calcium cheese (Figure 22). In the very high-calcium cheese, the fat pools were less regular in shape and were branched, unlike those present in the other cheeses. These differences appear to have been caused by the drastic water loss of the protein matrix. Hence, the irregular shape and large size of the fat reservoirs in the very high-calcium cheese occurred because the milk fat adapted to the stretched arrangement of the protein, as was also observed in the scanning electron micrographs (Figure 21) The slight linear orientation was produced during the cheddaring step (Hall et al., 1972; Kalab et al., 1978; Taranto et al., 1979) and the pressing step (Auty et al., 2001).

4.5.4 In vitro digestion of cheese

4.5.4.1 Matrix degradation index

Physical disintegration during in vitro digestion progressed differently ($P < 0.0001$) depending on the calcium level in the cheeses (Figure 23a). When measured at the end of the oral phase (5 min), MDI values were higher for the calcium-enriched cheeses than those obtained for the control ($P = 0.0002$) (Figure 23a). These higher values are explained by the brittle texture of the cheeses salted with $CaCl_2$, with the result that some crumbs became detached in the simulated saliva and were lost through the sieve. In contrast, the control did not yield fine particles during this stage of digestion. A similar study, which used the same in vitro digestion model, suggested that the brittleness of aged Cheddar cheese was responsible for MDI values that were three times higher after the oral phase in comparison with the values for less-brittle mild and low-fat Cheddar cheeses (Lamothe et al., 2012). Unfortunately, the low deformation conditions used for texture analysis did not allow proper evaluation of cheese brittleness.

Towards the end of the gastric phase, the cheese with very high calcium had the lowest MDI ($P < 0.0001$). Surprisingly, the high-calcium cheese had the highest MDI after 120 min ($P < 0.0001$) (Figure 23a), despite having a lower moisture-to-protein ratio (1.37) than the control had (1.62). Within similar matrices, higher moisture-to-protein ratios would be expected to accelerate disintegration by enabling faster diffusion (e.g. of digestive enzymes or calcium ions) through the cheese owing to an increase in the relative pore width of the protein matrix (Guinee et al., 2004a). During the gastric phase, the digestion of dislodged particles arising from the weaker milled-curd bonding could explain the faster disintegration of the high-calcium cheese in comparison with the control. The resistance of the very high-calcium cheese could be due to its appreciably low moisture-to-protein ratio (1.24), which slowed down the rate at which the cheese particles disintegrated. After 30 min of the intestinal phase had passed, the cheeses had disintegrated considerably, although the very high-calcium cheese was more resistant ($P < 0.0001$) than the others (Figure 23a). The leap after the addition of intestinal fluids was due to the action of trypsin and chymotrypsin (contained in the pancreatin extract), which rapidly completed the hydrolysis of the hydrated and exposed protein matrix. After 300 min of in vitro digestion, at the end of the intestinal phase, the control and the high-calcium cheeses had disintegrated completely (MDI values of 99.6% and 99.2% ± 0.5%, respectively). In contrast, the very high-calcium cheeses reached a lower MDI (94.6% ± 0.5%) than the other cheeses ($P < 0.0001$), all ripening conditions combined. For the very high-calcium cheeses, the residue recovered from the MDI test was highly hydrated but still contained a significant portion of the solids from the original sample.

Ripening had a small but statistically significant effect on reducing MDI values ($P = 0.0018$) during the in vitro digestion of the experimental cheeses (Figure 23b). After 120 and 150 min of digestion (i.e. at the end of the gastric phase and 30 min into the intestinal phase, respectively), the cheeses ripened for 4 weeks were slightly more resistant to disintegration (i.e. MDI variations below 5%) than were those ripened for 1 or 2 weeks ($P < 0.0001$). As previously mentioned, this difference

could be due to the better bonding of the milled curds after 4 weeks of ripening, although no effect was observed after the digestion was completed (P > 0.5).

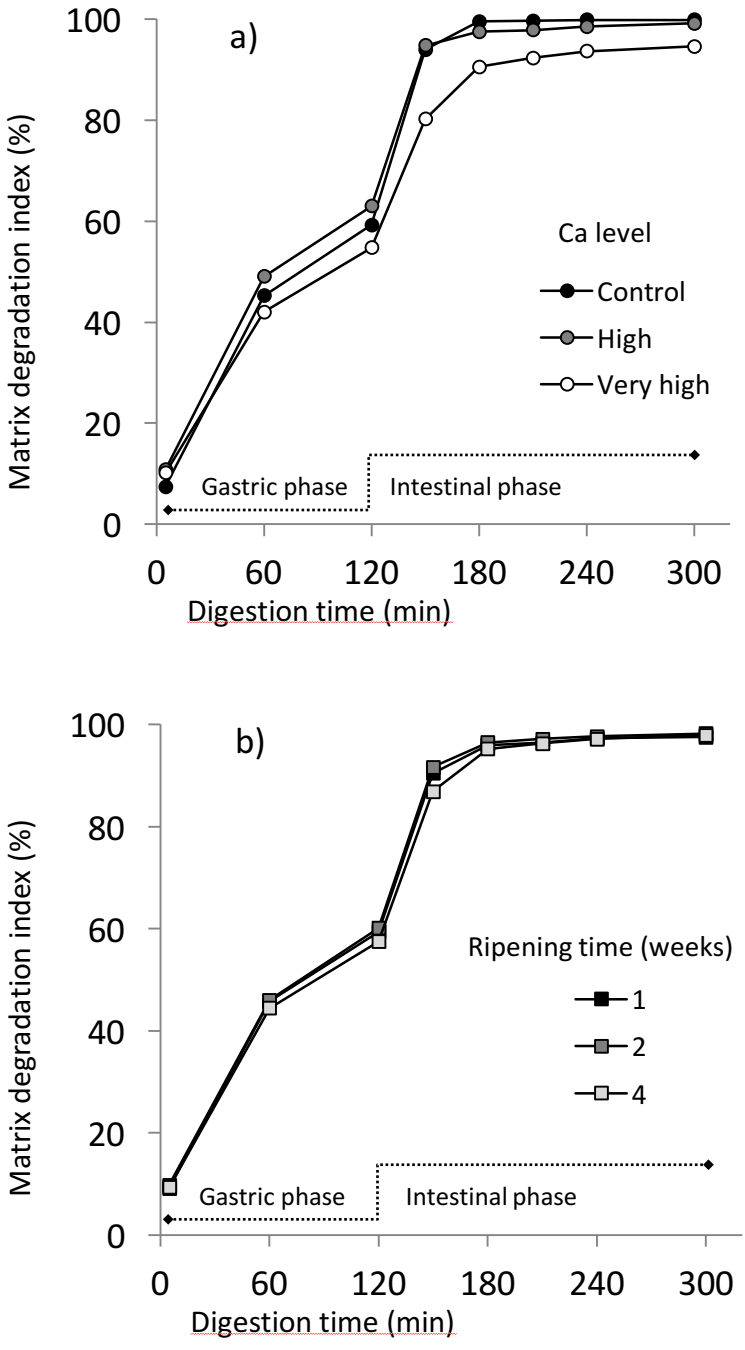

Figure 23. Matrix degradation index values during the in vitro digestion of a) cheeses with different calcium levels (all ripening times combined) and b) cheeses ripened for 1, 2, or 4 weeks (all calcium levels combined). The plots begin after the completion of the oral phase (5 min). SEM = 0.51%.

4.5.4.2 Lipolysis

Before the addition of the simulated intestinal fluids, the NEFA detected in the chyme were lower than 0.01%. No lipases were used in the oral or gastric phase, so lipolysis did not occur until the intestinal fluids were added. Once the intestinal phase began, lipolysis increased during digestion depending on the calcium level in the cheese ($P = 0.0005$) (Figure 24). Cheese ripening had no significant effect on lipolysis ($P > 0.05$). During the first 30 min under intestinal conditions, fatty-acid release was abundant for all the cheeses owing to the rapid lipolysis of the free milk fat that had detached from the cheese matrices during the gastric phase and during the rapid degradation at the beginning of the intestinal phase. This observation is in accordance with a previous study with full-fat Cheddar cheeses using the same digestion model, where at least 60% of the fat was free after only 30 min of intestinal digestion (Lamothe et al., 2012). The high amount of free fat was readily accessible to the pancreatic lipase in the chyme. Lipolysis progressed faster for the calcium-enriched cheeses than for the control ($P < 0.0001$), and the effect was consistent for the first 90 min of intestinal digestion (Figure 24). During the last 90 min of in vitro digestion, lipolysis progression slowed down for all cheeses and seemed to reach a plateau (Figure 24). The final lipolysis rates after 300 min of digestion were 73.6%, 77.9%, and 72.5% (SEM = 1.61%) for the control, high-calcium, and very high-calcium cheeses, respectively, with a statistical difference between the last two ($P = 0.0148$).

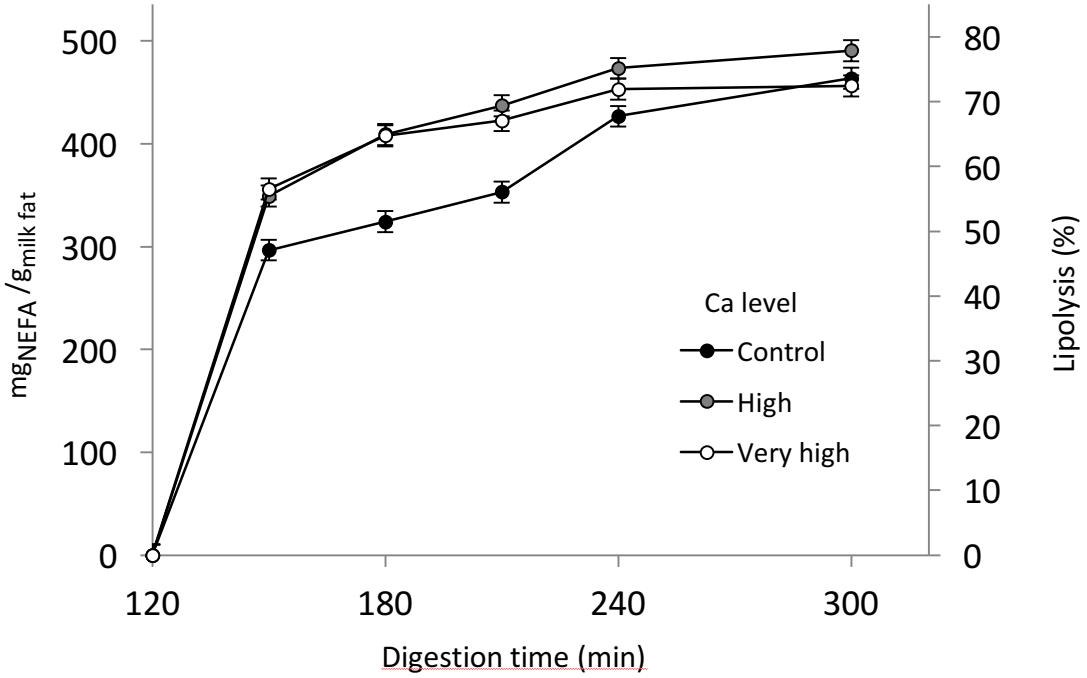

Figure 24. Evolution of non-esterified fatty acids (NEFA) and lipolysis during the intestinal phase of the in vitro digestion of control, high-calcium, and very high-calcium cheeses. SEM = 1.61%.

Calcium has been shown to increase lipolysis rates, an effect that can be explained by the depletion of free FA at the lipid–water interface. Calcium soaps are produced with free FA in the vicinity of neutral pH, and they precipitate under intestinal pH conditions, freeing the lipid–water interface so that lipase may access its substrate (Devraj et al., 2013; Hu et al., 2010). In the very high-calcium cheese, some fat may have still been trapped in the cheese matrix residue towards the end of digestion and would have been protected from the lipase. The effect of calcium on lipolysis may also be attributed to a change in cheese microstructure that increases free fat release from the matrix during digestion. The calcium-enriched matrices presented a high degree of fat coalescence interconnecting the fat pools in comparison with the control (Figure 21 and Figure 22). During in vitro digestion, the molten fat (at 37°C) would have readily exited the partially digested matrix, emptying into the chyme as free fat and enabling its lipolysis. This phenomenon

would explain the high lipolysis rates even if physical disintegration of the protein matrix was not complete.

At the end of digestion, lipolysis did not reach completion (i.e. 100% of the potential NEFA), probably because the in vitro system used does not mimic nutrient absorption and thus reaction products accumulate in the chyme, eventually halting the enzymatic reaction. Nonetheless, the results are comparable to those obtained for Cheddar cheese with the same in vitro digestion model (Lamothe et al., 2012).

4.6 Conclusion

Adding calcium chloride during the salting step of cheese manufacture had a significant impact on cheese structure and its behavior during in vitro digestion. Enrichment with $CaCl_2$ had a major effect by reducing the moisture content of the cheeses during the pressing stage. Such moisture differences, combined with the presence of different levels of calcium within the protein matrix, resulted in modified texture parameters and cheese microstructure.

Under the conditions set for this study, short-term ripening had only a limited effect on cheese texture and digestion, and that effect differed depending on the calcium level in the cheese. Although short-term ripening of the cheeses caused variations in the extent of proteolysis and the solubilization of CCP, the impact of such processes on cheese structure was lower than expected, and the cheeses did not show any major changes in texture properties during the ripening period, explaining the similarities observed on MDI and lipolysis during in vitro digestion of the different cheeses. Higher calcium levels led to faster lipolysis during the first half of the intestinal digestion, possibly by enhancing lipase activity. The protective effect of the matrix on lipolysis was observed for the very-high calcium cheese towards the end of the digestion, where less NEFA were detected even in the presence of higher calcium levels, when compared to the other cheeses.

This study revealed that enrichment of Cheddar curds with $CaCl_2$ during salting produced major modifications to the final cheese matrix by modifying its composition, structure and physicochemical evolution during short-term ripening. Such modifications affected the behavior on the cheese matrices during their in vitro digestion, and are a clear example that the food matrix and its microstructure could help control the release of lipid nutrients from dairy rennet gels. This study also provides insight into technological processes that can be used to achieve such nutrient modulation. Further works on cheeses with similar composition and structure are in course to better understand the net effect of calcium on fatty acid bioaccessibility. Eventually, the findings of this study could lead to the discovery of

novel nutritional aspects that could be adapted for the food industry to, among other things, control nutrient release, deliver bioactive molecules, and build evidence to substantiate health claims.

4.7 Acknowledgements

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Chapter 5. Effect of calcium on fatty acid bioaccessibility during in vitro digestion of Cheddar-type cheeses prepared with different milk fat fractions

This chapter is a research paper reporting a second series of model Cheddar cheeses standardized with creams prepared with milk fat fractions with different fatty acid profiles. A partial substitution of sodium chloride with calcium chloride was done during the salting step. The effect of both parameters on cheese composition, microstructure and in vitro digestion, with emphasis on the effect of calcium soaps on fatty-acid bioaccessibility, is presented. The paper has been published in the Journal of Dairy Science (doi 10.3168/jds.2016-11902). The role of the coauthors is as follows: Erik Ayala Bribiesca is the main author and did the experimental design, laboratory experiments, statistical analysis of the data, and writing of the paper. Sylvie Turgeon and Michel Britten supervised the project and assisted in proofing the manuscript. All coauthors authorized the inclusion of the paper in this thesis.

5.1 Résumé

Cette étude visait à comprendre l'effet du calcium sur la bioaccessibilité des lipides laitiers à partir de fromages de type Cheddar. Les fromages ont été préparés avec des huiles de beurre contrôle, oléine et stéarine et enrichis ou non en calcium au salage. La désintégration des fromages et la lipolyse ont été suivies en cours de digestion in vitro. Les savons de calcium présents dans les chymes ont été dosés par des extractions à différents pH. Les fromages préparés avec la stéarine étaient plus résistants à la désintégration que les autres. Les fromages enrichis en calcium présentaient une lipolyse plus rapide que les fromages sans enrichissement. L'analyse des chymes a montré que le calcium produit des savons avec les acides gras à longue chaîne, mais non pas avec ceux à courte chaîne. Bien que la production accrue de savons de calcium entraîne une lipolyse plus rapide, la biodisponibilité des acides gras impliqués serait réduite.

5.2 Abstract

This study aimed to better understand the effect of calcium on the bioaccessibility of milk fat from Cheddar-type cheeses. Olein, stearin and control AMF were used to prepare Cheddar-type cheeses, enriched or not with calcium using $CaCl_2$ during the salting step. The cheeses were digested in vitro, and their disintegration and lipolysis rates were monitored during the process. Calcium soaps were assessed in digested samples by extractions at different pH conditions. The cheeses with the stearin were more resistant to disintegration than the other cheeses. The Ca-enriched cheeses had faster lipolysis rates than the regular calcium cheeses. Analysis of the digestion products showed that calcium interacted with long-chain FA, producing calcium soaps, whereas no interaction with short-chain FA was detected. Although higher calcium levels resulted in faster lipolysis rates, the calcium soaps produced are expected to reduce the bioavailability of FA by hindering their absorption.

5.3 Introduction

Digestion is the process that allows the body to absorb nutrients from foods. However, not all ingested nutrients can be digested, absorbed and used for normal body functions. Thus, the total amount of a nutrient in a food does not necessarily reflect the amount that can be absorbed. Nutrient bioaccessibility is the fraction of an ingested component that is released from the food matrix into the juices of the gastrointestinal tract. Bioaccessibility is an important factor influencing nutrient bioavailability (Versantvoort et al., 2005; Watzke, 1998).

The bioaccessibility of lipids depends greatly on the ability of the digestive system to free lipids from the food matrix and then transform them into an absorbable form. TAG, which represent around 95% of dietary lipids, are hydrolyzed by digestive lipases to produce MAG and FA as end products (Carey et al., 1983; Jones et al., 2006). MAG can be incorporated into mixed micelles for intestinal uptake in the jejunum and the ileum without further hydrolysis. Absorption of FA depends partly on the length of their carbon chain (Duchateau et al., 2009). SCFA and MCFA can be absorbed directly by the intestinal mucosa, whereas long-chain fatty acids LCFA, including unsaturated fatty acids (UFA), must be assembled into mixed micelles to be transported for absorption (Bauer et al., 2005; Guéguen et al., 2008; Jones et al., 2006). Furthermore, some LCFA may bind to calcium as insoluble soaps, significantly reducing their absorption rates (Guéguen et al., 2008) (Astrup et al., 2010; Golding et al., 2010). Nevertheless, the effect of calcium is not straightforward, because it may also increase FA bioaccessibility by enhancing lipolysis (Hu et al., 2010).

Lipolysis takes place at the lipid/water interface between the fat droplets and the aqueous digestive fluids. Once the lipids have been released from the food matrix, the next stage in lipid digestion is the formation of emulsified fat droplets. This creates a lipid/water interface allowing the adsorption of lipase so that it can interact with the TAG in the fat droplet (Jones et al., 2006). Adsorption of the enzyme to the interface causes a conformational rearrangement, exposing the

catalytic site (Golding et al., 2010). Lipolysis products are removed from the interphase by incorporation into mixed micelles with bile salts. However, as LCFA accumulate on such interface, they eventually limit the access of lipase to the remaining substrate (Bauer et al., 2005; Favé et al., 2004). At this stage of lipid digestion, calcium can play a major role in FA bioaccessibility. Under pH conditions close to neutrality, such as those found in the jejunum and ileum, calcium reacts with saturated LCFA to form their corresponding calcium salts, commonly referred to as calcium soaps (CS) (Owen et al., 1995). The removal of FA from the fat droplet surface as CS allows lipase to access more substrate, increasing lipolysis rates (Hu et al., 2010; Patton et al., 1979). However, such CS form crystalline structures with extremely low water solubility (Harrison, 1924; Pereira et al., 2012), which disturbs their incorporation into mixed micelles and results in delayed FA uptake.

Fat absorption from the lumen is generally quite efficient, with only about 4% of the ingested fat escaping into the feces (Carey et al., 1983). An increase in fat excretion when fat is taken in combination with high amounts of calcium has been shown in various studies (Christensen et al., 2009). A meta-analysis reported that an increase of dairy calcium intake of 1241 mg/d resulted in an increase of fecal fat of 5.2 (1.6 to 8.8) g/d (Christensen et al., 2009). In such studies, fat loss was mainly related to LCFA loss as CS.

Cheddar cheese is recognized as a good dietary source of Ca. Calcium content of cheese may be controlled during the acidification process of the curd and by adding $CaCl_2$ before renneting. From a technological point of view, calcium has a major role in structuring the protein matrix of Cheddar cheese (Lucey et al., 1993; Ong et al., 2013). In a previous study on digestion of Cheddar cheese, $CaCl_2$ was added to curds during the salting step (Ayala-Bribiesca et al., 2016). Results have shown that cheeses with different structures exhibit different breakdown patterns and lipolysis rates during in vitro digestion (Ayala-Bribiesca et al., 2016; Lamothe et al., 2012).

Different types of milk fat can also be used to modify the composition of the milk in cheese manufacture. Olein and stearin are AMF fractions with melting points that are, respectively, lower and higher than that of a regular AMF. Olein fractions have a higher ratio of low-melting-point TAG, which are generally rich in UFA and SCFA. Stearin fractions are high in TAG with higher saturated LCFA content (Lopez et al., 2006).

The objective of this study was to determine the effect of calcium added as $CaCl_2$ at the salting step on the in vitro digestion and lipid bioaccessibility of Cheddar-type cheeses prepared with milk containing different milk fat fractions. A better understanding of the role of calcium in the bioaccessibility of FA in cheese will enable the development of technological strategies to manufacture functional food matrices.

5.4 Materials and methods

5.4.1 Characterization of AMF

Three different AMF were used to prepare the creams for cheese-making. The control AMF was prepared from commercial butter, which was melted at 80°C, and the lipid phase was recovered by aspiration. Commercial olein and stearin AMF fractions (Anhydrous Milkfat Premium and Specialty Milkfat 42, respectively) were kindly provided by Fonterra Ltd. (Auckland, New Zealand).

Thermal analyses of AMF were performed by differential scanning calorimetry (DSC) using a Q2000 calorimeter (TA Instruments, Saint Quentin en Yvelines, France). The AMF samples were placed in 50-µl aluminum pans and sealed hermetically. An empty pan was used as reference, and the equipment was calibrated with lauric acid (melting point = 43.7°C, $\Delta H_{melting}$ = 8.53 kcal/mol). The samples were conditioned for 5 min at 70°C to melt all fat crystals and nuclei and then cooled to −50°C at a rate of 1°C/min, followed by heating to 70°C at a rate of 1°C/min. The final melting point of each fraction was determined from their thermograms, from the intersection of the baseline and the tangent to the last endothermic drop (Lopez et al., 2006).

For the fatty acid analysis, samples from each AMF were methylated (Luddy et al., 1968). All standards and reagents were obtained from Sigma Aldrich (St. Louis, MO, USA). The solvents (HPLC-grade) were obtained from Fisher Scientific (Markham, ON, Canada). Heptanoic (C7:0) and nonadecanoic (C19:0) acids were added to the methylation tubes as internal standards to correct from 4- to 12-carbon FA and from 14- to 18-carbon FA, respectively. The AMF samples were treated with 0.4 N potassium methanolate in anhydrous methanol at 65°C for 2 min, mixed in a vortex mixer and heated for another 30 s. After the samples were cooled on ice, 300 µl of 14% BF_3 in methanol was added and the mixture was heated at 65°C for 2 min, mixed in a vortex mixer and cooled again on ice. This preparation sequence ensures the complete methylation of esterified and free FA present in the samples (Luddy et al., 1968). To extract the methyl esters, 0.6 g of

Na_2SO_4 was added to each tube, followed by 5 ml of *n*-hexane and mixed for 1 min with a vortex mixer. The methylation tubes were then centrifuged for 2 min at 3,000 × *g*, and the supernatant phase was kept for analysis.

Fatty acid profile analysis was performed by gas chromatography (GC) (6890A gas chromatograph with a flame-ionization detector (FID); Agilent Technologies, Mississauga, ON, Canada). The inlet was a split-splitless type and kept at 250°C with a split ratio set to 25:1. Helium was used as carrier gas (1.2 ml/min) and as make-up gas (25 ml/min). The column was fused silica and 30 m × 0.25 mm (internal diameter) with a liquid phase of type DB-FFAP (d_f [film thickness] = 0.25 µm) (J&W; Chromatographic Specialties Inc., Brockville, ON, Canada). After the automated injection, the oven temperature was kept at 50°C for 3 min, increased at a rate of 15°C/min to 200°C, and kept at 200°C for 15 min, for a total run time of 28 min. Hydrogen (35 ml/min) and compressed air (350 ml/min) were used for the FID, which was kept at 300°C. The molar percentage of each FA was calculated with respect to the total FA recovered.

5.4.2 Preparation and characterization of the creams

The day before cheese production, each AMF was melted at 65°C in a double-wall vat. Raw skim milk (0.27% fat; Laiterie Chalifoux, Sorel, QC, Canada) was added until a 36% fat mixture was obtained. The vat was mixed with a vane agitator, heated, and held at 65°C for 15 min before the mixture was pumped through the homogenizer (Two-phase, 200-L/h capacity, SN 185188; Rannie, Copenhagen, Denmark). The homogenizer was set to 25 bar (approximately 360 psi), which was the equipment's lowest effective setting. After 2 passes through the homogenizer, the creams were rapidly cooled to 15°C and kept overnight at 4°C.

Fat droplet size in the AMF creams was measured and compared with a native pasteurized cream by laser diffraction using a Mastersizer 2000 particle size analyzer (E ver. 5.54, SN MAL1023324; Malvern Instruments, Malvern, UK) equipped with a Hydro 2000MU sampler (Malvern Instruments). The cream samples were diluted at a 1:200 ratio in a dissociation buffer. The buffer was

composed of 8 M urea, 50-mmol/L EDTA, and 10-mmol/L dithiothreitol, adjusted to pH 7 with 1-mol/L NaOH, and filtered at 0.45 µm (Robin et al., 1991). All chemicals were obtained from Sigma Aldrich. The buffer with sample was then added to distilled water directly in the sampler to an approximate 1:3,000 ratio, until an obscuration rate of 10% was obtained for the analysis. Specific surface area, surface-weighted median diameter, and volume-weighted median diameter were recorded.

5.4.3 Cheddar-type cheeses

5.4.3.1 Cheese production

Cheeses were manufactured in the pilot plant at Agriculture and Agri-Food Canada's Research and Development Centre (Saint-Hyacinthe, Canada). Raw skim milk (0.27% fat) purchased from a local company (Laiterie Chalifoux) was separated into 3 batches, which were standardized to a final fat mass fraction of 4.5% ± 0.1% using the creams prepared with the different AMF. Each standardized milk was immediately pasteurized at 74.5°C for 18 s and then transferred into separate cheese vats (Model 4 MX, 250 L, double O type; Kusel Equipment, Watertown, WI, USA) set to maintain the milk at 32°C. The vats were filled with 225 kg of milk and 45 ml of a 450 g/L $CaCl_2$ solution (Cal-Sol 45%; Fromagex, Rimouski, QC, Canada). The starter (50 U/1,000 L, FD-DVS Flora Danica, 100103; Chr. Hansen, Hørsholm, Denmark) was added directly into the vats. When the milk reached pH 6.54 ± 0.02 and 14.5 ± 0.5°D), commercial microbial chymosin solution (ChyMax; Chr. Hansen, Hørsholm, Denmark) at 0.085 ml/kg, diluted in 30 volumes of distilled water, was added to each vat. Once set, the curd was cut and then cooked by increasing the temperature to 38°C at a rate of 0.2°C/min, after which the temperature was maintained. The whey was drained about 3 h after the gel was cut, when the pH of the whey was 6.06 ± 0.04. Cheddaring lasted about 2 h, and the curds were milled when their pH reached 5.38 ± 0.03.

The milled curds from each vat were separated into 2 containers and salted with NaCl or a mixture of NaCl and $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich), in which half of the

NaCl was replaced with $CaCl_2$•$2H_2O$ in a 1:1 ionic-strength contribution ratio, as calculated with Equation 2:

Equation 2. Ionic strength of a solution.

$$\mu = \frac{1}{2}\sum_i c_i Z_i^2$$

where μ is the ionic strength of the solution, and c_i and Z_i are, respectively, the concentration and the charge of the ion i (Barrow, 1979). The amounts of salts added to produce a regular-Ca or a high-Ca cheese were, respectively, 2.42% NaCl or 1.21% NaCl + 1.02% $CaCl_2$•$2H_2O$ (i.e., equivalent to the ionic strength of a 4.32% NaCl-in-moisture content in the milled curd, for both salting conditions). The salted curds were then packed into 13-kg-capacity stainless steel molds lined with synthetic cheesecloth and were pressed overnight at 2.75 bar (approximately 40 psi) at room temperature. When removed from the molds, the cheeses were vacuum-packed and kept at 4°C for 84 d, after which they were cut into smaller blocks that were individually vacuum-packed. The individually wrapped blocks were kept at 4°C until required for the experiments, which were completed within 21 d.

5.4.3.2 Cheese composition

All reagents for composition analyses were obtained from Fisher Scientific. Cheese pH was measured in a slurry prepared from 10 g of cheese and 10 g of distilled water. Moisture was quantified by difference from total solids, obtained by oven-drying at 100°C for 16 h. Protein was quantified by the Kjeldahl method (N x 6.38). Fat was quantified by Mojonnier ether extraction. For mineral analysis, the dry solids were ashed at 550°C for 16 h.

Main cations (i.e., Ca, Na, K, and Mg) and P were quantified by ICP-OES from the cheese ashes. The ashes were dissolved in 0.23 mol/L HNO_3, centrifuged for 15 min at 3,000 × g, and filtered through a 0.45 µm polyethersulfone filter. Commercial standards (Fisher Scientific) were diluted in the same acidic matrix and filtered.

The spectrometer (Prism 010-00084-1; Teledyne Leeman Labs, Hudson, NH, USA) was equipped with a Peek Mira Mist Nebulizer (PMM4000; Burgener Research, Mississauga, ON, Canada). The emission lines used for element quantification for Ca, Na, K, Mg, and P were 317.933, 589.592, 766.490, 279.080, and 213.618 nm, respectively. Detection was performed in radial mode on the argon-plasma torch.

5.4.3.3 Cheese structure.

The microstructure of the experimental cheeses was observed by scanning electron micrography and CLSM imaging. Cheese hardness was measured by axial compression of cheese cylinders.

Cheese samples were prepared as reported in a previous study (Ayala-Bribiesca et al., 2016), and scanning electron micrographs of typical fields were recorded using an XL30 scanning electron microscope (Philips, Eindhoven, Netherlands).

For confocal laser scanning microscopy, thick slices (approximately 0.5 mm) of fresh cheese at 4°C were cut with a razor blade and mounted in a hybridization well affixed on a microscope slide to stabilize the cover slip over the thick slice of cheese. Fat and protein in the samples were simultaneously stained over ice with a 0.01% aqueous solution of Nile Blue A (Sigma-Aldrich). After 2 min, the excess liquid was removed, and the sample was covered with Fluoromount-G and a cover slip and observed under a Zeiss 510 Meta confocal laser scanning microscope equipped with a Zeiss 40×/NA 1.4 Plan Apochromat objective lens (Carl Zeiss GmbH, Jena, Germany). Fat was detected at 488-nm excitation of an Argon/2 laser and with a 530- to 600-nm band-pass emission filter, and protein was detected at 633-nm excitation of an HeNe laser and with a 650-nm long-pass emission filter. The slides were kept over a refrigerated surface during observation to prevent fat mobility and coalescence. The images were processed with Zen software, v.2009 (Carl Zeiss GmbH), with green and red pseudo-colors assigned to fat and protein, respectively.

Cheese hardness was obtained from the peak force of a compression cycle (Bourne, 1978) using a TA-XT2 texture analyzer (Stable Micro Systems, Surrey, UK) and according to the parameters used in a previous study (Ayala-Bribiesca et al., 2016). Cylindrical samples (radius = 5 mm; height = 10 mm) were equilibrated for 30 min at 22°C for the temperature at which the cheese would enter the mouth or at 37°C for the temperature of digestion, and the samples were then compressed (axial compression) to a 30% strain with a probe moving at 0.4 mm/s.

5.4.4 In vitro digestion

The in vitro digestion was based on a static gastrointestinal model for the fed state simulating oral, gastric, and small-intestine digestion and was carried out in 50-ml tubes with head-over-heels agitation (Versantvoort et al., 2005), with the modifications presented in a previous study (Ayala-Bribiesca et al., 2016).

The tubes contained 3.0 g of cheese cut into 3-mm sided cubes and 2.5 g of glass beads, in agreement with considerations explained elsewhere (Ayala-Bribiesca et al., 2016). Oral, gastric, and intestinal fluids were sequentially added at 0, 5, and 120 min, respectively. The total duration of digestion was 270 min, and the system was maintained at 37°C throughout the process. A new digestion tube was sampled for analysis at each time point (i.e., after 5, 60, 120, 135, 150, 180, 240, and 270 min). The in vitro digestions were performed three times.

Physical disintegration of the cheeses was quantified using the matrix degradation index (MDI), obtained from the amount of cheese solids dispersed in the digestion fluids, as previously described (Lamothe et al., 2012). Briefly, large cheese particles were separated using a metallic sieve with 1.5-mm mesh. The drained liquid was used for FA and CS analyses. The retained solids were washed twice with 5 ml of unused digestive fluids at 37°C (saliva, gastric, or intestinal fluid, depending on the time point of sampling). The cheese solids were then transferred to a tinfoil dish, dried, and weighed. The mass of dry solids was used to obtain the MDI, using Equation 3.

Equation 3. Matrix degradation index.

$$\text{MDI}\ (\%) = 100 \times \frac{CS_0 - CS_t}{CS_0}$$

where CS_0 is the mass of cheese solids originally present in the digestion tube, and CS_t is the mass of cheese solids remaining at time t. Physical disintegration of the matrix was quantified after 5, 60, 120, 135, 150, 180, 240, and 270 min.

The extent of lipolysis during in vitro digestion was measured using a NEFA enzymatic colorimetric kit (Roche Diagnostics, Indianapolis, IN, USA). The chyme samples were handled and prepared as previously described (Ayala-Bribiesca et al., 2016). Absorbance was measured at 546 nm with a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) equipped with a tungsten lamp. Oleic acid was used as the calibration standard. Samples were analyzed after 120, 135, 150, 180, 240, and 270 min of in vitro digestion. Given that pancreatic lipase cleaves only sn_1- and sn_3-FA from the triglyceride molecule (Jones et al., 2006), theoretically only 2/3 of the TAG FA could be released. Lipolysis was expressed as the mass fraction (%) of NEFA released from the AMF present in the cheese sample, assuming an average molecular weight of 247 g/mol for milk FA (Lamothe et al., 2012). In parallel, the pH of the liquid fraction of the chyme was recorded at 60, 120, 135, 150, 180, 240, and 270 min.

5.4.5 Fatty acid bioaccessibility

At the end of digestion (270 min), aliquots of the chyme juice (approximately 1 ml) were transferred into two 50-ml Teflon high-speed centrifuge tubes (05-562-16B, Cat. # 3114-0050; Fisher Scientific, Ottawa, ON, Canada). The masses of chyme juice added were recorded, and 10 ml with distilled water and 80 µl of 5 N H_2SO_4 were added to the tubes. Preliminary tests (not shown) confirmed that the pH of the tube contents was always $\leq$ 2.0. Such acidic conditions dissociate any CS and protonate NEFA, enabling their transfer into the organic phase (de Jong et al., 1990). The lipids were extracted by adding 10 ml of ethanol and 10 ml of a 1:1 volumetric mixture of diethyl ether and hexane. Once the solvents had been added, the tubes were tightly closed, mixed with a vortex mixer, and transferred into an

orbital shaker, where they were mixed in the horizontal position at 150 rpm for 5 min in a chamber at 37°C. The tubes were then centrifuged at room temperature for 3 min at 3,500 rpm (Sorvall ST-40R centrifuge, TX-750 rotor, 3608 round bucket; Thermo Fisher Scientific, Waltham, MA, USA). The supernatant organic phase was removed with a Pasteur pipette and placed in 40-ml amber glass vials containing 2 g of Na_2SO_4. Two more extractions with 10 ml of 1:1 diethyl ether and hexane followed. The extracts were pooled in the vials, and tridecanoic acid (C13:0) was added as an internal standard. The FA were separated by solid-phase extraction (SPE) using commercially available aminopropyl cartridges (NH_2 Sep-Pak Plus, WAT020535; Waters, Taunton, MA, USA) that were first conditioned with a 10-ml wash of *n*-hexane. After the conditioning step, the contents of each vial were emptied into the solid-phase extraction cartridge funnels. The eluted solvent was recovered, used to rinse the vials, and eluted once again. Non-polar lipids were removed from the solid-phase extraction cartridges by eluting 10 ml of a 2:1 chloroform–isopropanol mixture. The FA were desorbed with 4 ml of 2% formic acid in diethyl ether solution. All elutions were performed at a rate of 2 ml/min. The FA in the solutions were quantified by GC (6890A gas chromatograph equipped with an FID; Agilent Technologies, Mississauga, ON, Canada). The inlet was a split type and kept at 250°C with a split ratio set to 10:1. Hydrogen was used as carrier gas (9 ml/min), and nitrogen was used as make-up gas (25 ml/min). The column was fused silica and 15 m × 0.53 mm internal diameter with a liquid phase of type DB-FFAP (d_f = 1 µm) (J125-3212; Agilent Technologies). After the automatic injection, the oven temperature was kept at 115°C for 3 min, increased at a rate of 10°C/min to 240°C, and kept at 240°C for 15 min, for a total run time of 30.5 min. The FID was kept at 300°C. Individual FA were identified and calibrated with their respective commercial standards (Sigma Aldrich). Overall bioaccessibility, as related to the extent of lipolysis, refers to the sum of individual FA in relation to total FA originally present in the AMF, and the bioaccessibility profile refers to the mass fraction (%) of each FA released after in vitro digestion and recovered in the acidic extraction in relation to the same FA originally present in the AMF used to prepare the experimental cheese.

5.4.5.1 Calcium enrichment and fatty acid solubility

To evaluate the effect of calcium enrichment on the precipitation of individual FA, an extraction of chyme juice was performed at pH 7, which represents conditions that can be normally found in the small intestine (Diem et al., 1970). Chyme juice collected at the end of in vitro digestion was adjusted to pH 7.00 ± 0.02 with 5 N NaOH. Less than 150 µl of NaOH solution per 10 ml of chyme juice was required to obtain the target pH, and thus the dilution effect was negligible. The samples were submitted to the same extraction as detailed for FA bioaccessibility, except that no acid was added to the extraction tube. Loss ratios for each FA between the extraction pairs (at pH 2 and pH 7) were compared among the different AMF types and the different calcium levels of the experimental cheeses using Equation 4, as follows:

Equation 4. Loss ratio of fatty acids extracted under different pH conditions.

$$LR = 1 - \frac{\mathrm{FA}_7}{\mathrm{FA}_2}$$

where, for chymes obtained after the digestion of each experimental cheese, *LR* is the loss ratio for each FA between the extraction pairs, FA_2 is the amount of the FA quantified in the organic phase of the extraction performed at pH 2, and FA_7 is the amount of the FA quantified in the organic phase of the extraction performed at pH 7.

Lastly, the impact of the calcium enrichment of the Cheddar-type cheese on CS formation was estimated by gravimetrically comparing the extraction rate differences between the high-calcium cheeses prepared with each AMF type and their respective regular-calcium counterparts. To that end, a second series of extractions at pH 2 and 7 was performed on chyme juice collected at the end of digestion. To extract gravimetrically quantifiable masses of lipids, the chyme juice samples were increased to approximately 10 ml. Once the sample was weighed, extractions proceeded as detailed for the FA analyses, except that no water was added and that, for the acidic extractions, 600 µl of 5 N H_2SO_4 was required to

reach pH 2. The organic phases of 3 extractions were pooled in a pre-weighed tinfoil dish. The solvents were left to evaporate for 12 h at room temperature under a fume hood, and the cups were then weighed. The mass fraction of fat lost between each extraction pair was computed by subtracting the mass fraction of fat extracted from the chyme juice at pH 7 from that obtained through the extraction at pH 2 (Equation 5).

Equation 5. Mass fraction of fat lost between extractions at neutral and acidic pH conditions.

$$ML = \mathrm{m}_2 - \mathrm{m}_7$$

Where *ML* is the mass fraction of fat lost between the extraction pairs, for each AMF and calcium level; m_2 is the mass fraction of fat extracted at pH 2; m_7 is the mass fraction of fat extracted at pH 7.

The increment in CS due to calcium enrichment (Δ_{CS}) was defined as the mass loss between the high and low calcium levels of cheeses prepared with each AMF type (Equation 6).

Equation 6. Increment in calcium soaps due to calcium enrichment.

$$\Delta_{\mathrm{CS}} = \mathrm{ML}_{\mathrm{highCa}} - \mathrm{ML}_{\mathrm{regularCa}}$$

Where Δ_{CS} is the increment of fat loss as CS due to calcium enrichment of cheeses with each AMF; ML_{highCa} is the mean of the mass fraction of fat lost for high calcium levels for each AMF; and $ML_{regularCa}$ is the mean of the mass fraction of fat lost for regular calcium levels for each AMF.

5.4.6 Statistical Analysis

Results for AMF thermal analysis and cream characterization were analyzed using a one-way ANOVA, and comparisons were performed using the LSD with a significance threshold of $P \leq 0.05$. Cheese physicochemical analyses were performed on triplicate samples, unless specified otherwise. The data were analyzed for statistical differences by a two-way ANOVA in a split-plot array with the AMF type nested in the main plot and the salting condition in the sub-plot.

Multiple comparisons were performed using the LSD with a significance threshold of $P \leq 0.05$. The in vitro digestion results and cheese hardness data were analyzed for statistical differences using a three-way ANOVA in a split-plot array with the AMF type and salting condition in the main plot and the digestion time or the compression temperature for digestion or hardness, respectively, nested in the sub-plot. Multiple comparisons were performed using the LSD with a significance threshold of $P \leq 0.05$. The FA composition of the bioaccessible lipid fraction was analyzed using a two-way ANOVA in a completely random design for the AMF and calcium level. The LSD was used for the multiple comparisons with a significance threshold of $P \leq 0.05$. All statistical analyses were performed with SAS 9.3 TS Level 1M0 software (SAS Institute, Cary, NC, USA).

5.5 Results and discussion

5.5.1 AMF characteristics

Thermograms obtained by DSC (Figure 25) revealed similar thermal behavior between the control and olein AMF, whereas the behavior of the stearin fraction was distinct. The control AMF presented the typical aspect of a milk fat thermogram (Lopez et al., 2006). Olein and stearin AMF thermal behavior may vary according to the specific conditions of the milk fat fractionation progress (Kaylegian et al., 1995). Crystallization events (Figure 25a) started at different temperatures ($P < 0.0001$) for all AMF types (Table 12), with the highest temperature for the stearin AMF and the lowest for the olein AMF. Final melting points (Table 12) determined from the last endothermic event (Figure 25b) also differed for all AMF ($P < 0.0001$). The olein AMF final melting point was slightly lower (by approximately 1°C) than that of the control AMF (35.43°C), whereas the stearin AMF final melting point was considerably higher (by approximately 7°C), in agreement with their crystallization behavior. The small exothermic peak of control AMF at 16°C (Figure 25a) would explain the slight shift in the melting profile for the olein AMF. For all practical purposes, both the olein and control AMF were expected to be completely melted during the in vitro digestion process (37°C), but not the stearin AMF.

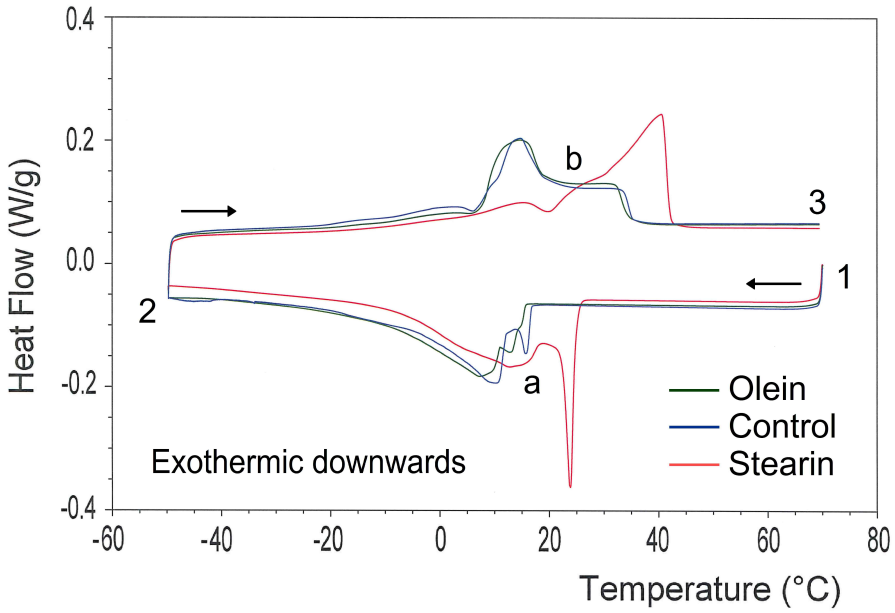

Figure 25. Differential scanning calorimetry thermograms of the control, olein, and stearin anhydrous milk fats recorded during a crystallization and melting cycle from 70 to −50°C (1 to 2) and −50 to 70°C (2 to 3) at a rate of 1°C/min. Exothermic (a) and endothermic (b) events are shown for the crystallization and melting spans, respectively.

Table 12. Main thermal events obtained from differential scanning calorimetry thermograms recorded at 1°C/min for olein, control, and stearin anhydrous milk fats.

AMF	Crystallization start (°C)	Final melting point (°C)
Olein	15.60 [c]	34.70 [c]
Control	16.81 [b]	35.43 [b]
Stearin	25.31 [a]	42.32 [a]
SEM	0.02	0.03

[a–c] Different letters denote different means within the columns.

The FA profiles of the AMF are presented in Table 13. Differences in individual FA reflected the nature of the olein and stearin fractions, although the olein was closer in composition to the control than the stearin was. This closeness would explain

the similar behavior during the DSC analysis, where only small shifts in the melting and crystallization events were observed between the olein and the control, whereas greater differences were observed for the stearin in comparison with the control. The main differences among the AMF are a) higher SCFA and MCFA in the olein, b) higher amount of UFA in the control, possibly explained by the different origins of the AMF, and c) higher LCFA in the stearin, replacing mainly UFA and SCFA, in comparison with the other AMF. The specific composition of fractionated AMF varies according to the crystallization intervals used during manufacture (deMan et al., 1980; Kaylegian et al., 1995), which suggests that the olein fraction was depleted only of some high-melting point TAG. Nevertheless, the overall variation between the olein and stearin AMF was consistent with what was expected (deMan et al., 1980; Kaylegian et al., 1995).

Table 13. Fatty acid composition of the olein, control, and stearin anhydrous milk fats.

	Fatty acids in AMF (molar %)			
Fatty acid	Olein	Control	Stearin	SEM
C4:0	10.88 [a]	10.15 [a]	6.94 [b]	0.31
C6:0	5.66 [a]	4.98 [b]	3.31 [c]	0.08
C8:0	2.69 [a]	2.28 [b]	1.52 [c]	0.03
C10:0	5.34 [a]	4.37 [b]	3.29 [c]	0.06
C12:0	6.63 [b]	4.41 [c]	7.15 [a]	0.09
C14:0	13.41 [b]	11.76 [c]	14.41 [a]	0.04
C15:0	1.19 [b]	1.02 [c]	1.49 [a]	<0.01
C16:0	28.25 [c]	30.67 [b]	35.17 [a]	0.10
C17:0	0.52 [c]	0.53 [b]	0.72 [a]	<0.01
C18:0	10.34 [b]	10.14 [c]	13.22 [a]	0.06
C18:1	13.70 [b]	17.70 [a]	12.21 [c]	0.05
C18:2	0.85 [b]	1.70 [a]	0.57 [c]	0.11
C18:3	0.54 [a]	0.27 [a,b]	0.00 [b]	0.08

[a–c] Different letters denote statistically different means within the rows.

5.5.2 Preparation of creams

The dispersion of the AMF in skim milk yielded creams with fat droplets having similar specific surface areas, surface-weighted median diameters, and volume-weighted median diameters (Table 14). In all cases, the fat droplets in AMF creams were smaller than those present in a cream containing native fat globules ($P < 0.001$) (Table 14). These results confirm the resemblance of the standardization creams with respect to their fat droplet size. No changes in the median values were observed from the preparation of the creams to their incorporation into the milk for the cheese-making process (results not shown).

Table 14. Characterization by laser diffraction of fat droplets in creams obtained by mild homogenization of olein, control, and stearin anhydrous milk fats in skim milk.

Milk fat in cream	Specific surface area	Surface-weighted diameter [1]	Volume-weighted diameter [1]
	m^2/g	$d_{(3,2)}$ in µm	$d_{(4,3)}$ in µm
Native [2]	2.18 [b]	2.76 [a]	3.77 [a]
Olein	2.83 [a]	2.13 [b]	2.51 [b]
Control	2.81 [a]	2.14 [b]	2.59 [b]
Stearin	2.81 [a]	2.13 [b]	2.72 [b]
SEM	0.06	0.05	0.12

[1] Median values are reported for surface- and volume-weighed diameters. [2] A native cream is included as a reference. [a,b] Different letters within columns indicate statistical differences.

5.5.3 Cheddar-type cheeses

5.5.3.1 Cheese composition

Cheese composition is presented in Table 15. In all cases, the experimental cheeses had a composition within the expected ranges for a regular full-fat Cheddar cheese (Health Canada, 2010a; Minister of Justice of Canada, 2014), although their compositions differed slightly (Table 15). Differences among the cheeses were found for moisture (AMF type, $P = 0.0062$), protein (AMF type, $P =$

0.0036), fat (AMF type, P = 0.0371, and calcium level, P = 0.0416), ash (AMF type × calcium level, P = 0.0049), and pH (AMF type × calcium level, P = 0.0017).

Table 15. Composition, as mass fraction, and pH of experimental cheeses prepared with different anhydrous milk fats and salted with NaCl (regular calcium level) or NaCl + $CaCl_2$ (high calcium level).

AMF	Ca level	Moisture	Protein	Fat	Ash	pH
		%	%	%	%	
Olein	Regular	35.5 [a,b]	23.5 [a,b]	33.1 [c]	3.60 [a]	5.45 [a]
	High	35.8 [a]	23.7 [a]	32.6 [c]	3.45 [b]	5.17 [c]
Control	Regular	34.9 [a,b,c]	22.1 [c]	33.7 [b,c]	3.61 [a]	5.44 [a]
	High	34.1 [c]	22.5 [b,c]	35.3 [b,c]	3.30 [c]	5.19 [c]
Stearin	Regular	34.9 [a,b,c]	22.0 [c]	33.7 [b,c]	3.51 [a,b]	5.43 [a]
	High	34.4 [b,c]	22.3 [c]	35.9 [b]	3.48 [a,b]	5.25 [b]
SEM		0.22	0.19	0.56	0.026	0.01

[a–c] Different superscript letters denote significant differences within means reported for each analysis.

The cheeses prepared with the olein AMF had a slightly higher moisture and protein contents than the cheeses prepared with the other AMF did. Nevertheless, the moisture-to-protein ratio of the cheeses was between 1.51 and 1.58, suggesting similar protein hydration levels. In a previous study, Cheddar cheeses were salted by adding $CaCl_2$ instead of replacing NaCl on an ionic-strength basis, and major variations in moisture-to-protein ratios were obtained (i.e., from 1.24 to 1.62) (Ayala-Bribiesca et al., 2016). Incorporating $CaCl_2$ by replacing NaCl was successful in obtaining cheeses with limited moisture-to-protein variations.

The olein AMF cheeses had the lowest fat content, although the variations were small and significant only in relation to the cheeses prepared with the stearin AMF with the high calcium level. Ash was more abundant in the cheeses with the regular-calcium level in comparison with the high-calcium cheeses, although that difference was not significant for the cheeses prepared with the stearin AMF.

Differences in ash content between the high- and regular-calcium cheeses could be explained by the different masses of salt added. A lower mass of $CaCl_2$ than of NaCl is required to obtain an equivalent ionic strength. Finally, the cheeses with $CaCl_2$ added during salting had lower pH values than those salted only with NaCl. As expected, this difference was due to the reaction of calcium with phosphate and citrate, freeing H^+ into the aqueous phase (Philippe et al., 2003).

5.5.3.2 Mineral profile

Salting conditions modified the mineral profiles of the experimental cheeses (Table 16) in terms of their calcium ($P = 0.0002$), potassium ($P = 0.0172$), and sodium ($P < 0.0001$) concentrations. No significant differences were detected for magnesium or phosphorous. The AMF types did not influence the mineral composition of the experimental cheeses. The calcium concentration in the regular-calcium cheeses was similar to the value (7,210 µg/g) reported for commercial Cheddar cheese (Health Canada, 2010a). The calcium concentration in the high-calcium cheeses increased 33% (by 2,330 µg/g in comparison with the regular-calcium cheeses) when $CaCl_2$ was added during the salting step. As expected, the sodium concentration decreased in the high-calcium cheeses in comparison with the regular-calcium cheeses owing to the lower amount of sodium added during the salting step. Higher potassium losses after the salting step were observed in the high-calcium cheeses in comparison with the regular-calcium cheeses. These losses occurred after the salts were added and during the pressing step. Potassium is present mainly as free ions, so it is more diffusible during the osmotic drainage of cheese than are magnesium and phosphorous, which are partly bound to proteins in colloidal associations (Gaucheron, 2005).

Table 16. Major minerals in experimental regular-calcium cheeses (salted with NaCl) and high-calcium cheeses (salted with NaCl + $CaCl_2$) (all anhydrous milk fat sources combined).

Salting conditions	Major minerals (µg/g)				
	Ca	K	Mg	Na	P
NaCl	7,136	387	280	5,867	3,863
NaCl + $CaCl_2$	9,466 ***	277 *	273 n.s.	2,881 ***	4,303 n.s.
SEM	68	16	3	39	218

*P < 0.05, ***P < 0.001, n.s. not significant. Selected minerals were determined by inductively coupled plasma–optical emission spectroscopy.

5.5.3.3 Cheese structure

The scanning electron micrographs revealed similar cheese microstructures for all the experimental cheeses (Figure 26).

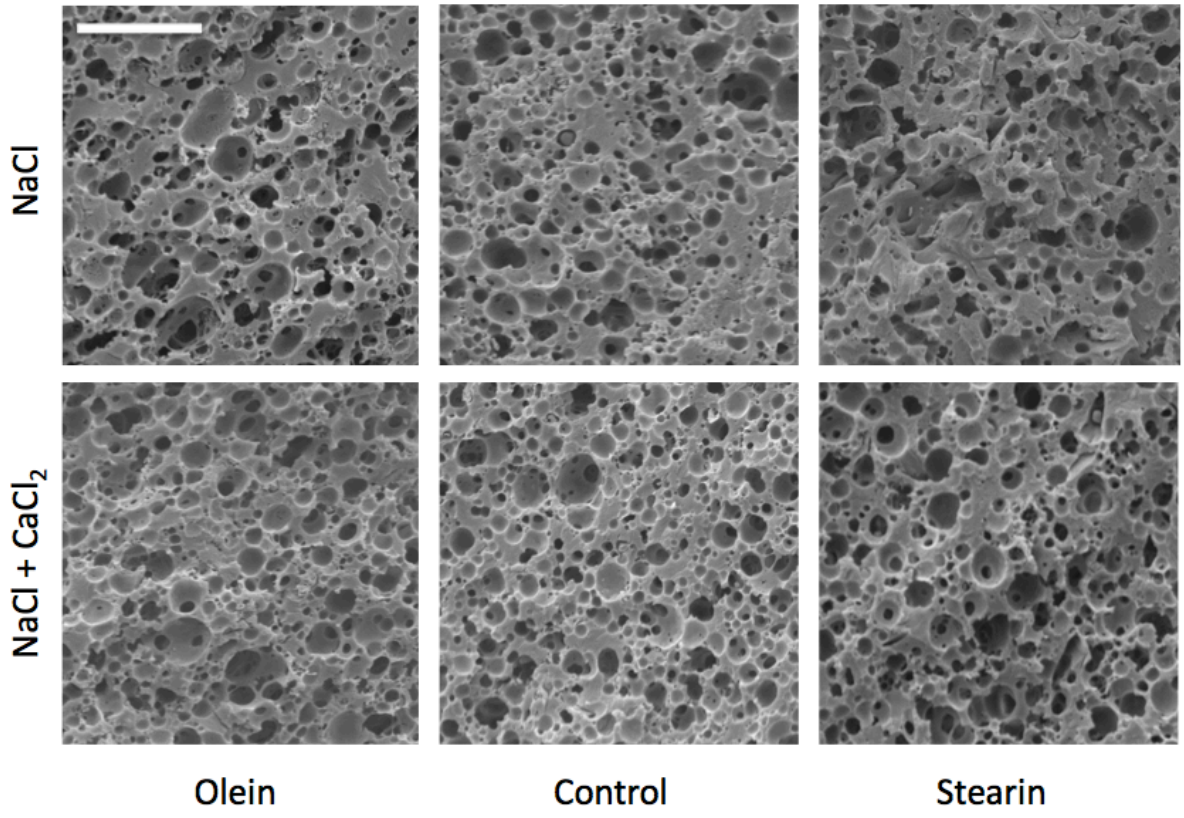

Figure 26. Scanning electron micrographs of Cheddar-type cheeses prepared with olein, control, or stearin anhydrous milk fats and with regular or high calcium levels for salting conditions with, respectively, NaCl or NaCl + $CaCl_2$. Voids represent the space once occupied by fat. Bar = 10 µm.

The cheeses presented similar features (Figure 26), with a continuous protein matrix embedded with small and abundant fat globules. The size of the fat droplets shown in the scanning electron microscopy images (Figure 26) are in accordance with the results reported for fat droplet size (Table 14). The fine fat dispersion is explained by the mild homogenization performed to disperse the AMF, in contrast to commercial or regular Cheddar cheeses, where the fat is present as native MFG. Furthermore, the distribution of the fat globules within the protein matrix was homogenous and coalescence was limited, as confirmed by the numerous individual fat droplets depicted in the confocal laser scanning microscopy images (Figure 27).

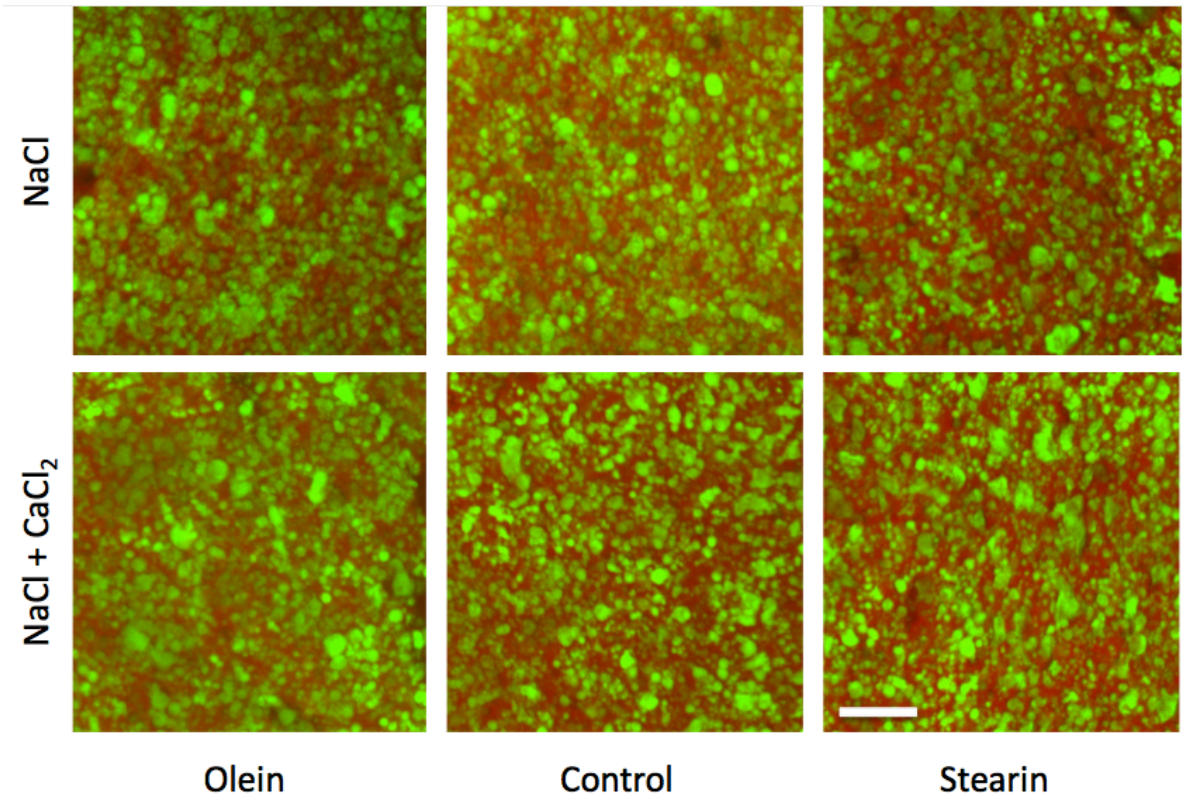

Figure 27. Confocal laser scanning microscopy images of Cheddar-type cheeses prepared with olein, control, or stearin anhydrous milk fats and with regular or high calcium levels for salting conditions with, respectively, NaCl or NaCl + $CaCl_2$. Green and red channels represent fat and protein, respectively. Overlapping channels show the distribution of fat within the protein matrix. Bar = 20 µm.

Cheese hardness varied according to the analysis temperature and the AMF type ($P = 0.0013$) used for the cheese preparation (Figure 28), but no effect was detected for the different calcium levels. At 22°C, the cheeses prepared with the stearin AMF were more than 50% harder ($P < 0.0001$) than those prepared with the olein and control AMF. This behavior is explained by the different melting profiles of the different AMF types (Figure 25b): the olein and control fractions have a large endothermic event before 20°C, indicating that an important part of their fat was liquid at the analysis temperature of 22°C. In contrast, the major endothermic event of the stearin fraction begins at 20°C, and therefore, most of its fat is still solid at 22°C. Analysis of hardness at 37°C did not reveal any significant differences among the cheeses, although the cheese samples with the stearin AMF retained their shape better than the other cheeses did. Indeed, the DSC thermograms (Figure 1b) show that the olein and control AMF were molten at 37°C, whereas the stearin AMF still had some solid fat at that temperature.

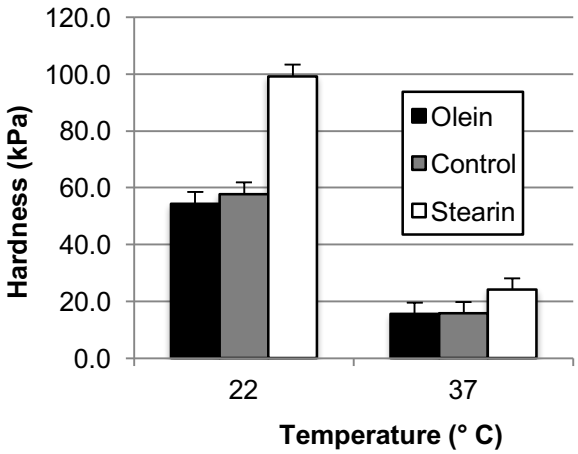

Figure 28. Hardness at 22 and 37°C of Cheddar-type cheeses prepared with olein, control, or stearin anhydrous milk fats. SEM = 0.32.

5.5.4 In vitro digestion

During in vitro digestion, the MDI varied over time differently for the AMF types ($P < 0.0001$), whereas no effect was detected for the different calcium levels. The MDI after the oral and gastric phases was consistently lower ($P < 0.0001$) for the

cheeses prepared with the stearin AMF than for the cheeses prepared with the other AMF types (Figure 29). With respect to the cheeses recovered in the mesh, those prepared with the stearin preserved their shape for longer during digestion (results not shown). Although no statistical difference was found for cheese hardness at 37°C, the DSC results indicate that part of the stearin AMF would be solid at the digestion temperature, a fact that could explain the greater resistance to disintegration of those cheeses. As the intestinal phase progressed, the gap separating the MDI of the cheeses prepared with the stearin AMF from the MDI of the other cheeses quickly decreased. The pancreatic enzymes helped complete the disintegration of all matrices within 1 h of the start of intestinal digestion (Figure 29). As previously suggested (Ayala-Bribiesca et al., 2016), the cheese matrix swells with fluid during gastric digestion, and thus pancreatic proteases can act more quickly when the intestinal fluids are added. The cheeses prepared with the olein and control AMF behaved the same way throughout digestion. The DSC results show that the thermal properties of the olein and control AMF were similar in comparison with those of the stearin AMF, further suggesting that AMF was the main factor involved in the different disintegration profiles, especially given that the overall composition and structure of the cheeses did not differ greatly. The results obtained for MDI agree with those previously obtained using the same in vitro system for Cheddar-type cheeses, where the matrices underwent only partial disintegration during the gastric phase and quickly accelerated their disintegration as the intestinal fluids were added (Ayala-Bribiesca et al., 2016; Lamothe et al., 2012).

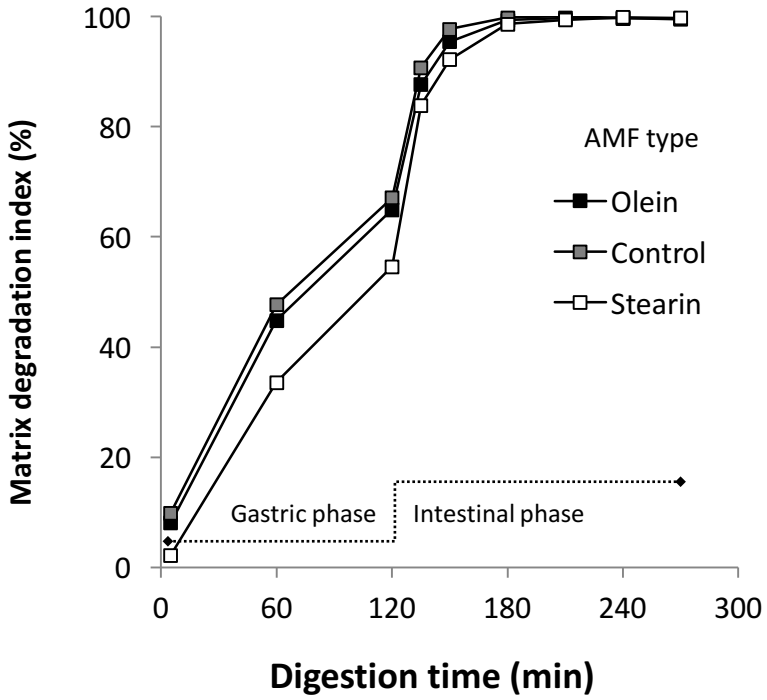

Figure 29. Matrix degradation index of Cheddar-type cheeses prepared with olein, control, or stearin anhydrous milk fats during in vitro digestion. SEM = 0.9.

Lipolysis during in vitro digestion of the Cheddar-type cheeses was affected by time, AMF type, and calcium level in a statistically significant triple interaction ($P < 0.0001$). Lipolysis during the oral and gastric phases was considered negligible, given that no lipase was added in the simulated fluids for those phases. Small amounts of NEFA (in statistically equivalent amounts for all the cheeses) were detected before the addition of the simulated intestinal fluids. Although lipolysis is low in young Cheddar cheeses made from pasteurized milk (Collins et al., 2004; Hickey et al., 2007), the presence of NEFA in the experimental cheeses can be explained by the occurrence of some lipolysis during the dispersion of the AMF in raw milk before the mild homogenization treatment. The lipases naturally present in raw milk (Deeth et al., 1995; O'Mahony et al., 2013) came in contact with the exposed AMF droplets (i.e., with no protective MFGM), allowing the enzymes to access the TAG (Armand, 2008; Favé et al., 2004) before being denatured by pasteurization (Keenan et al., 1995b).

As expected, lipolysis increased sharply after the intestinal fluids were added (Figure 30). After 15 min of intestinal digestion, lipolysis was slower for the cheeses prepared with the stearin AMF, regardless of the calcium level, than for the other cheeses ($P \leq 0.0006$). In contrast, the cheeses with the olein AMF and the high calcium level had the highest extent of lipolysis during the first 30 min of intestinal digestion ($P < 0.0001$).

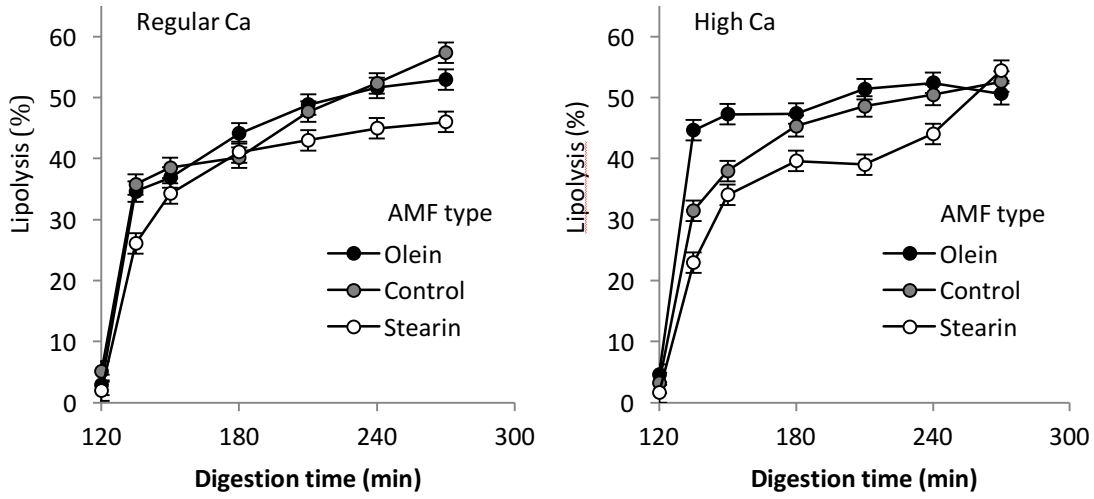

Figure 30. Progression of lipolysis (% as the mass fraction of non-esterified fatty acids in relation to the anhydrous milk fat) during the in vitro digestion of Cheddar-type cheeses prepared with olein, control, or stearin AMF and with regular or high calcium levels for cheeses salted with, respectively, NaCl or NaCl + $CaCl_2$. Bars indicate SEM.

The faster lipolysis rates observed for the olein and control AMF during the first hour of intestinal digestion appear to be due to the higher SCFA and MCFA contents in those AMF in comparison with the stearin AMF. SCFA are almost completely positioned in the sn_1 and sn_3 positions of the TAG (Christie, 1995; Michalski, 2008). This makes them preferentially accessible to cleavage by digestive lipases, which are specific to those positions (Armand, 2008). Furthermore, SCFA and MCFA are relatively water-soluble under intestinal pH conditions (Bugaut, 1987; Marten et al., 2006), so they do not accumulate at the lipid/water interface like LCFA (Armand, 2008). Lipase can thus access its substrate more easily and rapidly, in addition to the fact that TAG containing such

FA partition preferentially onto the fat droplet surface (Favé et al., 2004). As well, MCFA are released by pancreatic lipase faster than LCFA are (Mu et al., 2005). In sum, the higher amount of shorter FA (which are more easily cleaved by lipases and have higher water solubility in comparison with longer FA) appeared to have allowed lipolysis to be higher and faster for the control and olein AMF than for the stearin AMF.

The control AMF contained considerably more oleic acid (C18:1) and less MCFA than the olein AMF did; these differences could explain the lower lipolysis at the beginning of the intestinal phase. Oleic acid is present mainly in the sn_{1-} and sn_{3-} positions of milk fat (Christie, 1995), which were more abundantly occupied by SCFA and MCFA in the olein AMF. The fact that oleic acid is not water-soluble, in contrast to SCFA and MCFA, would explain the delayed lipolysis of the control AMF in comparison with the olein AMF. The faster lipolysis of the control AMF in comparison with that of the stearin AMF appears to be due to the physical state of the fat. Lipolysis of solid fat has been shown to be slower than that of liquid fat (Berry et al., 2007; Bonnaire et al., 2008), because the digestive lipases have less access to solid fat (Michalski et al., 2013).

The cheeses with the stearin AMF had considerably more LCFA, which are more prone to remain in the fat droplet because of their longer aliphatic moiety and lower water affinity.

Towards the end of digestion, lipolysis of the stearin AMF increased considerably for the cheese with a high-calcium level and the stearin AMF ($P = 0.0007$) in comparison with the regular-calcium cheese with the same AMF. This clearly shows the effect that calcium has on FA release by depleting LCFA from the lipid/water interface and thus allowing further lipolysis (Patton et al., 1979; Sassene et al., 2014). At the end of digestion, the final lipolysis rates were similar for all conditions (Figure 30) except the cheese with the stearin AMF and the regular calcium level ($P \leq 0.007$), which had the only final rate under 50%. Therefore, the overall combined effect of AMF type, calcium level and time explain the triple interaction of these three parameters on lipolysis.

During the in vitro digestions, the pH varied over time differently for the AMF types ($P < 0.0001$). Although the pH of chymes (Figure 31) was influenced mainly by the intrinsic conditions provided by the digestion fluids, the cheeses prepared with olein caused a slightly higher buffering effect ($P = 0.008$) towards the end of gastric digestion than the other cheeses did, because of their overall faster disintegration. During the intestinal phase, the pH of the chymes from the cheeses prepared with the stearin AMF was higher than the pH of the other cheeses ($P < 0.003$) (Figure 31), possibly owing to slower lipolysis, which provided less H^+ as free FA were released (Li et al., 2011). Lastly, the effect of the salting conditions ($P = 0.0015$) on the pH of chymes was that the cheeses with the high-calcium level had a lower pH, with an overall difference of 0.07 pH units (data not shown). That difference could be explained by the lipolysis-enhancing effect of calcium (Hu et al., 2010).

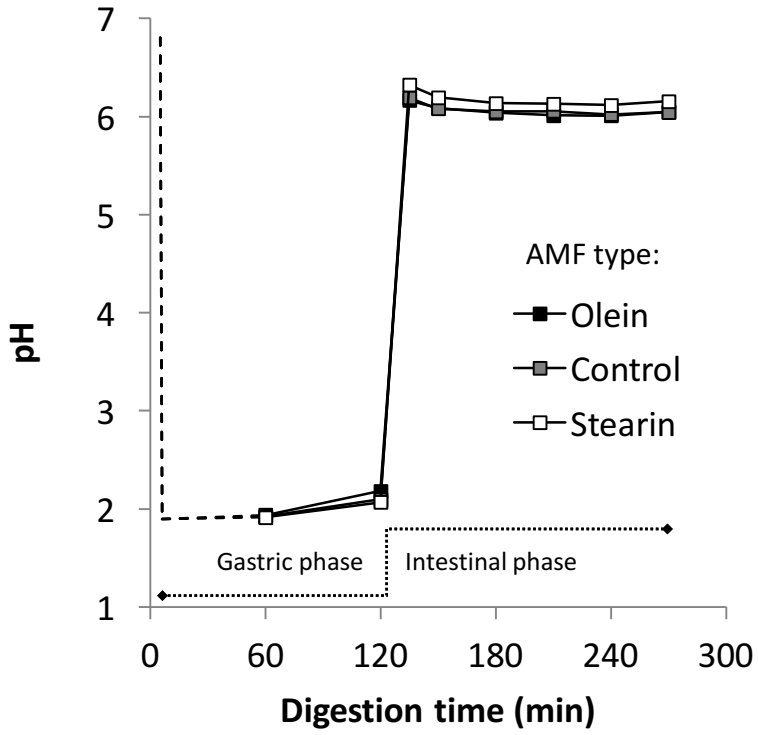

Figure 31. Change in pH during the in vitro digestion of Cheddar-type cheeses prepared with olein, control, or stearin anhydrous milk fats (AMF). The dotted line indicates the expected pH before the first sampling. SEM = 0.02.

5.5.4.1 Fatty acid bioaccessibility

The overall bioaccessibility of FA varied according to the AMF type and the calcium level of the experimental cheeses (P = 0.0096). When regular-calcium levels were present, the olein and control AMF had equivalent overall bioaccessibility, whereas the stearin AMF had a lower value (Table 17). The overall bioaccessibility values were in agreement with the final lipolysis rates obtained at the end of the in vitro digestion of the respective cheeses (Figure 30, regular Ca). The calcium enrichment of the cheeses had different effects on overall bioaccessibility depending on the AMF present. As expected, the high-calcium level in the cheeses

prepared with the stearin AMF enhanced lipolysis and thus induced an increase in overall bioaccessibility. This would be explained by the removal of fatty acids from the surface of fat droplets (as CS), so that lipase could access more substrate. High calcium had no effect on the cheeses with the olein AMF, possibly because of their higher content of SCFA, which can easily be released into the aqueous phase of the chyme. Surprisingly, the calcium enrichment of cheeses with the control AMF slightly reduced their overall bioaccessibility in comparison with their regular-calcium counterparts. Nevertheless, the overall FA bioaccessibility of all the cheeses with the high calcium level was equivalent, and the values are in agreement with the results obtained for the final lipolysis rates (Figure 30, high Ca).

Table 17. Overall bioaccessibility of fatty acids after in vitro digestion of experimental cheeses prepared with different anhydrous milk fat (AMF) types.

AMF	Ca level	Overall bioaccessibility *
		%
Olein	Regular	56.8 [a]
	High	55.7 [a,b]
Control	Regular	57.5 [a]
	High	55.5 [b]
Stearin	Regular	48.6 [c]
	High	52.4 [b]
SEM		0.9

* Overall bioaccessibility was defined as the mass fraction (%) of fatty acid released from triacylglycerols during digestion in relation to the total amount of fatty acid present in the respective AMF. [a–c] Different letters indicate significant differences.

Overall bioaccessibility was broken down for the individual FA (i.e. defined as the mass fraction (%) of FA released from TAG during digestion in relation to the total amount of the corresponding FA present in the respective AMF). On such basis, AMF type influenced saturated LCFA bioaccessibility ($P \leq 0.0002$) (Figure 32). Additionally, an AMF type × calcium level interaction was found for the bioaccessibility of SCFA ($P \leq 0.0314$), MCFA ($P \leq 0.0096$), and oleic acid ($P = 0.0002$) (Figure 33).

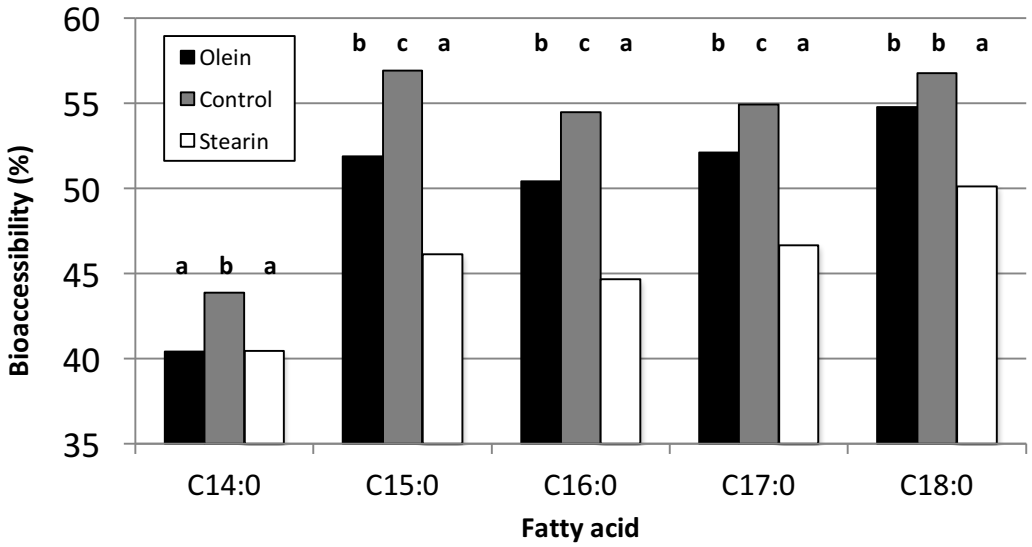

Figure 32. Bioaccessibility of saturated long-chain fatty acids after the in vitro digestion of Cheddar-type cheeses prepared with different AMF types. Different letters indicate significant differences within bar groups. SEM ≤ 0.7 for all FA.

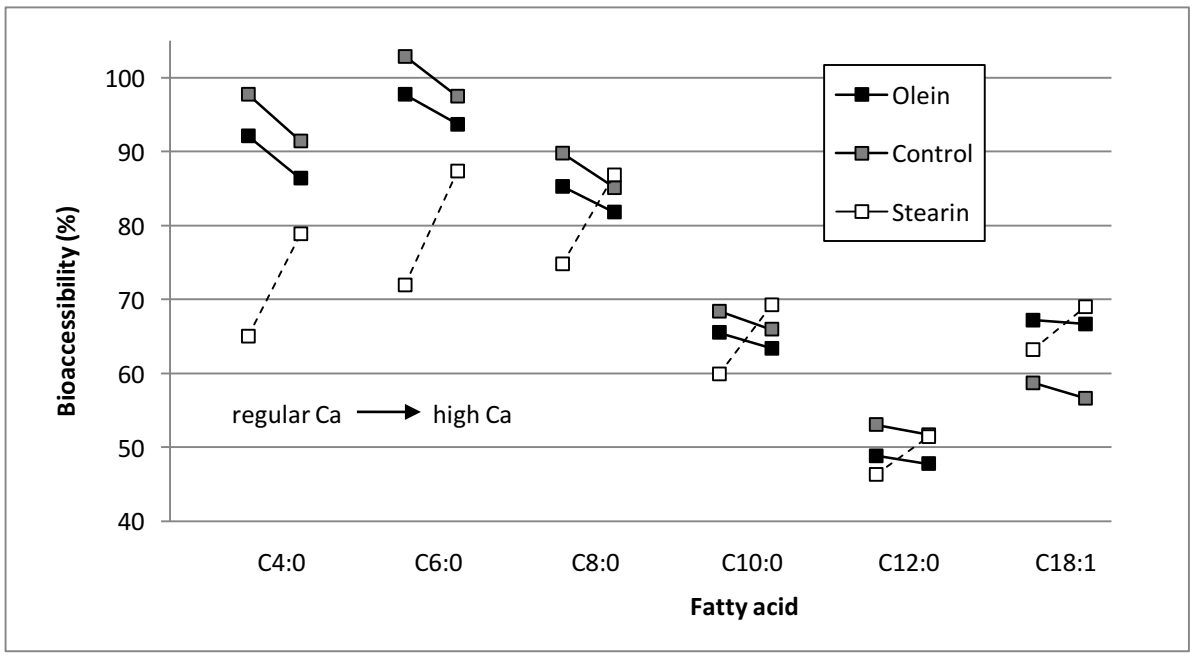

Figure 33. Bioaccessibility of short-chain, medium-chain, and unsaturated fatty acids after the in vitro digestion of Cheddar-type cheeses prepared with different calcium levels and different AMF types. Dashed connectors indicate significant differences between regular and high calcium levels (left and right markers, respectively), and solid lines indicate statistically equivalent means. SEM = 3.9, 3.0, 2.3, 1.7, 1.0, and 1.4 for C4:0, C6:0, C8:0, C10:0, C12:0, and C18:1, respectively.

The calcium level did not appear to have a significant effect on LCFA bioaccessibility, a somewhat surprising result given that higher release rates of LCFA were expected in the presence of calcium. One possibility is that the available calcium in the chyme was depleted as LCFA soaps. Then, the calcium-induced lipolytic enhancement of the stearin AMF was reflected in higher release rates of SCFA, MCFA, and oleic acid, when comparing the high calcium to the regular calcium level (Figure 33). No significant effect for calcium level was observed for any FA of the other AMF types. It should be remembered that bioaccessibility was defined in this study as the amount of individual FA released during in vitro digestion (or the sum of the amounts of all the FA, when referring to overall bioaccessibility); that amount is related to the extent of lipolysis and therefore does not account for FA residues that are absorbable as sn_2-monoacylglycerols or for the loss of bioaccessibility due to the formation of CS. For these reasons, the exact amount of bioaccessible FA is uncertain, because the acidic conditions during fat extraction dissociated the CS formed during the in vitro digestion of the cheeses.

The main difference in the overall behavior of the stearin AMF with respect to the other AMF types was most likely related to the physical state of the fat. The stearin AMF had solid portions of fat at 37°C, which slowed down the lipolysis process overall (Lopez et al., 2013), as observed for the progression of lipolysis during in vitro digestion. The stearin AMF was also the richest in LCFA in comparison with the other AMF, and thus the calcium that was present formed soaps. The formation of calcium soaps enabled enzyme access to other FA, as reflected by significant increases in SCFA, MCFA, and oleic acid (Figure 33). Although the results did not show a significant effect of calcium level on the bioaccessibility of individual LCFA, the effect on total fatty acids was significant.

Differences in FA bioaccessibility among the AMF could also be related to oleic acid (C18:1). Oleic acid is the second most abundant FA in milk fat (after palmitic acid, C16:0) and it is almost completely esterified in the sn_1 and sn_3 positions (Jensen et al., 1995; Kaylegian et al., 1995; Michalski, 2008). The fact that high

lipolysis rates were reached during the in vitro digestions suggests that most of the oleic acid was released. Oleic acid is less prone to precipitate with calcium in comparison with saturated LCFA such as myristic (C14:0), palmitic (C16:0), and stearic (C18:0) acid (Björklund-Jansson et al., 2005; Garrett, 2013). Nevertheless, oleic acid is highly hydrophobic, so it would probably accumulate at the interface.

5.5.4.2 Calcium enrichment and fatty acid solubility.

Neither the AMF types nor the calcium level caused any differences in the loss ratios between each extraction pair for any FA. As previously mentioned, the lipids lost during extractions at pH 7 include not only NEFA bound as CS but also dissociated water-soluble FA, whose water solubility increases as the chain length decreases. The GC analysis failed to detect any NEFA from C4:0 to C8:0 in the extracts obtained from chymes adjusted at pH 7, indicating that none of those FA partitioned to the organic phase at that pH (Table 18). At the same time, partitioning into the supernatant organic phase increased proportionally with the chain length for the other saturated.

Table 18. Differences in fatty acid extraction rates (reported as loss ratios) between solvent extraction from chymes adjusted at pH 7 and 2.

Fatty acid	Loss ratio * pH 7:pH 2	SEM
C4:0	1.000	-
C6:0	1.000	-
C8:0	1.000	-
C10:0	0.912	0.006
C12:0	0.794	0.011
C14:0	0.584	0.019
C15:0	0.444	0.023
C16:0	0.292	0.029
C17:0	0.153	0.032
C18:0	0.060	0.041
C18:1	0.086	0.047
Total FA	0.363	0.029

* Loss ratios represent the ratio of fatty acids remaining in the aqueous phase after the solvent extraction of chymes at pH 7, in relation to the same fatty acid recovered under extraction conditions at pH 2. When available, SEM obtained from the statistical model are reported.

These results show that the proportion of FA that are soluble at pH 7 does not depend on their relative amount with respect to the other FA. This finding appears to be in accordance with the Henderson–Hasselbalch equation, for a given pH, because the common ion among FA is H^+, which determines the amounts of dissociated and protonated FA according to their specific pKa. Hence, protonated FA would transfer into the organic phase, whereas the rest would remain soluble in the aqueous phase or as insoluble CS.

Equation 7. Henderson-Hasselbach equation.

$$\mathrm{pH} = \mathrm{pKa} + \log_{10}\left(\frac{[A^-]}{[HA]}\right)$$

The extraction pairs included 2 pH conditions for chymes obtained at the end of the in vitro digestion of each experimental cheese. Under acidic conditions (pH 2), all the lipids present in the chyme were extracted into the solvents, whereas

conditions close to neutrality (pH 7) would exclude water-soluble FA (e.g., dissociated SCFA and MCFA) and CS, which are insoluble in both water and the solvents used. Mass losses between the extraction pairs represented around 1/3 of the total fat from the cheese added to the digestion tubes. No significant differences were found among the experimental cheeses, although the results do suggest a tendency towards higher mass losses when high-calcium levels were present, as opposed to regular-calcium levels (Table 19). Consequently, the effect of calcium enrichment translated into an overall increment in CS, with Δ_{CS} values suggesting higher sensitivity to calcium increase in the stearin AMF than in the other AMF types (Table 19).

Table 19. Mass fractions of fat lost (ML) between extraction pairs performed at pH 2 and 7 and increment in calcium soaps due to calcium enrichment (Δ_{CS}) in chyme juices obtained after 270 min of in vitro digestion of Cheddar-type cheeses prepared with different anhydrous milk fat types and with a regular- or a high-calcium level.

AMF type	Ca level	ML	Δ_{CS}
		mg/g	mg/g
Olein	Regular	340	41
	High	381	
Control	Regular	367	14
	High	382	
Stearin	Regular	319	94
	High	413	
SEM		30	-

For the cheeses prepared with the stearin AMF, the Δ_{CS} was close to the expected values, considering the calcium increment between the 2 calcium levels used (i.e. ≈ 7 mg or 0.175 mmol, conveyed by 3 g of cheese in the digestion tube, potentially binding, for example, up to 89.6 mg of palmitic acid, at a 1:2 stoichiometric ratio). However, this was not the case for the Δ_{CS} values for the other cheeses. No statistically significant differences for mass losses were detected. Mass losses of water-soluble FA were probably confounded with those of insoluble CS, limiting the sensitivity of the extraction technique to quantify the latter.

A noteworthy observation is that during the extractions there was the presence of a white layer between the solvent supernatant and the aqueous phase, strongly suggesting the presence of CS. Although CS are insoluble in the solvents used, early research on the solubility of CS (Harrison, 1924) reported that they are easily dispersible in diethyl ether, which was one of the organic components. During the extractions at pH 7, shaking would have dispersed CS preferentially into the organic phase, which then congregated into a layer over the aqueous phase during centrifugation (Figure 34). That white layer was absent when the extractions took place at an acidic pH, where the CS are dissociated and where protonated FA solubilize into the solvent. Further tests in an ongoing study with chymes obtained after the in vitro digestion of calcium-free model dairy matrices did not produce such a white layer, even when extraction was performed at pH 7 (results not shown); that finding supports the idea that the layer is composed mainly of CS.

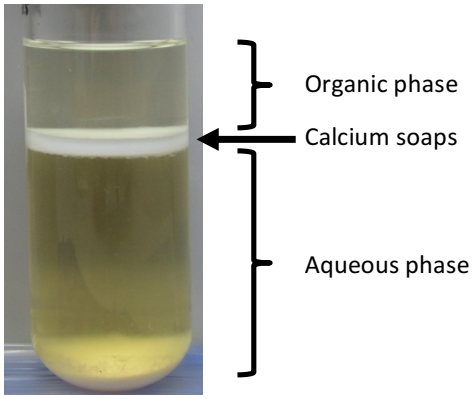

Figure 34. Typical layering of chymes during solvent extractions at pH 7 (after centrifugation). The arrow points to the white layer of calcium soaps.

5.6 Conclusion

The in vitro digestion of Cheddar-type cheeses prepared with different AMF types and calcium levels led to different disintegration and lipolysis profiles. The most distinctive factor among the cheeses was the incorporation of a stearin AMF, with a high proportion of solid fat at digestion temperatures. Such solid fat is believed to be the main reason for the observed variations in cheese texture and the progression of digestion, given that the cheeses with the stearin AMF were more resistant to disintegration. The calcium level, in turn, did not seem to modify cheese characteristics and did not affect cheese disintegration during digestion but did have an impact on lipolysis during in vitro digestion. That impact was related to the depletion of LCFA from the lipid/water interface, which allowed the progression of lipolysis. Towards the end of the digestions, lipolysis extent was similar for all the cheeses except those with the regular calcium level and the stearin AMF. During the digestion of the latter cheeses, the low lipolysis rate was due to the higher proportion of LCFA, resulting in exacerbated accumulation of lipolysis products. Calcium enrichment was found to counteract such accumulation, apparently by binding LCFA to form CS and resulting in higher SCFA, MCFA, and oleic acid release rates. Nevertheless, the bioaccessibility of FA from the cheeses prepared with the stearin AMF was lower than that of the cheeses with the control or the olein AMF. This study confirms that calcium can play a modulating role in FA bioaccessibility and that such a role also depends on the FA profile of the fat. Studies on the interaction of calcium with FA under in vitro and in vivo digestive conditions are currently underway to provide a better understanding of the effect of calcium on free FA bioaccessibility. In parallel, another study is underway to develop a more sensitive technique to quantify CS. In the near future, this project will enable the development of dairy matrices that, after ingestion, can modify metabolic responses and serve as effective carriers for bioactive molecules and for specifically tailored nutrition.

5.7 Acknowledgements

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Chapter 6. Postprandial lipemia and fecal fat excretion in rats is affected by the calcium content and type of milk fat present in Cheddar-type cheeses

This chapter is presented as a research paper presenting the continuation of the in vitro study presented in the previous chapter. The same model cheeses were used to perform an oral lipid tolerance test using the rat as a model. The paper presents the metabolic responses obtained during postprandial lipemia and confirms the loss of some fatty acids, namely as calcium soaps, through feces. The research paper has been submitted for publication. The role of the coauthors is as follows: Erik Ayala Bribiesca is the main author and did the experimental design, laboratory experiments (except blood sampling and necropsies), statistical analysis of the data, and writing of the paper. Geneviève Pilon and André Marette participated in the design and supervision of the in vivo protocol (Project No. 2011-091-1, Sirul 93470, approved by the Research Ethics Committee of Université Laval). Sylvie Turgeon and Michel Britten supervised the project and assisted in proofing the manuscript. All coauthors authorized the inclusion of the paper in this thesis.

6.1 Résumé

Cette étude visait à mieux comprendre l'effet du calcium sur la biodisponibilité, chez le rat, des lipides laitiers à partir du fromage. Des fromages de type cheddar ont été fabriqués avec des huiles de beurre contrôle, oléine ou stéarine et salés avec ou sans $CaCl_2$. La lipémie postprandiale des rats a été suivie après l'ingestion du fromage et les savons de calcium ont été quantifiés dans les matières fécales. Les fromages enrichis en calcium ont provoqué un pic de triglycérides plus élevé et plus rapide que leurs témoins, à l'exception des fromages avec stéarine. Parallèlement, des savons de calcium étaient plus abondants dans les fèces des rats ayant ingéré les fromages enrichis en calcium, en particulier celui avec stéarine. Cette biodisponibilité réduite s'explique par l'affinité des acides gras saturés à longue chaîne pour le calcium. Ces résultats montrent que la biodisponibilité des lipides peut être régulée par le calcium et par le profil d'acides gras présents dans le fromage cheddar.

6.2 Abstract

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

6.3 Introduction

When food enters the body, nutrients must be released from the food matrix and broken down into an absorbable form. The food matrix acts as a nutrient-release regulator as it disintegrates during the digestion process, which has an impact on the effectiveness of nutrient absorption (Norton et al., 2015). Since processing modifies the structure and composition of foods, it can affect their digestibility and, therefore, nutrient bioavailability (McClements et al., 2009a; McClements et al., 2009b; Parada et al., 2007).

Dairy foods have been extensively studied for their nutritional quality and, more recently, for their versatility, which makes them suitable for use in designing food products with enhanced health advantages such as bioactive molecule transport, regulated nutrient release and overall health maintenance (Fruekilde et al., 2004; Jaejoon et al., 2011; Turgeon et al., 2011). Among dairy foods, cheese has received special attention because of its widespread consumption. Interestingly, cheese also represents the main type of solid and hard dairy products, as opposed to fluid or semi-solid products such as beverages and yogurt. Cheese is recognized as a nutrient-dense food and, from a technological viewpoint, it is a highly customizable matrix. The structure, composition and sensory properties of cheese can be modified to enhance its technological and nutritional functionality, particularly for lipids and lipid soluble molecules (Banville et al., 2000; Michalski et al., 2004; Michalski et al., 2007; Stratulat et al., 2014). Interest on the health effects of lipids, especially milk fat, has led to a wide array of in vivo and clinical studies on their metabolic fate of lipids as determined by their physicochemical and structural characteristics in foods (Clemente et al., 2003; Fruekilde et al., 2004; Michalski et al., 2005; Phan et al., 1999; Vors et al., 2013). Since cheese can be designed to carry fat with different characteristics, it is an excellent model for studying the digestion of solid matrices and the impact of their structure and composition on lipid bioavailability.

Cheddar cheese is recognized as a source of calcium, which is intrinsically present in milk and is also added during cheese manufacture in the form of $CaCl_2$ (Lawrence et al., 2004; Wolfschoon-Pombo, 1997). Calcium plays a key role in determining cheese texture (Lucey et al., 1993; Ong et al., 2013; Ong et al., 2015), but it also plays a major nutritional role in the digestion of milk fat. In the digestive system, calcium enhances lipolysis (i.e. release of fatty acids (FA) from triacylglycerols (TAG)), but limits the absorption of saturated long-chain fatty acids (LCFA). When lipolysis takes place, LCFA accumulate at the lipid droplet surface, which limits lipase access to its substrates (Favé et al., 2004). At intestinal pH (i.e. close to neutrality), LCFA react with calcium, producing insoluble salts, normally referred to as calcium soaps (CS) (Patton et al., 1985). The CS are readily removed from the interface, which enables lipolysis to continue on the newly exposed triglycerides of the fat droplet (Patton et al., 1979). This precipitation drives the increase in lipolysis rates (Armand et al., 1992), which in turn it results in a reduced absorption of LCFA bound to calcium (Hu et al., 2010; Karupaiah et al., 2007; Lorenzen et al., 2014). The balance between these effects determines the net impact on lipid bioavailability.

It is generally accepted that CS have considerably lower bioaccessibility than the respective fatty acids (Boyd et al., 1932; Gacs et al., 1977; Sheikh et al., 1987), but not all of them are lost into feces. Owing to the acidic pH, CS do not form during gastric digestion, and both calcium and FA can be absorbed when they pass into the duodenum (Guéguen, 1992). As the chyme continues through the small intestine, lipolysis produces new FA that can form CS as pH increases. Some CS are absorbed because they can co-solubilize with fatty acids within the mixed micelles and thus reach the enterocyte boundary for absorption (Jandacek, 1991). The overall process is less efficient than that for the corresponding free FA (Graham et al., 1983), and results in some CS being lost through feces. In humans, diet supplementation with calcium has shown to increase fecal excretion of fat in the form of CS, but such excretion varied with the source of calcium used, with dairy-sourced calcium leading to higher excretion rates (Bendsen et al., 2008; Lorenzen et al., 2011; Soerensen et al., 2014).

Postprandial lipemia is a complex and highly dynamic process that depends on various physiological, genetic and dietary factors that shape the digestion, absorption and metabolic handling of lipids (Armand, 2008; Berry, 2009). The content, composition and structure of fat in a meal are major determinants of postprandial lipemia (Berry, 2009). The gradual absorption of FA from CS defers their incorporation into chylomicrons, in comparison with free FA, which can translate into a less abrupt lipemic response. The reduction of the postprandial lipemic peak may influence the metabolism of fatty acids and have a beneficial effect on hyperlipemia and reduce cardiovascular complications in the long term (Lefevre et al., 2004; Michalski, 2008; Raynal-Ljutovac et al., 2011; Su et al., 2009).

Previous studies on in vitro digestion of cheese have shown that different structures lead to different behaviour during digestion, demonstrating the potential role of the cheese matrix as a lipid-release modulator (Ayala-Bribiesca et al., 2016; Lamothe et al., 2012). Additionally, Cheddar-type cheeses with different levels of calcium and different anhydrous milk fats (AMF) (i.e. control, olein or stearin, the last two respectively with lower and higher amounts of LCFA than the control), showed different profiles of lipid bioaccessibility during in vitro digestion (Ayala-Bribiesca et al., 2017). In this study, cheeses prepared with olein AMF and enriched with calcium showed a 30% increase in fatty acid bioaccessibility at the beginning of the intestinal digestion, in relation to the regular calcium control. Final lipolysis rates were higher for cheeses prepared with the control AMF and the olein AMF than for those prepared with the stearin AMF. Nevertheless, cheeses prepared with the stearin-AMF reached the same final lipolysis levels as the other cheeses when they were enriched with calcium (Ayala-Bribiesca et al., 2017). This levelling of final lipolysis rates can be explained mainly in terms of calcium soap formation when high calcium levels and high amounts of saturated LCFA were present. In vivo studies are required to confirm the physiological repercussions of the interaction between calcium and different fatty acids observed in vitro.

Bioavailability in vivo is mainly estimated by balance studies, which compare the ingested and the excreted amount of a nutrient. However, the spatial, temporal and physiological interactions of the digestive, secretive and absorptive processes that take place in living organisms are often treated as a black box (Jandacek et al., 2004; van Aken, 2010). In vitro approaches are useful for elucidating the mechanisms governing food digestion even if they fail to fully recreate and control all the processes occurring in a living organism (Minekus et al., 2014). Hence, conclusions derived from in vitro tests are often limited to nutrient bioaccessibility. For this reason, in vivo models are required to better assess the metabolic effect of foods, as well as to validate the relevance of in vitro models. After such a validation, data obtained through in vitro models can be used to screen and target experimental conditions before moving back to in vivo models (Failla et al., 2005; Golding et al., 2010).

The aim of this study was to better understand the effect of calcium on bioavailability of milk fat from a cheese matrix using the rat model. To achieve this, Wistar rats were fed experimental Cheddar-type cheeses prepared with different AMF fractions and enriched or not with calcium. The main objective was to assess the effect of calcium on postprandial lipemia caused by the consumption of Cheddar-type cheeses prepared with a control AMF and olein and stearin AMF fractions. Simultaneously, CS excreted in the feces were quantified to assess the extent of interaction of calcium with FA from the different AMF fractions. Finally, this work was also intended to evaluate the capability of a relatively simple in vitro digestion system used in previous studies (Ayala-Bribiesca et al., 2017) to predicting lipid-related physiological responses.

6.4 Materials and methods

6.4.1 Cheddar-type cheeses

The cheeses were the same as those used in a previous in vitro study, in which detailed information on their manufacture, composition and microstructure is provided (Ayala-Bribiesca et al., 2017). Briefly, Cheddar-type cheeses with different fatty acid profiles were obtained by standardizing their fat content with three different AMF (control, olein and stearin, with final melting points of 35.43, 34.70 and 42.32°C, respectively). Olein AMF had the highest ratio of SCFA and MCFA, the control AMF had the highest amount of UFA and the stearin AMF was the richest in saturated LCFA (Figure 35).

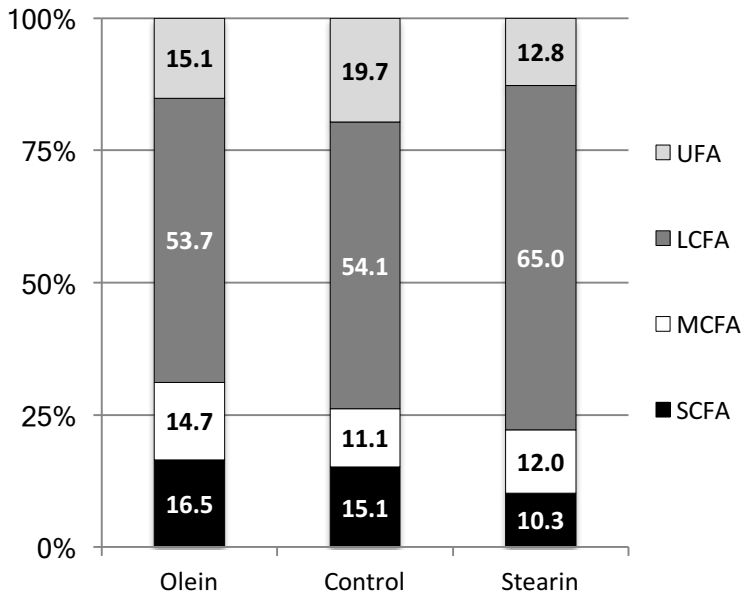

Figure 35. Fatty acid subclass composition (molar %) of different anhydrous milk fats (AMF) used to prepare the Cheddar-type cheeses. Calculated from data presented by Ayala-Bribiesca et al. (2017). Subclasses UFA, LCFA, MCFA and SCFA stand, respectively, for unsaturated fatty acids (C18:1, C18:2 and C18:3), long-chain fatty acids (C18:0 to C14:0), medium-chain fatty acids (C12:0 to C8:0) and short-chain fatty acids (C4:0 and C6:0). SEM < 0.1% for all subclasses.

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

All the Cheddar-type cheeses had a composition within the expected ranges for a regular full-fat Cheddar cheese (Health Canada, 2010a; Minister of Justice of Canada, 2014), except for the high calcium content of cheeses that included $CaCl_2$ in the salting step (Table 20). Composition differences among cheeses were within the expected variations for the pilot equipment used.

Table 20. Experimental conditions used for the different Cheddar-type cheeses and their moisture, fat and calcium content.

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

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

6.4.2 In vivo protocol

The in vivo protocol (Project No. 2011-091-1, Sirul 93470, approved by the Research Ethics Committee of Université Laval) was carried out using 72 young adult male Wistar rats (10 weeks old, Strain code 003, Charles Rivers Laboratories, Montreal, Canada). Upon arrival, the animals were acclimated for 10 days on an ad libitum diet of commercial chow and free access to water. After the

acclimation, rats were randomly assigned to each experimental cheese (n = 12) and were then trained to rapidly ingest the cheese meal. As shown in Table 21, a total of 5 rats were excluded during the study due to refusal to ingest the cheese meal (2 rats) or incomplete results during the postprandial follow-up (3 rats).

Table 21. Experimental cheeses and the respective number of rats that completed the in vivo protocol.

AMF in cheese	Salting conditions	n
Olein	NaCl	11
	$NaCl + CaCl_2$	11
Control	NaCl	10
	$NaCl + CaCl_2$	12
Stearin	NaCl	11
	$NaCl + CaCl_2$	12

Training was done in two stages (Figure 36). During the first stage (i.e. days 1 thru 4), an 11.5-hour fasting period was followed by a 10-g meal of experimental cheese. Leftovers were removed 30 min later and weighed. Food removal coincided with the end of the 12 h dark cycle set. A second 10-g cheese meal was presented 7 h later, followed by ad libitum access to chow after 30 min. Access to chow and any cheese leftovers were removed after 4.5 h, to restart the fasting period. This cycle was repeated for a total of 4 days. During the second stage (i.e. days 5 thru 9) the cheese meals followed the same sequence as in the first stage. The second stage differed from the first one in that when the second cheese meal was removed, only a 10-g portion of chow[15] was left in the cage and no additional food was given to the rats until the next day.

[15] A small amount of chow was given to the rats to avoid the risk of constipation and to prevent any nutritional deficiencies. Preliminary tests showed that rats ate the chow before the fasting period began, so it was not necessary to remove any leftovers from the cages.

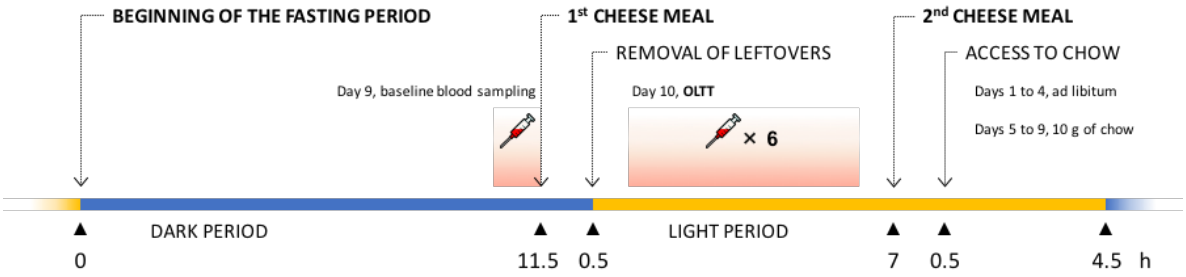

Figure 36. Training protocol for the ingestion of cheese meals prior to the oral lipid-tolerance test (OLTT).

Water was always accessible. Rats were weighed daily to ensure proper weight gain. Towards the end of the training sequence, the rats usually consumed all the cheese meals within 15 min. On day 9, a sample of blood was taken before feeding the cheese meal, to have a fasting baseline. Throughout the protocol, the cages were searched to remove any remaining food or feces every time rats were handled. From day 7, feces were collected directly from the animals before the first meal (at the end of the fasting period) and kept at -20°C until required for analysis.

On day 10, an oral lipid-tolerance test (OLTT) was performed after the fasting period using the experimental cheeses as the fat source (Figure 36). The cheese meal was reduced to 7 g to ensure full ingestion of the amount presented and the cages were searched to identify any residue. Cheese ingestion levels were monitored every day before the OLTT and were reported as the mass fraction (%) of ingested meal relative to the total amount of cheese presented.

During the postprandial phase, blood samples obtained by puncture of the saphenous vein were collected in EDTA-preconditioned vials every hour for a total of 6 hours. No lubricant was used during saphenous sampling in order to prevent contamination of blood with exogenous lipids. Glycemia was monitored at the same time points as the OLTT. Analyses performed on blood samples included total TAG, non-esterified fatty acids (NEFA) and total cholesterol, determined spectrophotometrically after preparation with commercially available kits (Randox, Kearneysville, USA). Incremental areas under the curve (iAUC) for the postprandial TAG rise were calculated for each animal by the trapezoidal method using each

animal's fasting TAG concentration as the baseline. To compute iAUC, missing values during the postprandial phase (i.e. 5% occurrences, randomly distributed, due to hemolysis or untimely sampling and attributed to the absence of lubricant) were imputed from the group's mean for the corresponding time point.

After the OLTT, a second meal of 10 g of cheese was presented, followed by the 10-g portion of chow that was left in the cage. On day 11, a cheese meal of 7 g was presented for 30 min and necropsies were performed after 1 h by complete blooding at the tail and heart puncture under isoflurane anesthesia. Livers were weighed to detect any abnormal growth due to steatosis. Stomachs were harvested to weigh the gastric contents 1 h after the ingestion of the cheese meal.

6.4.3 Calcium soap quantification

Feces were analyzed to quantify the amount and profile of fatty acids lost as calcium soaps. All solvents were of HPLC grade and obtained from Fisher Scientific (Toronto, Canada), and all other chemicals and standards were obtained from Sigma Aldrich (Oakville, Canada). Fecal samples corresponding to days 8–11 of the in vivo protocol were lyophilized to prevent loss of volatile fatty acids (Tangerman et al., 1996). Fecal samples were pooled for each rat and ground in a porcelain mortar. Lipid extraction was based on the Folch procedure (Folch et al., 1957), and according to conditions reported by Bourlieu et al. (2015). A sample of dried feces (i.e. ≈ 50 mg) was precisely weighed in a methylation tube and mixed with 500 µl of water and 200 µl of 2.0 N HCl. Once the sample was dispersed, 3000 µl of Folch solvent mixture (i.e. 2:1 chloroform-methanol) was added, followed by 200 µl of internal standard containing C7:0, C13:0 and C19:0 dissolved in chloroform. The sample was vigorously mixed using a vortex, then 125 µl of 125 mM NaCl and 750 µl of Folch solvent mixture were added, and the tube was vortexed again. Samples were allowed to stand for 30 min to permit phase separation. Both phases were recovered. The lower chloroform phase containing the lipids was conditioned in a solid-phase extraction (SPE) cartridge to separate out NEFA for GC analysis, whereas the upper aqueous phase was conditioned for Ca, Mg and P quantification.

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

For the aqueous phase analysis, samples of exactly 500 µl were diluted with 10 ml of 0.23 M HNO_3, centrifuged for 15 min at 3000 × *g* and filtered with a 0.45-µm PES syringe filter. Ca, Mg and P were quantified from the filtrate using an ICP-OES (Prism 010-00084-1 spectrometer from Teledyne Leeman Labs, Hudson, NH, USA, equipped with a Burgener Research Peek Mira Mist Nebulizer, PMM4000). Emission lines used for element quantification of Ca, Mg and P were 317.933, 279.080 and 213.618 nm, respectively. Detection was done in radial mode on the argon-plasma torch. Commercial standards (Fisher Scientific, ON, Canada) were diluted in the same acidic matrix and filtered. Results were presented assuming that soluble minerals were entirely in the aqueous phase. For calculations, the total volume of the latter was approximated to that dictated by the theoretical partition of the methanol in the ternary mixture with water and chloroform (Folch et al., 1957). That volume (i.e. 1720 µl for the conditions set in this study) was representative of the amounts recovered during the collection of the aqueous phases.

6.4.4 Statistical analysis

All statistical analyses were performed with SAS 9.3 TS Level 1M0 (SAS Institute, Cary, NC, USA). The amount of cheese meal ingested was analyzed by analysis of variance (ANOVA) in a split-plot array with the AMF type and salting condition in the main plot and the time after ingestion of the cheese in the sub-plot. Cheese composition, rat weight before necropsy, stomach contents, iAUC and NEFA and calcium in feces were analyzed using a two-way ANOVA for the AMF and calcium level using the GLM procedure. Postprandial responses were transformed ($\log_{10}$) and analyzed with a repeated-measures ANOVA using the MIXED procedure[16]. Normality of data was verified by the Shapiro-Wilk test. Multiple comparisons were done using least square difference (LSD) with a significance threshold of $P \leq 0.05$.

[16] Model information used for the MIXED procedure. Covariance Structure: Variance Components; Estimation Method: REML; Residual Variance Method: Parameter; Fixed Effects SE Method: Kenward-Roger; Degrees of Freedom Method: Kenward-Roger.

6.5 Results and discussion

6.5.1 In vivo protocol

6.5.1.1 Training stage

The ingestion rates increased during the first stage of the training protocol ($P < 0.0001$) and stabilized during the second stage of the training with a sustained ingestion level around 90% of the amount presented, all cheeses confounded. A drop of the ingestion rate was observed on day 9 and was attributed to the baseline blood sampling, after which the rats were irritated and did not consume the cheese meal as they had during the preceding days (data not shown). Nevertheless, the resulting overall ingestion rates for the OLTT on day 10 were over 98% (SEM 0.89), with no differences observed among the experimental cheeses ($P = 0.3064$).

Weight progression throughout the protocol was normal for all rats, according to the technical specifications of the supplier (data not shown). Mean weight increased from 389 g (SEM = 2.4) to 409 g (SEM = 2.9) during the 10 days of the protocol, with no differences among treatments ($P > 0.4$). At necropsy, livers had a healthy aspect, with no visual signs of steatosis and their weight was similar for all treatments (13.01 g, SEM = 0.14), equivalent to 3.18% (SEM = 0.02) of the body weight.

6.5.1.2 Postprandial responses

Overall, the cheese test meal was ingested in under 15 min. Although this feeding strategy lacks the narrow timing achieved by gavage, it avoids the need to blend the food to a liquid state, which would have destroyed the original food matrix. No differences were detected for gastric contents 1 h after cheeses had been ingested (9.85 g, SEM = 0.12).

Glucose levels changed during the postprandial phase ($P < 0.0001$) and evolved in a similar way for all cheeses (Figure 37a), with no effect detected for calcium level

or AMF type. This was expected, since the Cheddar-type cheeses had a similar macronutrient composition and contained very small amounts of sugars. One hour after the cheese meal was consumed, there was a drop of glucose to 4.87 mmol/L, which gradually climbed back to its fasting level after 4 h.

Total cholesterol concentration varied over time ($P < 0.0001$), although it was similar for all experimental cheeses, with no effect detected for calcium level or AMF type. The rise in cholesterol rise after a fatty meal is part of the normal lipid digestion and handling process and occurs due to its synthesis and endogenous turnover (Sethi et al., 1993). The FA composition of the different AMF did not differ drastically and, once combined with endogenous lipids, resulted in similar postprandial cholesterol behavior (Figure 37b). Dairy products have been reported to induce an increase in HDL cholesterol, in part related to their SCFA and MCFA contents (German et al., 2006; Parodi, 2016), but the cholesterol analysis that was performed does not provide data on specific lipoproteins.

NEFA levels were equivalent for all AMF, but varied significantly according to time ($P < 0.0001$) and according to calcium level ($P = 0.0263$). No statistical interactions were detected. One hour after cheese ingestion, overall circulating NEFA reached a peak (2.9 mEq/L) and decreased thereafter, reaching baseline values after 5 h (Figure 37c). Normally, NEFA decrease after a meal containing fat and carbohydrates due to the secretion of insulin, which inhibits lipolysis within adipocytes (Lairon et al., 2007). However, the experimental cheese meal did not contain large amounts of carbohydrates. The postprandial rise in circulating NEFA occurs because some of them escape uptake and re-esterification in adipose tissue. This rise is exacerbated immediately after a meal, when chylomicrons are hydrolyzed by lipoprotein lipase (Berry, 2009; Lairon et al., 2007). Rats that consumed the high-calcium cheeses presented overall levels of NEFA (1.67 mEq/L) that were slightly higher ($P = 0.0263$) than those that consumed the regular-calcium cheeses (1.52 mEq/L) (SEM = 0.04). It has been shown that this increase in NEFA includes mainly fatty acids of dietary origin, in this case, the cheese test meal, because lipolysis of endogenous lipids is halted in the

postprandial state (Berry, 2009). For this reason, higher calcium levels during the digestion process probably enabled an overall increase in lipolysis and lipid uptake. This would have led to higher chylomicron secretion and metabolism, explaining the rise in circulating NEFA.

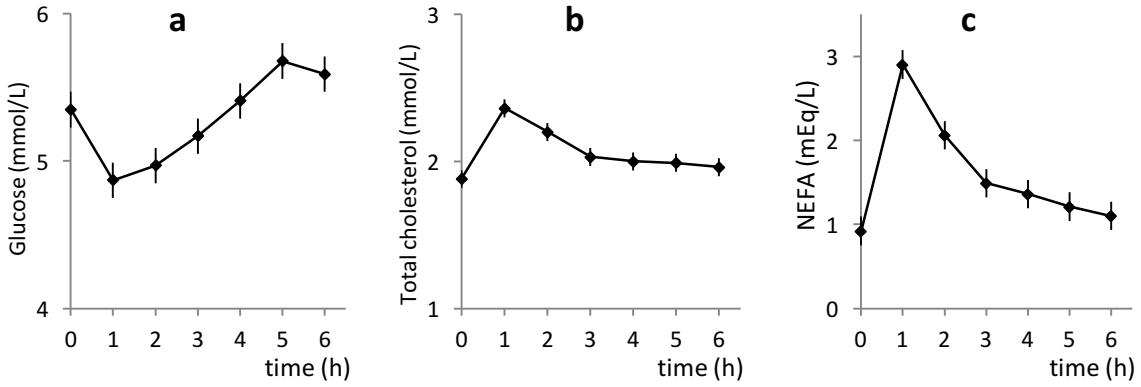

Figure 37. Postprandial progression of (a) glucose (b) total cholesterol and (c) NEFA concentrations in plasma after the ingestion of a cheese meal. Bars indicate SEM.

Finally, TAG levels varied in time ($P < 0.0001$) and differed in magnitude according to AMF type ($P = 0.0023$) and to calcium level ($P = 0.0054$). No statistical interactions were detected. As expected, TAG concentrations increased rapidly after the ingestion of the cheese meal, for all treatments. Peak values were observed during the two first hours of the OLTT, with a sustained PPL level afterwards that was about twice that of the fasting TAG concentration (Figure 38). The AMF type in cheese resulted in different overall TAG concentrations in plasma, with the stearin AMF causing lower levels (1.43 mmol/L) than olein (1.84 mmol/L) and the control (1.72 mmol/L), which were statistically equivalent (SEM = 0.09). The lower TAG levels induced by the stearin AMF compared to the other AMF types can be explained by the higher content of saturated LCFA of the former, resulting in a higher melting point (42.32°C), which is above the rats' body temperature. During digestion, solid fats have slower lipolysis and absorption rates than liquid fats (Berry, 2009; Berry et al., 2007), which can explain the decreased postprandial response of the stearin AMF. Whether the TAG levels remained

above the baseline for longer periods in the stearin AMF treatments compared to the others is unknown because the OLTT lasted only 6 h. For treatments with stearin AMF, the TAG concentration seemed to show a rising trend after the drop that occurred after the first TAG peak (Figure 38). By comparing the iAUC calculated for the different treatments, it was found that the AMF type in cheeses had a significant effect (P = 0.0384), with the stearin inducing the lowest TAG levels after the 6 hours of the OLTT (Table 22). Although the effect of calcium on iAUC fell short of the significance threshold (P = 0.0583), a trend was observed towards higher values with high calcium cheeses (5.52 mmol × h/L) compared to regular-calcium cheeses was observed (4.60 mmol × h/L) (SEM = 0.35).

Table 22. Incremental area under the curve (iAUC) for TAG appearance in blood 6 h into the postprandial phase after ingestion of experimental cheeses prepared with different types of anhydrous milk fat (AMF).

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

Despite the lack of statistical significance of the iAUC, cheeses with high calcium content led to significantly higher circulating TAG levels (1.80 mmol/L) than those observed after ingestion of cheeses with regular-calcium content (1.53 mmol/L) (SEM = 0.07). This difference points to the effect that calcium has in terms of enhancing lipolysis and the repercussions on fat absorption. Looking at the TAG concentrations during the OLTT (Figure 38), there appears to be a trend towards high calcium levels inducing an exacerbated height of the lipemia peak for cheeses containing olein AMF (with higher SCFA and MCFA ratios). Based on the same logic, the effect of high calcium on stearin AMF, with higher saturated LCFA content, would lead to a less abrupt rise in TAG, possibly with a delay in the

lipemia peak, as suggested by the TAG peaks occurring during the first two hours of the OLTT (Figure 38). These trends are consistent with the effect of calcium on lipid digestion and its increased affinity for saturated LCFA under intestinal conditions, leading to higher lipolysis rates (Hu et al., 2010; Zangenberg et al., 2001), but coupled with slower absorption of the resulting calcium soaps (Boyd et al., 1932; Jandacek, 1991; Lorenzen et al., 2007). Unfortunately, the OLTT performed does not make possible to determine whether the net TAG clearance before the return to baseline TAG conditions is equivalent for the different calcium levels or the different AMF types present in the cheeses.

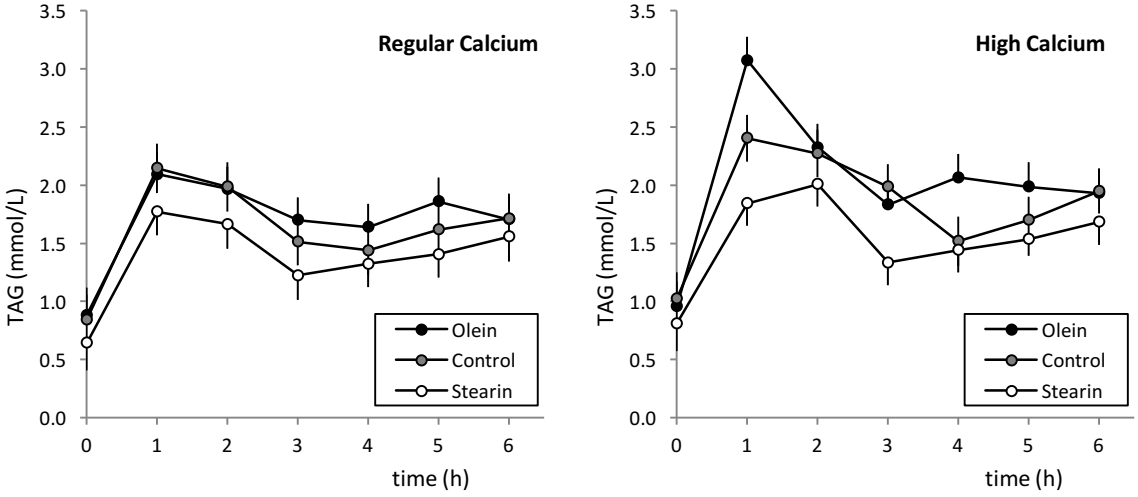

Figure 38. Postprandial progression of TAG concentration in plasma after the ingestion of Cheddar-type cheese with regular or high calcium content and prepared with olein, control or stearin AMF. Bars indicate SEM.

6.5.2 Quantification of fecal calcium soaps

At beginning of the protocol, fecal samples were dark in colour, which is typical for the chow diet (Jandacek et al., 2004). As the protocol advanced, feces became representative of the diet with the cheese meals, as evidenced by the change to a very light brown. From day 8, most pellets collected were pale and were kept for analysis. Total FA excretion was affected by the AMF type ($P < 0.0001$) and the calcium content ($P < 0.0001$) of the cheeses. Feces from rats fed stearin AMF cheeses presented total FA excretion levels that were 29% higher than for rats

consuming cheeses prepared with the control or the olein AMF (Table 23). Concurrently, higher calcium levels in cheese led to higher FA excretion rates than regular calcium levels (Table 24), which is reflected in the 31.5% increase in total FA.

Table 23. Mass fraction of long chain fatty acids and total fatty acids (FA) recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses prepared with a control, olein or stearin AMF.

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

^{a,b} Different index letters indicate significant differences within columns. SEM was obtained from the statistical model.

Table 24. Mass fraction long chain fatty acids and total fatty acids (FA) recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses with regular or high calcium content.

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

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

When broken down into individual fatty acids, differences in excretion rates are mainly due to differences in excretion of saturated LCFA. Volatile acids in feces were similar for all treatments (C2 3.7, SEM = 0.44; C3 0.3, SEM = 0.12; C4 0.6 SEM = 0.26, in mg/g of dry feces). These acids were likely produced by the intestinal microbiota (Diem et al., 1970; Pray et al., 2012) because dietary SCFA, such as butyric acid from milk fat, are preferentially hydrolyzed by digestive lipases

and rapidly absorbed (German, 2008). No MCFA (C6, C8 and C10) were detected in feces, indicating that their absorption was complete. Excreted levels of saturated LCFA (C12 to C18:0) were influenced by calcium content and AMF type present in the cheeses (in all cases, $P \leq 0.01$). Rats consuming the cheeses prepared with the stearin AMF excreted higher amounts of saturated LCFA than those consuming the cheeses prepared with the other AMF (Table 23). This agrees with studies showing that fats with different melting points (i.e. due to different TAG structure and FA composition) result in different digestion and absorption behaviours, and in lower absorption levels for higher melting points (Berry, 2009).

Independently, higher calcium levels in cheese led to higher saturated LCFA excretion levels than those induced by cheeses with regular calcium levels (Table 24). Excretion of UFA (C18:1, C18:2, C18:3) was similar among treatments, except for oleic acid ($P < 0.0001$), which was excreted in higher amounts by rats fed the high calcium cheeses (Table 24). These results are in agreement with the selectivity of calcium for fatty acids, which preferentially binds to saturated LCFA like palmitic and stearic acid, and they agree with the lower affinity of calcium for PUFA, MCFA and SCFA owing to their higher water solubility (Mu et al., 2005; Owen et al., 1995).

Analysis of the aqueous extract of the feces revealed an increase in total calcium and phosphorous for groups consuming the high-calcium cheeses compared to the regular-calcium cheeses (Table 25). Magnesium levels were equivalent for all groups. A calcium increase in dry feces of 7.3 mg/g, equivalent to 12.5% for the regular calcium group, was observed. Such increase was in stoichiometric excess if considered solely as CS with respect to the FA increase of 20.5 mg/g observed for the same treatments (i.e. calcium to FA ratio of 0.35, when 0.07 is the calcium ratio in calcium palmitate). Consequently, calcium lost through feces did not consist solely of CS, but may have included calcium phosphates, as suggested by the simultaneous rise in phosphorous level.

Table 25. Mass fraction of Ca, Mg and P recovered in feces, on a dry basis (mg/g), from rats that were fed Cheddar-type cheeses with regular or high calcium content.

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

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

When analyzed together, FA and calcium excretion rates point to the formation of CS in the intestine through LCFA complexation by calcium ions, with palmitate and stearate as the predominant soaps. Moreover, the highest excretion levels by far for a single fatty acid were those obtained for palmitic acid. This fatty acid has already been identified as being responsible for elevated plasmatic cholesterol levels (Grundy et al., 1990). The reasons for the increased excretion of oleic acid are unclear, but it was related to calcium and not to the relative amount found in the different AMF types. Oleic acid is less prone to produce CS than saturated LCFA and it is almost completely absorbed with or without the presence of calcium (Mattson, 1979). The increase in oleic acid excretion is probably caused by CS interfering with micelle formation and the lipid uptake, or the effect of lipase activity on endogenous fats in the large intestine, which is enhanced in the case of the diet with high calcium levels. Although it is not possible to affirm that excreted fatty acids consisted exclusively of CS, based on the increase in excretion, calcium certainly played a role in reducing the bioavailability of saturated LCFA and oleic acid.

While calcium enrichment of cheeses led to higher overall TAG levels after 6 hours into the postprandial phase, it increased the amount of FA excreted as calcium soaps, which points to a lower FA bioavailability, which was not observed within the time frame of the OLTT. This result shows that the effect of calcium is not straightforward and that it depends on the transit of chyme within the digestive

system. This way, the increased concentration of calcium enabled higher lipolysis rates. The rapid release of fatty acids could explain the increase in the lipemic response during the OLTT, especially considering that calcium has a lower affinity for FA that have a more unsaturated or shorter carbon chain. This means that calcium could form soaps with stearic or palmitic acid, removing them from the fat droplet surface and enabling lipase to continue its lipolytic effect, permitting the release of other FA. Even if the CS produced have slower absorption rates, UFA or shorter FA could still be absorbed and they would be the FA causing the exacerbated lipemia in the presence of high calcium levels. Then, as digestion continued and chyme advanced towards the distal ileum, where pH conditions approach neutrality, CS would have been less prone to be absorbed, resulting in the higher excretion levels observed in this study.

Finally, the results obtained through the in vivo model are in agreement with those obtained following the in vitro digestion of the same experimental cheeses (Ayala-Bribiesca et al., 2017). Disintegration of the experimental cheeses during in vitro digestion was slower for those containing the stearin AMF, when compared to those with the control or the olein AMF (Ayala-Bribiesca et al., 2017), which would partially explain the decreased lipemic response observed in vivo for cheeses with the stearin AMF. Additionally, the in vitro lipolysis patterns of the experimental cheeses, analogous to intestinal digestion, depict slower lipolysis rates for the stearin AMF than those for the olein and control AMF. This supports the PPL patterns observed during the OLTT, where cheeses containing the control and the olein AMF induced higher lipemia, as indicated by the increase in TAG, than cheeses containing the stearin AMF. Lastly, it is interesting to note that with the high-calcium cheese, the extent of in vitro lipolysis for the stearin AMF increased towards the end of the digestion and eventually reached the same levels as for the olein and control AMF (See Figure 30 in Chapter 5) (Ayala-Bribiesca et al., 2017). While this demonstrates the enhancing effect of calcium on lipolysis, it would seem contradictory given the lower lipemic responses obtained in vivo for cheeses with the stearin AMF. Such discrepancy can be explained by the lack of an absorption process in the in vitro model. In a closed in vitro vessel, lipolysis products

accumulate and the medium eventually saturates, halting lipolysis. In the presence of higher calcium levels, the excess calcium further drives the extent of lipolysis by reacting with FA to produce CS. In vivo, CS also occur and some are excreted because their absorption is less efficient than that of the corresponding FA. This reduced bioaccessibility of CS could also explain the decreased lipemic response of the stearin AMF (rich in LCFA), as observed in vivo.

6.6 Conclusion

The results presented show the potential of solid dairy matrices to act as nutrient-release regulators during the digestive process. The study demonstrated that it is possible to modify PPL and to increase or decrease fatty acid bioavailability, which could be of interest for specific health requirements. In vivo digestion of Cheddar-type cheeses prepared with different AMF types and calcium levels led to different postprandial responses. The cheese containing the stearin AMF led to lower circulating TAG levels than cheeses prepared with olein or control AMF, as well as to higher excretion levels than the other AMF types. The higher amount of solid fat at body temperature of the stearin AMF, with respect to the other AMF is believed to be a major reason for variations observed on PPL. This agrees with the results obtained in vitro for the same cheeses (Ayala-Bribiesca et al., 2017), where the use of stearin AMF led to slower disintegration rates and lower lipolysis rates when compared to olein or control AMF. Further analysis of the transporter lipoproteins is required to determine if any of the experimental cheeses would result in a better postprandial lipoprotein profile.

Cheeses with high calcium content induced higher lipemia levels during the OLTT than those with regular calcium content (higher bioavailability during the early postprandial period), but also led to higher excretion rates (decreased net bioavailability). Furthermore, excreted FA results indicate that the net bioaccessibility of fatty acids from cheeses prepared with the stearin AMF was lower than for cheeses with the control or the olein AMF. The results presented agree with those obtained in vitro in a previous study, where calcium was reported to bind LCFA like palmitic and stearic acids to form CS, resulting in higher SCFA, MCFA and oleic acid release rates (Ayala-Bribiesca et al., 2017).

This work illustrates the complementarity of in vitro and in vivo studies. While in vitro approaches are useful for screening and identifying promising experimental conditions, the relevance of the resulting findings can only be validated through in

vivo trials. Once validated, the reliability of the predictions derived from the in vitro model increases.

This study shows that FA bioavailability depends on the composition and characteristics of the fat, and it can be modulated by the amount of calcium ingested with the fat. Studies should be undertaken to further elucidate the kinetics of calcium and fatty acids and the associated effect on PPL. By understanding and harnessing this modulation capacity, it will be possible to design foods to meet specific nutritional criteria, with a targeted effect on lipid bioavailability and tailored release and absorption kinetics for lipid-soluble nutrients and molecules.

6.7 Acknowledgements

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Chapter 7. General discussion

The main objective of this project was to better understand the role of calcium on fatty acid bioaccessibility by its effect on the Cheddar cheese matrix and lipolysis during digestion. This study describes the process by which Cheddar cheese releases fatty acids from milk fat during digestion and the influence that calcium can have on their bioaccessibility and the resulting metabolic responses using the rat model.

7.1 Completion of the specific objectives

7.1.1 Assessment of in vitro bioaccessibility of fatty acids from cheese matrices enriched with calcium

Adding calcium chloride during the salting step of cheese manufacture modified the texture parameters and cheese microstructure, which led to different disintegration rates during in vitro digestion. In the presence of high calcium levels, lipolysis was faster in the beginning of the intestinal phase, when compared to regular calcium levels. Such effect was explained by calcium-driven enhancement of lipolysis through the production of calcium soaps. Conversely, final lipolysis rates were lower for cheeses with very-high calcium levels than for the cheese with high calcium levels. This was explained by the protein matrix resisting disintegration and, hence, preventing lipases to access part of the fat. The differences among lipolysis rates were interpreted as differences on fatty acid bioaccessibility.

7.1.2 Assessment of the impact of calcium on fatty acid bioaccessibility from cheeses prepared with different milk fats under simulated digestive conditions.

Cheddar-type cheeses were prepared with control, olein and stearin AMF and regular and high calcium levels. When digested in vitro, the cheeses showed different disintegration and lipolysis profiles. Cheeses prepared with a stearin AMF were harder and more resistant to disintegration than cheeses prepared with the other fats.

Calcium level did not affect disintegration, but did influence lipolysis. High calcium increased the lipolysis extent of cheeses prepared with the stearin AMF, with respect to their regular calcium control. This was explained by the formation of calcium soaps, which would reduce the accumulation of LCFA at the lipid/water interface during lipolysis. Cheeses prepared with olein AMF showed faster lipolysis in the presence of high calcium levels than any other cheese. The same effect of

calcium soaps was provided as an explanation, through the removal of LCFA from the fat-water interphase, providing access to shorter fatty acids.

7.1.3 Modulation of bioavailability of cheese lipids contained by modifying the composition and structure of their matrices.

The experimental cheeses with different AMF types and calcium levels were used in an in vivo study with rats. The cheeses led to different postprandial responses, as well as different fecal fat excretion rates. Cheeses with olein AMF led to the highest TAG rise, with the opposite occurring with cheeses containing stearin AMF. Calcium enrichment of cheeses modified the postprandial evolution of TAG. High-calcium cheeses with olein AMF caused a fast and exacerbated rise in TAG, while a delayed peak was caused by those with stearin AMF. The study confirmed that fatty acid bioavailability can be modified by the type of fat and the calcium content of the cheese. Cheeses with stearin caused higher levels of fat excretion than the other cheeses. Independently, cheeses with high calcium levels led to an increased fecal fat that the regular calcium controls.

7.2 Confirmation of the hypothesis

The hypothesis of this project was that the concentration of calcium and the FA profile in Cheddar cheese influence the mechanical disintegration and soap formation in the gastro-intestinal environment, modulating both lipolysis rate and FA bioaccessibility.

Through the completion of the three specific objectives, it is possible to affirm that calcium influences bioavailability of milk FA from Cheddar-type cheeses. The effect of calcium on hardening the matrix was shown to be mainly mediated by an osmotic dehydration of the matrix when $CaCl_2$ was added over NaCl. No hardening was seen when NaCl was replaced with $CaCl_2$ on a constant ionic strength basis. The structuring effect of calcium mainly takes place during milk clotting. Hence, when added after the curd formation, the osmotic effect of the calcium salt seems to be more important than its structuring effect. In vitro digestions showed that calcium was key to increase lipolytic activity on available fat (i.e. released from the matrix into the digestive fluids). This increase was explained by the formation of calcium soaps with LCFA, which are generally recognized to have lower bioaccessibility than their respective fatty acids. Yet, by increasing the lipolysis rate of milk fat, fatty acids that are less prone to produce calcium soaps (i.e. SCFA, MCFA and UFA) were released faster. The differences in bioaccessibility were hence explained in terms of timing. High calcium levels led to fast bioaccessibility of short and unsaturated fatty acids and slow bioaccessibility of longer fatty acids. The postprandial responses showed that calcium modified the lipemic profiles according to its concentration and the type of milk fat present in the Cheddar-type cheeses. In vivo, it was shown through fecal fat quantification that the bioavailability of LCFA involved was lower.

7.3 Original aspects and contributions to science

This project showed how different cheese matrices and their composition can modify the way they are digested in vitro and that such differences translate into different metabolic responses in vivo. The physical constitution of the cheese, with different hardness and different fat distribution within the continuous protein gel was shown to modify the disintegration process and fat release. Whether due to a lower moisture content or the presence of milk fat with high melting point, harder matrices resulted in longer disintegration than softer matrices.

The formation of calcium soaps in the intestinal environment and its impact on increasing fatty acid excretion has been widely described. Nevertheless, there seems to be two perspectives regarding the role of calcium on lipid digestion. In vitro research focuses on enhanced lipolysis in the presence of calcium, but pays less attention to its implications on lipid bioaccessibility. In vivo research considers calcium to cause lower FA absorption (or higher excretion), without much reference to its effect on lipolysis. An original aspect of this project is that it conciliates the perspectives from both experimental approaches by coupling in vitro and in vivo experiments on the same matrices.

Furthermore, it was shown that calcium causes, possibly by removing long-chain fatty acids from the fat droplets, a high and rapid release of short- and medium-chain FA. Because those FA do not react with calcium under intestinal conditions, they are readily absorbed. On the other hand, higher concentration of saturated LCFA, led to the formation of calcium soaps. During the postprandial phase, this led to a slower absorption of those FA, as confirmed by the delayed rise of plasma TAG and the net reduction on FA uptake, as indicated by the higher fecal loss of fatty acids.

In vitro models are useful to gain understanding of the underlying processes occurring during digestion and to identify the most promising conditions to test with in vivo protocols. For these reasons, another original aspect of this project was the coordination of the in vitro and in vivo experiments with the same model cheeses.

While in vitro approaches are useful for screening and identifying promising experimental conditions, the relevance of such findings can only be validated through in vivo trials. This work succeeds in illustrating the complementarity between in vitro and in vivo studies. First, the in vitro study produced data on the processes taking place during the digestion of the model cheeses. Second, the data obtained in vitro were coherent with the metabolic responses observed in vivo. By such means, the in vivo trial supported the relevance of the in vitro approach, increasing the reliability of the predictions derived from the in vitro model.

Finally, the extraction method that yielded a white layer separating the organic and the aqueous phases is an original and promising starting point to develop a simple in vitro method to quantify calcium soaps at different pH conditions. Preliminary analyses (not included in this thesis) on the interphases described after the solvent extractions indicate it is predominantly composed of calcium soaps.

7.4 Perspectives

This project provides a better understanding of calcium and fat interaction during cheese digestion and their impact on lipid bioaccessibility. This may enable the development of technological strategies to manufacture food matrices that can modulate lipid digestion and absorption. Specifically concerning dietary lipids, such modulation may aid in preventing the overflow of the metabolic capacity during the postprandial period and limit the undesirable effect of hyperlipidemia on cardiovascular health.

General conclusion

Much is known on the physiology related to lipid digestion and absorption, but including the structure of the food matrix into the equation is gaining increased attention. Research has demonstrated that the food matrix plays an important role on nutrient effectiveness, where its structure can be as important as its composition. This project provides progress in understanding the factors that affect lipid bioaccessibility and bioavailability from dairy matrices, namely those related to the presence of calcium and the FA composition of milk fat. In vitro experiments were helpful to better understand the in vivo responses. The results give an insight on calcium effect on lipid bioavailability during cheese digestion, but much is yet to be understood and confirmed by clinical studies.

The cheese matrix is considerably more complex than simplified model solutions and emulsions, but it was shown to be a convenient model to study lipid digestion. Cheddar cheese was chosen for its high calcium and fat content. Similar experiments using other cheeses (e.g. with lower calcium levels) could add valuable input to that presented in this project. The texture of Cheddar cheese allowed to standardize the particle size for the in vitro studies, as opposed to crumbly or very soft cheeses. Nevertheless, further rheological and textural characterization of the matrices would be required to better understand the factors influencing the disintegration process during digestion.

Lipolysis is a very efficient process and differences among treatments are reduced towards the end of the in vitro digestion. Hence, limiting data to endpoints leaves important information behind. Concerning the calcium-FA interaction, it would be of interest to study the evolution of the free FA profile as the digestion takes place. This would allow to differentiate LCFA as they are released, and relate such release kinetics to their precipitation as calcium soaps. Strategies aimed to differentiate endogenous and dietary FA could also be useful in describing the metabolic fate of the different FA, as well as the provenance of FA that form the calcium soaps.

Fundamental studies on calcium soaps would be useful to better predict their role on lipolysis enhancement and fatty acid bioaccessibility. More research on their behavior during digestion and absorption is required to elucidate processes that are presently treated as a black box. The extraction method to estimate CS formed under intestinal pH conditions developed in this project should be refined and the interphase should be characterized to confirm the presence of calcium soaps.

Calcium and fat are present in many foods of a normal diet. Regardless of their source, they are repeatedly in interaction during the digestive processes. Many other factors influence lipid bioavailability (e.g. thermal history of the fat, presence of non-digestible lipids, and presence of fiber during the digestive process). In sum, further works should progressively integrate such factors to get a more detailed portrait of their influence on fat digestion and absorption.

Finally, the culinary preparation of food has been overlooked although it can drastically modify the composition and the structure of food matrices. In this scope, the span of possible matrices in a real (complex) meal greatly increases. More research in that direction would provide more realistic conditions such as those occurring during the simultaneous digestion of different foods.

The digestion mechanisms explored in this project may open new strategies for effective delivery of lipids. On a technological standpoint, the manufacturing processes implemented during the experimental phase may be used to exploit such strategies. This project consolidates aspects of nutrition and food sciences towards healthy and wholesome food innovation.

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Appendices

Appendix A. In vitro digestion system

Table 26. Composition of the simulated digestion fluids used in the Versantvoort in vitro digestion model (Versantvoort et al., 2005).

Constituents and concentrations of the various synthetic juices of the in vitro digestion model representing fed conditions

	Saliva	Gastric juice	Duodenal juice	Bile juice
Inorganic solution	10 ml KCl 89.6 g/l 10 ml KSCN 20 g/l 10 ml NaH_2PO_4 88.8 g/l 10 ml $NaSO_4$ 57 g/l 1.7 ml NaCl 175.3 g/l 20 ml $NaHCO_3$ 84.7 g/l	15.7 ml NaCl 175.3 g/l β.0 ml NaH_2PO_4 88.8 g/l 9.2 ml KCl 89.6 g/l 18 ml $CaCl_2 \cdot 2H_2O$ 22.2 g/l 10 ml NH_4Cl 30.6 g/l 6.5 ml HCl 37% g/g	40 ml NaCl 175.3 g/l 40 ml $NaHCO_3$ 84.7 g/l 10 ml KH_2PO_4 8 g/l 6.3 ml KCl 89.6 g/l 10 ml $MgCl_2$ 5 g/l 180 µl HCl 37% g/g	30 ml NaCl 175.3 g/l 68.3 ml $NaHCO_3$ 84.7 g/l 4.2 ml KCl 89.6 g/l 150 µl HCl 37% g/g
Organic solution	8 ml urea 25 g/l	10 ml glucose 65 g/l 10 ml glucuronic acid 2 g/l 3.4 ml urea 25 g/l 10 ml glucoseamine hydrochloride 33 g/l	4 ml urea 25 g/l	10 ml urea 25 g/l
Add to mixture organic + inorganic solution	290 mg α-amylase 15 mg uric acid 25 mg mucin	1 g BSA 2.5 g pepsin 3 g mucin	9 ml $CaCl_2 \cdot 2H_2O$ 22.2 g/l 1 g BSA 9 g *pancreatin* 1.5 g lipase	10 ml $CaCl_2 \cdot 2H_2O$ 22.2 g/l 1.8 g BSA 30 g bile
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

The inorganic and organic solutions are augmented to 500 ml with distilled water. After mixing of the inorganic and organic solutions, some further constituents are added and dissolved. If necessary, the pH of the juices is adjusted to the appropriate interval.

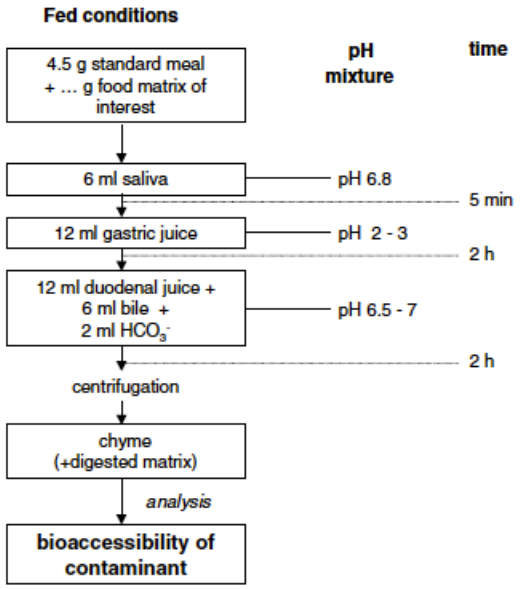

Figure 39. Schematic representation of the original Versantvoort in vitro digestion model (Versantvoort et al., 2005).

Appendix B. Glyceride-related lipid structure and nomenclature

Fatty acids

Table 27. Common names and molecular weights (MW) of some carboxylic acids. Compilation from various sources (Christie, 1995; Jensen et al., 1995; Kaylegian et al., 1995; Lide, 2005).

Abbreviation	Common name	MW (g/mol)	Solubility in water (g/100g water at 20°C)
C2	Acetic	60.05	miscible
C3	Propionic	74.08	miscible
C4:0	Butyric	88.11	miscible
C5:0	Valeric	102.13	2.5
C6:0	Caproic	116.15	0.967
C7:0	Enanthic	130.19	0.240
C8:0	Caprylic	144.21	0.080
C9:0	Pelargonic	158.24	0.0284
C10:0	Capric	172.26	0.015
C11:0	Undecylic	186.29	n.a.
C12:0	Lauric	200.31	0.0055
C13:0	Tridecylic	214.35	0.0033
C14:0	Myristic	228.37	0.0020
C14:1	Myristoleic	226.37	n.a.
C15:0	Pentadecylic	242.40	0.0012
C16:0	Palmitic	256.42	0.00072
C16:1	Palmitoleic	254.41	n.a.
C17:0	Margaric	270.45	0.00042
C18:0	Stearic	284.47	0.00029
C18:1 (cis)	Oleic	282.46	n.a.
C18:1 (trans)	Elaidic	282.46	n.a.
C18:2	Linoleic	280.44	n.a.
C18:3	Linolenic	278.48	n.a.
C19:0	Nonadecylic	298.50	n.a.
C20:0	Arachidic	312.52	n.a.
C22:0	Behenic	340.59	n.a.
C24:0	Lignoceric	368.63	n.a.

Data not available is identified as n.a.

Main fatty acids with dietary relevance.

Butyric and caproic acids occur in milk fats, and are not normally found in common vegetable oils. Caprylic, capric, lauric and myristic acids occur in milk fat, coconut and palm kernel oils. Palmitic acid is the most widely occurring FA, being present in practically all dietary fats: it is present in marine oils, in milk and fat deposits of land animals and in vegetable fats; main sources include palm oil, cottonseed oil, lard and beef tallow. Stearic acid is less abundant than palmitic acid, but it is also common in most fats. Stearic acid is a significant component in cocoa butter and shea butter, present in most animal fats and is a major component in the tallow of ruminant fats. Finally, oleic acid is also present in all fats and it is especially abundant in oils (Ratnayake et al., 2009). Polyunsaturated fatty acids were not included in this section because they are often present in low levels, but their nutritional importance is acknowledged.

Subclasses of fatty acids

Saturated fatty acids are grouped into subclasses as follows (Food and Agriculture Organization of the United Nations (FAO), 2010):

- Short-chain fatty acids: SCFA 3 to 7 carbon atoms.
- Medium-chain fatty acids: MCFA 8 to 13 carbon atoms.
- Long-chain fatty acids: LCFA 4 to 20 carbon atoms.
- Very-long-chain fatty acids: VLCFA 21 or more carbon atoms.

The following subclasses according to fatty acid saturation level are also common and used in this document:

- Saturated SFA
- Unsaturated UFA Any FA with one or more unsaturations
- Mono-unsaturated MUFA Any FA with only one unsaturation
- Polyunsaturated PUFA Any FA with more than one unsaturation

Triacylglycerols

International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAQ-IUB) TAG nomenclature "*Stereospecific numbering*" (sn) takes recognition of the fact that two primary carbinol groups of the parent molecule (glycerol) are not identical in their reactions with dissymmetric structures, which include nearly all biochemical processes and therefore must be distinguished in nomenclature (IUPAQ-IUB, 1968). Hence, sn_1 and sn_3 positions are not interchangeable for the same carbinol group (IUPAQ-IUB, 1968) (Figure 40). If the secondary hydroxyl group is shown to the left of C-2 in a Fischer projection (i.e. with the carbon in the mid or secondary position in the plane of the page and the primary or end carbons behind the plane of the page (Small, 1991)) (IUPAQ-IUB, 1968). The carbon atom above C-2 is called C-1 and the one below C-3; the use of such "stereospecific numbering" is indicated by the prefix sn before the stem-name of the compound (IUPAQ-IUB, 1968). Under this system, there can be no formal inversions as long as the four bonds of C-2 remain intact (IUPAQ-IUB, 1968).

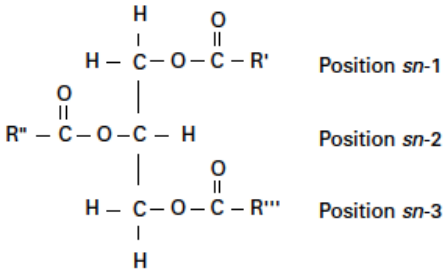

Figure 40. Structure of the triacylglycerol (TAG) molecule. Reproduced from Berry et al. (2005).

Appendix C. Solubility products of some calcium soaps and salts

Solubility products at 25°C (Garrett, 2013).

Calcium stearate $K_{sp} \approx 2 \times 10^{-20}$

Calcium palmitate $K_{sp} \approx 6 \times 10^{-18}$

Calcium oleate $K_{sp} \approx 1 \times 10^{-15}$

Calcium laurate $K_{sp} \approx 6 \times 10^{-13}$

Calcium carbonate $K_{sp} \approx 5 \times 10^{-9}$

Where $K_{sp} = [A]^c[B]^d$ for a reaction $AB \leftrightarrow cA + dB$ (Lide, 2005)

so, for $CaFA_2 \leftrightarrow Ca^{2+} + 2FA^-$

and $K_{sp} = [Ca^{2+}][FA^-]^2$

and from the reaction we know that, in molarity, $2[Ca^{2+}] = [FA^-]$

hence, solubility can be calculated from

$K_{sp} = [Ca^{2+}][FA^-]^2 = [Ca^{2+}][2Ca^{2+}]^2 = 4[Ca^{2+}]^3$

Solving for $[Ca^{2+}]$ gives the molar solubility, since every mole of $CaFA_2$ solubilized produces 1 mole of Ca^{2+}.

Appendix D. Stereospecific composition of milk fat

Table 28. Molecular weight (MW) and molar and mass fractions of milk fat fatty acids per their sn-position on the TAG (Walstra et al., 1984).

Fatty acid	MW	Mass	Molar fraction (%) in sn-				Mass fraction (%) in sn-			
	(g/mol)	(%)	1	2	3	1+3	1	2	3	1+3
4:0	88.1	4.4	2	1	97	99	0.09	0.04	4.27	4.36
6:0	116.1	2.4	4	12	84	88	0.10	0.29	2.02	2.11
8:0	144.2	1.4	13	42	45	58	0.18	0.59	0.63	0.81
10:0	172.3	2.7	17	50	33	50	0.46	1.35	0.89	1.35
12:0	200.3	3.3	24	50	26	50	0.79	1.65	0.86	1.65
14:0	228.4	10.9	27	56	17	44	2.94	6.10	1.85	4.80
14:1	226.4	0.8	10	45	45	55	0.08	0.36	0.36	0.44
15:0	241.4	0.9	40	53	7	47	0.36	0.48	0.06	0.42
16:0	256.4	30.6	46	42	12	58	14.08	12.85	3.67	17.75
16:1	254.4	1.4	40	37	23	63	0.56	0.52	0.32	0.88
17:0	270.4	0.4	33*	33*	33*	66	0.13	0.13	0.13	0.26
17:1	268.4	0.1	33*	33*	33*	66	0.03	0.03	0.03	0.07
18:0	284.4	12.2	58	20	22	80	7.08	2.44	2.68	9.76
18:1	282.4	24.9	43	25	32	75	10.71	6.23	7.97	18.68
18:2	280.4	1.8	40	40	20	60	0.72	0.72	0.36	1.08
18:3	278.4	0.7	39	32	29	68	0.27	0.22	0.20	0.48
20:0	312.5	0.2	33*	33*	33*	66	0.07	0.07	0.07	0.13
Total (%)		99.1					38.64	34.07	26.38	65.02

* Unavailable data, their distribution was computed as equimolar among the sn- positions.

From data in Table 28, an average MW for overall milk fatty acids is 247.1 g/mol, while 246.02 is the average MW of fatty acids in positions sn_1- and sn_3- (which are the positions that pancreatic lipase acts on to release fatty acids during digestion).

Appendix E. Peer-reviewed communications

Research papers

Ayala-Bribiesca, E., Turgeon, S. L., Pilon, G., Marette, A., & Britten, M. (Submitted). Postprandial lipemia and fecal fat excretion in rats is affected by the calcium content and type of milk present in Cheddar-type cheeses*, in submission process.*

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2017). Effect of calcium on fatty acid bioaccessibility during in vitro digestion of Cheddar-type cheeses prepared with different milk fat fractions. *Journal of Dairy Science, 100*(4), 2454-2470.

Ayala-Bribiesca, E., Lussier, M., Chabot, D., Turgeon, S. L., & Britten, M. (2016). Effect of calcium enrichment of Cheddar cheese on its structure, in vitro digestion and lipid bioaccessibility, *International Dairy Journal, 53*, 1-9.

Oral presentations

Ayala-Bribiesca, E., Turgeon, S. L., Pilon, G., Marette, A., & Britten, M. (2016). Role of calcium on lipid digestion and absorption from cheese matrices, *IDF cheese Science and Technology Symposium Programme*, Dublin, Ireland.

Ayala-Bribiesca, E., Turgeon, S. L., Marette, A., Pilon, G., & Britten, M. (2014). Role of calcium on lipid digestion and absorption from cheese matrices, *IDF World Summit*, Tel Aviv, Israel. Last minute cancellation of the event for safety reasons.

Ayala-Bribiesca, E., (2014). Impact du calcium sur la digestion et l'absorption des lipides du fromage, *Colloque de la relève scientifique du Centre québécois de valorisation des biotechnologies*, Quebec City, Canada.

Ayala-Bribiesca, E., (2013). Impact du calcium sur la bioaccessibilié des lipides laitiers à partir de matrices de type fromage, *Séminaires en nutrition de l'Université de Montréal*, Montreal, Canada. Invited speaker.

Ayala-Bribiesca, E., (2011). Effect of calcium on Cheddar cheese physical degradation and lipolysis during in vitro digestion. *4th International Symposium on Delivery of Functionality in Complex Food Systems*, Guelph, Canada.

Turgeon, S. L., Rinaldi, L., Ayala-Bribiesca, E. (2011). Matrices laitières : effet de la composition et des procédés sur leur microstructure et leurs propriétés nutritionnelles, *Colloque STELA*, Quebec City, Canada.

Poster presentations

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2017). Interaction of calcium with fatty acids from digested cheese: Ca soap extractions, *5th International Congress on Food Digestion (INFOGEST Network)*, Rennes, France.

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2015). Effect of added calcium on Cheddar cheese structure and in vitro digestion, *4th International Congress on Food Digestion (INFOGEST Network)*, Naples, Italy.

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2014). Des fromages qui contrôlent l'absorption des lipides. *Forum technologique NOVALAIT*, Drummondville, Canada.

Ayala-Bribiesca, E., Lussier, M., Turgeon, S. L., & Britten, M. (2013). Quantification and fatty acid profile of calcium soaps produced during in vitro digestion of cheese-type matrices, *Colloque STELA*, Montreal, Canada.

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2012). Calcium enrichment during cheddar cheese salting: Effect on structure and digestion. *FAST Program Symposium on Functional Foods and Natural Health Products*, Winnipeg, Canada.

Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2012). Ajout de calcium au salage du fromage cheddar : effet sur sa structure et sa digestion, *Forum technologique NOVALAIT*, Drummondville, Canada.

Ayala-Bribiesca, E., Lussier, M., Turgeon, S. L., & Britten, M. (2011). Effect of calcium on fatty acid precipitation during in vitro digestion of a model cheese. *Symposium INAQ sur la nutrition et le métabolisme : aspects cliniques et fondamentaux*, Quebec City, Canada.

Lussier, M., Ayala-Bribiesca, E., Turgeon, S. L., & Britten, M. (2011). Effect of calcium on fatty acid precipitation during in vitro digestion of a model cheese. *Journée de la recherche en sciences de la santé de l'Université de Sherbrooke*, Sherbrooke, Canada.