The Influences of Pectin and Apples, as a Pectin-rich Food Matrix, on Lipid Digestibility, Bioaccessibility and Postprandial Lipemia

by

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ABSTRACT

The Influences of Pectin and Apples, as a Pectin-rich Food Matrix, on Lipid Digestibility, Bioaccessibility and Postprandial Lipemia

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University of Guelph, 2019

Advisor: Associate Professor Dr. Amanda Wright

Pectin, a common dietary fibre, can potentially modulate lipid digestion and postprandial lipemic response, which contributes to cardiovascular risks. This thesis used in vitro models to study how pectin, both in purified form and food matrices, can affect lipid digestion. A human trial was also undertaken to investigate the effects of consuming whole apples on postprandial lipemia.

Firstly, the effects of pectin on the digestion of a soy lecithin-stabilized emulsion rich in docosahexaenoic acid (DHA) was studied using a static in vitro model. Different levels of pectin and gastric pH conditions were applied to represent realistic food composition and gastrointestinal dynamics in humans. Pectin led to changes in emulsion microstructure, which were reflected in lipid digestibility and DHA bioaccessibility results.

Based on above findings, a randomized crossover trial was conducted with 26 overweight and obese adults to understand how consuming ~200 g whole apples, as a pectin-rich food matrix, can modulate postprandial lipemia induced by a high fat dairy beverage. Apple consumption did not change gastric emptying, postprandial lipemic or glycemic responses, but increased insulinemia, which was potentially induced by fructose and may have counteracted potential lipid lowering effects of apples.
To study how whole apples affected emulsion stability, digestibility and bioaccessibility of the dairy beverages used in the human study, two in vitro digestion models (i.e. static with a gastric pH of 3.0 or 6.5 and dynamic (i.e. TIM-1) systems) were applied. Gastric pH was found to influence apples’ effects on emulsion stability and digestibility in the static model. In TIM-1, apples did not lower lipid bioaccessibility, in agreement with the no effect of apples observed in the human study.

In summary, pectin and apples impacted in vitro emulsion stability and the subsequent lipid digestion and bioaccessibility, depending on factors including gastric pH, pectin content and meal fat content. In humans, apples did not alter postprandial lipemia. Overall, this thesis provides valuable insight about pectin and apples’ functionality regarding lipid metabolism, which applies to food and nutrition scientists and industries. It also highlights the need to improve the physiological relevance of in vitro digestion methods for lipid research.
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<tr>
<td>AIS</td>
<td>alcohol insoluble solids</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>ANCOVA</td>
<td>analysis of covariance</td>
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<tr>
<td>ApoB48</td>
<td>apolipoprotein-48</td>
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<tr>
<td>BMI</td>
<td>body mass index</td>
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<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
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<tr>
<td>$C_{\text{max}}$</td>
<td>peak concentration</td>
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<td>CMRF</td>
<td>chylomicron-rich fraction</td>
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<td>CONSORT</td>
<td>Consolidated Standards of Reporting Trials</td>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>$D_{3,2}$</td>
<td>surface-based mean droplet diameter</td>
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<tr>
<td>$D_{4,3}$</td>
<td>volume-based mean droplet diameter</td>
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<tr>
<td>DE</td>
<td>degree of esterification</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<td>FA</td>
<td>fatty acids</td>
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<td>FAME</td>
<td>fatty acid methyl esters</td>
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<td>FFA</td>
<td>free fatty acids</td>
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<td>GAE</td>
<td>gallic acid equivalents</td>
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<td>GC</td>
<td>gas chromatography</td>
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<td>GI</td>
<td>gastrointestinal</td>
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<td>GIT</td>
<td>gastrointestinal tract</td>
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<td>HFM</td>
<td>high fat meal</td>
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<td>HGA</td>
<td>high gastric acidity</td>
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<td>HMP</td>
<td>high methoxyl pectin</td>
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<td>HNRU</td>
<td>Human Nutraceutical Research Unit</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>HOMA-IR</td>
<td>homeostatic model assessment of insulin resistance</td>
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<td>iAUC</td>
<td>incremental area under the curve</td>
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<td>LGA</td>
<td>low gastric acidity</td>
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<td>LMP</td>
<td>low methoxyl pectin</td>
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<td>NEFA</td>
<td>non-esterified fatty acid</td>
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<td>OFTT</td>
<td>oral fat tolerance test</td>
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<td>PPL</td>
<td>postprandial lipemia</td>
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<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
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<td>PSD</td>
<td>particle size distribution</td>
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<td>research ethics board</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SDF</td>
<td>simulated duodenal fluids</td>
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<td>SEM</td>
<td>standard error</td>
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<td>SFA</td>
<td>saturated fatty acids</td>
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<td>SGF</td>
<td>simulated gastric fluids</td>
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<td>TAG</td>
<td>triacylglycerol</td>
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<td>TIM-1</td>
<td>TNO gastrointestinal model (upper tract)</td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>time to reach peak concentration</td>
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<tr>
<td>TRL</td>
<td>triacylglycerol rich lipoproteins</td>
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<td>unsaturated fatty acids</td>
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CHAPTER 1. Literature Review

1.1. Introduction

This review will firstly outline the biodynamics of lipid digestion and absorption and consequently postprandial lipemia, and then give an overview of recent advances in in vitro digestion methods, including correlations between findings from these methods and human trials. Details of dietary fibres, including definitions, classification and postulated health benefits, which are relevant to their structures and physicochemical properties, particularly in relation to lipemia, will then be discussed. Lastly, current studies on the effects of apple and apple pectin-containing food consumption on lipid digestion and metabolism will be summarized.

1.2. Lipid digestion and absorption, and postprandial lipemia

1.2.1. Overview of gastrointestinal tract (GIT) and digestive physiology

The GIT is the largest endocrine organ in the human body, starting from the mouth and the esophagus, stomach, and then the small intestine (duodenum, jejunum, ileum), which is followed by the large intestine (colon), rectum, and lastly terminating at the anus (Johnson, 2014). The GIT is responsible for the breakdown and absorption of various foods and liquids and the excretion of waste products (Johnson, 2014). Proper functioning of the GIT is essential for the body to obtain the nutrients required for health.

Generally it can be considered that there are two types of digestion: mechanical (physically breaking down large pieces of foods into smaller pieces) and chemical (enzymes breaking down macromolecules into smaller molecules for absorption) (Johnson, 2014). When food is ingested, mastication in the mouth reduces the particle size and assists in the mixing of food particles with saliva, which lubricates the bolus and contains digestive enzymes (e.g.
salivary amylase) to initiate chemical digestion. This so-called bolus mixture is pushed through the pharynx, and then the esophagus, before it reaches the stomach which is lined with a mucous membrane consisting of glands secreting gastric juices that contain mucus, enzymes (e.g. pepsin for protein digestion), and hydrochloric acid (maintains a stomach pH of around 2 during the fasted state). Mechanical mixing forces and gastric juices help with the transformation of the bolus into chyme, a semi-liquid substance (Johnson, 2014).

The process by which ingested food enters and leaves (i.e. gastric emptying) the stomach can be affected by food composition (Ehrlein & Schemann, 2005). For example, gastric emptying of liquids starts immediately after eating and proceeds at an exponential rate, while it is slower and mainly linear for viscous contents (Figure 1.1) (Ehrlein & Schemann, 2005). This slower gastric emptying is partly caused by enhanced resistance to the flow of viscous chyme. Also, with increased chyme viscosity, the depth of the peristaltic constrictions is diminished and the propulsion is reduced (Ehrlein & Schemann, 2005). Solid food particles have to be sufficiently broken down before they can be evacuated from the stomach, thus a lag phase is observed in gastric emptying after consumption of a solid meal (Ehrlein & Schemann, 2005). Furthermore, gastric emptying is inhibited by feedback control occurring in the small intestine; hydrochloric acid, enhancing or diminishing osmolality of the chyme, and an increased amount of nutrients entering the small intestine participate in this regulation all play roles in this regulation (Ehrlein & Schemann, 2005). Cholecystokinin (CCK) is one of the most important hormones involved in controlling gastric emptying; it can mediate satiety by acting on the CCK receptors distributed throughout the central nervous system and slowing down gastric emptying (Shillabeer & Davison, 1987). The release of CCK from I-cells of the intestinal epithelium is induced by luminal hydrochloric acid, amino acids and fatty acids with at least 12-carbon chains.
The feedback-inhibition induced by nutrients is impacted by the amount of nutrients entering the small intestine and the length of the intestine having contact with such nutrients (Moran & Dailey, 2012). The interactions among lipid digestion, CCK regulation and gastric emptying will be discussed later.

Given the importance of gastric emptying in relation to food structure and stability in GIT, scientists have been measuring gastric emptying in human trials. Scintigraphy is considered the best validated standard procedure for such purpose, but it is expensive, requires nuclear medical equipment and involves radiation exposure (Willems, Otto Quartero, & Numans, 2001). The procedures might affect gastric emptying by presenting mental stress to participants (Medhus, Lofthus, Bredesen, & Husebye, 2001). Alternatively, acetaminophen (i.e. paracetamol) absorption test can be used as an indirect assessment of gastric emptying (Willems et al., 2001). In the test, acetaminophen is well mixed in a liquid meal which is consumed orally, and it travels with the meal until it leaves the stomach and reaches the duodenum, where it is rapidly absorbed and enters the blood stream; it is assumed that gastric emptying is the rate-limiting step in delivering acetaminophen to its absorption site, so its appearance in the blood reflects of the rate of gastric emptying (Bartholomé et al., 2015; Willems et al., 2001). This method has been widely applied in numerous human trials, as it is economical, safe, easy to perform and non-invasive hence less stress to participants, and it has been validated by a few studies (Medhus et al., 2001; Medhus, Sandstad, Bredesen, & Husebye, 1999; Willems et al., 2001). Nevertheless, a recent publication argued that plasma acetaminophen concentration is an inappropriate biomarker for gastric emptying in their human trial, potentially due to encapsulation of acetaminophen in the solid bolus caused by phase separation of meals in the stomach (Bartholomé et al., 2015). This,
again, shed light on the significance of food structure and stability in GIT, and more caution could be applied when interpreting plasma acetaminophen data as a measure of gastric emptying.

Figure 1.1. Gastric emptying of solid and liquid radiolabeled meals, plotted as the radioactive fraction remaining in the stomach against time (Camilleri, Malagelada, Brown, Becker, & Zinsmeister, 1985).

The small intestine is the site where food is further digested and most of the nutrients are absorbed, especially lipids (further details in Section 1.2.2.) (Johnson, 2014). There are three sections comprising the small intestine: the duodenum (the section distal to the pylorus of the stomach), the jejunum (the middle section), and the ileum (the section connecting to the large intestine via the ileocecal valve). After a meal, gut distension induces the intestinal motility, which is modulated by the luminal nutrients. Peristalsis, segmentation, and stationary clusters of contractions are the three most frequently occurring types of contractile movements of the small intestinal walls during the postprandial period. After a meal, the distal intestinal segments are filled, inhibiting the proximal intestinal motility; as a result, the number of stationary segmenting contractions increases while the number of peristaltic waves propagating towards the end of the
small intestine decreases. This feedback-regulation reduced the flow rate of chyme along the small intestine (Johnson, 2014).

The almost completely digested food from the small intestine is transported into the cecum which connects to the large intestine (colon). There, complex carbohydrates can be fermented by the microbiota harbored inside the colon, while water and certain nutrients (electrolytes, vitamins produced by the microbiota, and products of microbiotic fermentation, including acetate, propionate and butyrate) are absorbed at the mucosa of the intestinal wall (Johnson, 2014). Of note, the microbiota and their metabolites can play important roles in lipid metabolism, as discussed later. The remaining waste, i.e. stool or feces, is passed along to and stored in the rectum until it is excreted (Johnson, 2014).

1.2.2. Lipid digestion

Lipids are a macronutrient class which provides energy and other essential functions for the human body. Over 95% of dietary lipids are triacylglycerols (TAG) and the remainder are phospholipids, free fatty acids (FFA), sterols (animal sourced cholesterol and plant sourced phytosterols) and other minor lipids (Garaiova et al., 2007). The following discussion will focus on TAG. A TAG molecule consists of a glycerol backbone with three acylated fatty acids. The positions of the fatty acids are numbered by the stereochemical numbering system i.e. sn-1, sn-2 and sn-3. The fatty acids are carboxylic acids with an aliphatic chain and can vary in terms of chain length, degree and type of unsaturation (number of double bonds), and configuration of double bonds (adjacent hydrogen atoms on the same (cis) or opposite (trans) side of the double bond). For example, butyric acid is a saturated fatty acid with an aliphatic chain of four carbon atoms; in comparison, docosahexaenoic acid (DHA) is a fatty acid with a 22-carbon chain, in which there are six cis-double bonds and the first double bond locates at the third carbon from
the methyl group (also called the omega end, therefore such fatty acids are named \(\omega\)-3 polyunsaturated fatty acids (PUFA)). The different fatty acid structures determine their physicochemical properties, including water solubility, melting points, oxidative stability, etc., and these properties can influence the effects of fatty acids in biological systems, such as substrate specificity, lipid peroxidation, membrane fluidity, and gene interactions (Rustan & Drevon, 2005). For example, increased incorporation of very long chain \(\omega\)-3 PUFA can change the melting point of core cholesteryl esters in low-density lipoproteins (LDL) (Nenseter et al., 1992), which is known to affect the cellular pathways of LDL recognition during the early stages of atherosclerosis (Prassl, 2011). It is widely acknowledged that the dietary lipids play an important role in human health, i.e. they are a major energy sources and humans must ingest certain essential fatty acids (i.e. linoleic acid and \(\alpha\)-linolenic acid) to maintain normal physiological functions. However, overconsumption of lipids has been associated with obesity and metabolic disorders. Further, not all lipids contribute equally and their digestion and absorption may be impacted by other dietary components.

When food enters the mouth, chewing breaks down large food particles and disperses any dietary lipids in the bolus, which is later transferred to the stomach. TAG are partially hydrolyzed in the stomach by lingual and gastric lipases, which are highly active at low pH (3.0-6.0) (Fink, Hamosh, & Hamosh, 1984; Hamosh, 1990). Those lipases preferentially cleave off the sn-3 fatty acids, forming sn-1,2 diacylglycerols. Also, their activity is generally higher towards short- and medium-chain fatty acids than towards long-chain fatty acids (Mu & Høy, 2004). Although, in adults, lingual and gastric lipases account for only 10-30% of TAG digestion; infants rely more on the activity of these pre-duodenal lipases, because they can penetrate and hydrolyze milk fat, i.e. the main fat source for infants (Hamosh, 1990). Any initial
digestion of lipids facilitates their further digestion in the duodenum by reducing the lipid droplet size and forming hydrolysis products that increase TAG solubilization (Mu & Høy, 2004). Also, other food components, such as complex polysaccharides and phospholipids, as well as the peptic digestion products of dietary proteins could serve as emulsifiers. They assist in emulsification of dietary lipids in the stomach, a process which is also facilitated by muscle contractions and subsequent propulsion of lipids through the small opening of the pyloric sphincter and the associated high shear forces (Gropper & Smith, 2013).

Lipid digestion mainly occurs in the duodenum. As the bolus enters the duodenum, the secretion of pancreatic lipases and bile acids from the liver is induced (Mu & Høy, 2004). With the help of segmentation and peristalsis, the lipids are mixed with and further emulsified by the amphiphilic bile acids, such that large lipid droplets are broken down into smaller ones, with the water-oil interface stabilized by bile acids to prevent coalescence (Bauer, Jakob, & Mosenthin, 2005). The increase in droplet surface area allows more interfacial adsorption of pancreatic lipases and colipases, thereby facilitating lipid droplet hydrolysis. Pancreatic lipases preferentially hydrolyze the sn-1 and sn-3 ester bonds, resulting in the formation of sn-2 monoacylglycerol (MAG) and FFA. Further hydrolysis of MAG by pancreatic lipases and cholesterol esterases can produce glycerol and FFA.

Pancreatic lipase activity is impacted by many factors. Firstly, the presence of a double bond near the carboxyl group in very long chain ω-3 PUFA (e.g. DHA) makes it difficult for these fatty acids to be cleaved from TAG molecules (Bottino, Vandenburg, & Reiser, 1967; Giang et al., 2016; Zhu, Ye, Verrier, & Singh, 2013). This may have implications in manufacturing interesterified fats containing very long chain ω-3 PUFA, especially for use by individuals with impaired digestion, such as pancreatic deficiency and gastric tube feeding.
Secondly, although surface-active constituents such as bile salts and phospholipids are required for optimal lipid absorption, they inhibit pancreatic lipase activity at higher concentrations, potentially by displacing the enzymes from the oil-water interface to the aqueous phase (Bauer et al., 2005). Colipase acts as a bridge between pancreatic lipase and the lipid droplet and stabilizes the enzyme at the interface, thereby reducing this inhibition (Crandall & Lowe, 2001). Furthermore, the accumulation of lipolytic products in the oil-water interface can impede lipase activity (Larsson & Erlanson-Albertsson, 1986), so removing them from the interface is necessary for maintaining lipolysis.

Lipolytic products are removed from the oil-water interface by micellar solubilization with bile salts, which form a structure with polar groups projecting into the aqueous phase and nonpolar hydrocarbon ends remaining in the centre. Bile salts need to reach a critical micellar concentration to form a micellar solution, which is reduced to 0.75 to 1 mM in the presence of endogenous MAG and phospholipid in vivo (Bauer et al., 2005). However, the solubilization of digested fat is now regarded as being more complicated than previously understood. For example, there are various different colloidal structures present in intestinal fluids, including phospholipid vesicles, which may display differences in solubilizing capacity/affinity towards specific compounds (Elvang et al., 2016). Also, the nature of these colloidal structures can fluctuate, over time, and depend on digestate composition. Overall, the high water solubility of the colloidal structures increases the fatty acid concentration in the small intestinal contents, thus improving the transfer of lipolytic products from the lumen, across the unstirred water layer near the intestinal wall (considered as a main barrier to lipid absorption because the diffusion rate is slow here), and finally to the absorptive site: the enterocyte apical membrane (Camilleri, 2006).
In vitro models have been widely applied to simulate human lipid digestion. Compared with in vivo methods, they are less expensive and labor-intensive, do not have ethical restrictions, and easy to sample at the site of interest; thus, they can be useful tools to screen a relative large number of samples (Minekus et al., 2014). Static in vitro digestion models usually include the gastric and small intestinal phases, with optional oral and/or large intestinal phases; the following in vivo physiological conditions are simulated, including the concentration of digestive enzymes (e.g. pepsin and pancreatic lipase), pH, salt concentrations and digestion time (Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014). Static digestion models generally apply constant ratios of meal to the abovementioned digestive components at each step of digestion (Minekus et al., 2014), while there are some discrepancies between experimental conditions in different studies. These discrepancies often impede the comparison of results between publications and therefore, a consensus of standardized static in vitro digestion procedure (Figure 1.2) was published (Minekus et al., 2014) and is still being further developed (Brodkorb, 2018). There are remaining issues requiring investigation, which preclude the application of one method for analysis of all samples. For example, of relevance to lipid digestion is the fact that FFA can accumulate at the lipid droplet surface and inhibit pancreatic lipase activity, thus it is suggested to use the pH stat method in which FFA are neutralized (Minekus et al., 2014). However, it is difficult to get accurate measurements with pH stat when the salt composition is complex, as suggested in the consensus, and the pH of digestate contents are not as strictly controlled in vivo as in the pH stat. Also, unless a back-titration step is applied with the pH stat to re-dissolve precipitated fatty acids, they may effectively underestimate the lipid hydrolysis.
There are also dynamic models (e.g. TNO Gastro-Intestinal Model (TIM-1)), which are more sophisticated and allow for the transport of digested meals and changes in enzyme and biosurfactant concentrations and/or pH over time to be incorporated in the modeling parameters (Minekus et al., 2014; Thuenemann, 2015). TIM-1, one of the most well-known dynamic models, includes jejunal and ileal compartments, from which dialysate samples mimicking intestinal absorption can be collected (Figure 1.3). Nevertheless, there are still issues associated with TIM-1, such as high maintenance and difficulty of processing solid meal samples.

Overall, the question of how accurately *in vitro* digestion models mimic the complex physicochemical and physiological processes occurring within animals or humans, remains. A few studies correlating *in vitro* and *in vivo* support that *in vitro* models appear to be a good predictive tool for the digestibility and bioavailability of some lipids and lipophilic nutrients, such as interesterified lipids using the TIM-1 (Thilakarathna et al., 2016), protein-stabilized corn oil emulsions (Li, Kim, Park, & McClements, 2012), and beta-carotene (when the static model was combined with Caco-2 cells uptake model) (Alminger et al., 2012). More such studies should be conducted to confirm the validity and applicability of *in vitro* digestion results.
Figure 1.2. A flow diagram of a simulated *in vitro* digestion method adapted from the standardized model published by Minekus et al. (2014). SGF and SIF are Simulated Gastric Fluid and Simulated Intestinal Fluid, respectively. Enzyme activities are in units per mL of final digestion mixture at each corresponding digestion phase.
1.2.3. **Lipid absorption and tissue distribution**

The movement of FFAs across the enterocyte apical membrane is the first step of lipid absorption; two mechanisms have been proposed: a protein-independent diffusion and a protein-dependent uptake (Iqbal & Hussain, 2009). When FFA concentration in the lumen is higher than inside the cell, diffusion occurs across these epithelial cells; when extracellular concentration of FFA are lower, several proteins, including cluster of differentiation 36 (CD36) and various FA transport proteins are involved in the uptake of FFA and MAG (Hussain, 2014). In the
enterocyte, these lipolytic products are used to synthesize TAG along the MAG pathway, which involves monoacylglycerol and diacylglycerol acyltransferases (Iqbal & Hussain, 2009).

The synthesized TAG are packed in the enterocytes into several different lipoproteins, resulting in TAG-rich lipoproteins (TRL), which are secreted into the lymph and then circulated systemically for further utilization throughout the body. The main intestinally-derived TRL for transporting TAG is the chylomicron (CM), which is secreted following the ingestion of fat (Mu & Høy, 2004). To form primordial CM particles, ApoB48, a truncated form of apolipoprotein B (ApoB), is lipidated with neutral lipids, such as TAG. As there is only one non-exchangeable ApoB48 for each CM particle, measurement of ApoB48 is a strategy used to monitor the secretion and metabolic fate of intestinal CM (Xiao & Lewis, 2012). After ingestion of dietary fat, the CM size can indicate the amount of lipids resynthesized in the enterocytes; the amount of lipid transported by CM can be several-fold higher in the postprandial versus fasting state (Iqbal & Hussain, 2009). In contrast, the increase in CM particle numbers during the postprandial state does exist but will be relatively less than the CM size does (Xiao & Lewis, 2012). TAG in CM are metabolized by lipoprotein lipases (LPL) bound to the capillary endothelium of adipose tissue and muscle. These hydrolyze CM-TAG and release FA for tissue uptake (Lambert & Parks, 2012). After hydrolysis, CM are converted into smaller and denser particles (i.e. remnants), which are taken up by the liver; some released FA are not utilized by tissues at this point but rather “spillover” into a FFA pool to be taken up by liver or other tissues later (Lambert & Parks, 2012). The TAG remaining in CM remnant particles are repackaged into another main type of lipoprotein called very low density lipoproteins (VLDL), which are constantly synthesized in liver and are the predominant TRL during fasting conditions (Lambert & Parks, 2012; Lopez-Miranda, Williams, & Lairon, 2007). The liver derived, ApoB100-containing
VLDL carry the recycled dietary FA from CM remnants, and they are secreted into circulation to allow lipids to be stored in tissues (Lopez-Miranda et al., 2007; Pirillo, Norata, & Catapano, 2014).

It should be noted that, carbohydrate consumption and hence insulin play important roles in the FA uptake into tissues; glucose infusion and postprandial insulinemia have been shown to up-regulate LPL, which suppresses lipolysis from adipose tissue and hepatic VLDL production (Lopez-Miranda et al., 2007), and results in up-regulation of enzymes for TAG synthesis and storage (Lambert & Parks, 2012). However, the insulin action is compromised in insulin resistance and type 2 diabetes, resulting in high TRL concentrations in these individuals (Lambert & Parks, 2012). High levels of TRL and their prolonged residence time in the circulation may contribute to a higher atherosclerotic risk, because TRL remnants are small and dense, allowing them to penetrate the arterial wall and be retained in the subendothelial space (Pirillo et al., 2014; Tomkin & Owens, 2012).

1.2.4. Postprandial lipemia and cardiovascular health

Postprandial lipemia refers to the metabolic events occurring after consumption of fat-containing food, including the rise in TRL and the fluctuation of other lipoproteins in blood, including high, low and very low density lipoproteins (HDL, LDL and VLDL, respectively), each of which has been associated with cardiovascular disease (CVD) risk (Pirillo et al., 2014). Given that humans spend most of their time in this dynamic, non-steady postprandial (fed) state, it is important to consider how postprandial lipemia contributes to CVD risk and how this differs between lipids and is impacted by different foods.
In recent years, several large epidemiological studies in Denmark (Freiberg, Tybjaerg-Hansen, Jensen, & Nordestgaard, 2008; Langsted, Freiberg, & Nordestgaard, 2008), USA (Bansal et al., 2007) and Japan (Iso et al., 2014) have demonstrated that, instead of fasting TAG level, postprandial TAG level is more strongly associated with the incidence of cardiovascular events, such as myocardial infarction, ischemic heart disease, ischemic stroke (Bansal et al., 2007; Freiberg et al., 2008; Iso et al., 2014; Langsted et al., 2008). As mentioned previously, the magnitude of the rise in postprandial lipemia, especially the presence of remnant particles, could promote pathogenesis and progression of atherosclerosis, as discussed in section 1.3.2. Thus, delayed clearance of CM from circulation could pose an important atherogenic risk factor (Bysted, Hølmer, Lund, Sandström, & Tholstrup, 2005). Furthermore, the fatty acid composition of TAG in CM closely resembles the composition of dietary lipids consumed (Mu & Høy, 2004). The compositional differences in circulating lipid fractions may contribute to different atherogenic risk potentials, as the quantity, degree of saturation, and chain length of fatty acids may affect LPL activity, and thus influence the production of atherogenic remnants (Tholstrup & Bysted, 2001). Additionally, different food components, including fibres, have been shown to modulate postprandial lipemia (see section 1.3.3). Such correlations between ingestion of food ingredients, postprandial lipemia, and atherogenicity make it interesting to compare the absorption and clearance of TRL following ingestion of meals of differing compositions.

1.3. Dietary fibre

1.3.1. Introduction

Dietary fibre (DF) is generally defined as plant-sourced carbohydrates which are resistant to enzymatic digestion in the human upper GIT, such that they reach the colon for bacterial fermentation (Dikeman & Fahey, 2006). In addition, novel ingredients (synthesized, modified or
natural ingredients without a history of safe use as DF) can be approved as DF in Canada, as long as there are generally accepted scientific evidence demonstrating that they exhibit one or more of the following physiological effects but not limited to: improving laxation or regularity by increasing stool bulk, reducing blood total and/or LDL cholesterol levels, reducing postprandial blood glucose and/or insulin levels, and providing energy-yielding metabolites through colonic fermentation (Health Canada & Bureau of Nutritional Sciences, 2012).

Many epidemiological and experimental results have indicated that increased DF consumption is consistently associated with decreased risk of developing chronic diseases, including CVD, cancers, diabetes and obesity. Firstly, in large epidemiological studies, DF intake is found to be inversely associated with risk factors for CVD, such as blood pressure, serum total cholesterol, serum insulin levels (Ning, Van Horn, Shay, & Lloyd-Jones, 2014; Zhou et al., 2015) and C-reactive protein (CRP) (Grooms, Ommerborn, Pham, Djoussé, & Clark, 2013; Ning et al., 2014), while it was positively associated with serum HDL cholesterol levels (Ning et al., 2014; Zhou et al., 2015). In interventional studies, DF supplementation significantly lowered total and LDL cholesterol (Kristensen & Bügel, 2011; Ravn-Haren et al., 2013) and inflammatory biomarkers such as plasminogen activator inhibitor-1, factor VII (Kristensen & Bügel, 2011) and CRP (Jiao, Xu, Zhang, Han, & Qin, 2015) in the fasting state.

Secondly, an inverse relationship has also been observed between DF intake and the development of several types of cancers. Per 10 g/d increase in total DF consumption decreases the risk of developing colorectal adenomas by 9% (Ben et al., 2014) and the subsequent colorectal cancer by 10% (Aune et al., 2011). Supplementation of lupin kernel fibres (17-30 g/d for 2 to 4 weeks) increased daily fecal dry matter and fecal weight, lowered fecal pH, enhanced SCFA formation, reduced fecal concentrations of total and secondary bile acids (Fechner,
Fenske, & Jahreis, 2013), and stimulated colonic *bifidobacteria* growth (Johnson, Chua, Hall, & Baxter, 2006; Smith et al., 2006) (further discussed in section 1.3.3). The 10 g/d increase in DF intake is also associated with reductions in the risk of developing gastric cancer (44%) (Zhang, Xu, Ma, Yang, & Liu, 2013), breast cancer (5%) (Aune et al., 2012), and all cancer mortality (9%) (Liu, Wang, & Liu, 2015).

Lastly, a significant reduction in the prevalence of diabetes has also been associated with higher levels of DF intake (Anderson et al., 2009; The InterAct Consortium, 2015; Yao et al., 2014), and this may be partially explained by body weight management (The InterAct Consortium, 2015). A high level of DF consumption (> 15.55g/100kcal) consumed by people who were at risk of developing type 2 diabetes was associated with more sustained and significant body weight reductions (≥5% of their baseline weight) and a 62% reduction in progression from prediabetes to diabetes, compared with individuals consuming the lowest intake of DF (< 10.85 g/1000 kcal) (Lindstrom et al., 2006). Similar results are observed in other epidemiological studies with individuals of diverse ethnic/racial backgrounds, indicating a general protective effect of DF consumption against diabetes progression and obesity development (Anderson et al., 2009). This may be attributed to DF’s ability to improve fasting plasma glucose and insulin levels, to prevent postprandial glycemia and insulinemia, and/or to delay gastric emptying and enhance the satiety, which were observed in several clinical trials with soluble DF (e.g. psyllium, pectin, konjac mannan, guar gum, etc.) (Anderson et al., 2009; Papathanasopoulos & Camilleri, 2010; Slavin, 2005). Also, including DF in diet generally decreases the diet’s metabolizable energy (ME, which is gross energy minus the energy lost in the feces, urine and combustible gases), as DF are resistant to GIT digestion (Baer, Rumpler, Miles, & Fahey, 1997). However, this relationship is affected by DF solubility; soluble DF
decreased ME with a low fat diet and increased ME with a high fat diet (Baer et al., 1997; Isken, Klaus, Osterhoff, Pfeiffer, & Weickert, 2010), while insoluble DF had the opposite effect (Isken et al., 2010). The mechanism by which DF solubility determines the effect on ME remains unclear.

1.3.2. Gap in the recommended current DF intake and strategies to address

Given all the evidence demonstrating DF’s health benefits, there has been rising awareness to improve DF intake. On this point, the average daily DF consumption of North American adults is 15 g/d; in contrast, the current dietary recommended intake value is 14 g/1000 kcal (based on the decreased risk of coronary heart disease with DF consumption), which equates to approximately 25 g/d for women and 38 g/d for men (Jones, 2014). Based on National Health and Nutrition Examination Survey (NHANES) 2003-2006 data, under 5% of the United States’ population meets the recommended intake (Jones, 2014) and the numbers are expected to be similar in Canada.

In support of addressing the current “fibre gap”, CODEX recommends recognizing and naming all substances that behave like fibre as DF, regardless of how they are produced, as long as they show physiological benefits (Jones, 2014). Also, consumer education programs that help the public to identify isolated DF in fortified food, to choose foods naturally rich in DF, and to model diets which achieve DF recommendations are suggested as strategies to address the “fibre gap” problem (Jones, 2014). In addition, DF supplementation of common food products, including pasta, bread and yogurt, may help to improve fibre intakes. Indeed, a large body of academic and applied research has addressed the fact that addition of DF to foods can impact texture, sensory properties, palatability and consumer acceptance. Importantly, careful consideration is rarely, but should also be given to the impacts that DF fortification could have
on the bioavailability and efficacy of other nutrients present in foods. For example, some DF have the potential to alter emulsion microstructure and therefore, impact lipid digestion. This includes by inducing emulsion droplet aggregation, thereby reducing the interfacial area for lipases; thickening the intestinal contents, thereby limiting contact between lipases and lipid droplets; and binding calcium ions and/or bile salts, which act to reduce the presence of accumulated FFA at lipid droplet surfaces (Dikeman & Fahey, 2006; Zhang, Zhang, Zhang, Andrew, & McClements, 2015). Such interactions and their impacts on lipid digestion and bioavailability require further investigation (see section 1.3.3).

1.3.3. Physicochemical properties of DF and their physiological functionalities

As discussed in section 1.3.1, the effects of DF intake on chronic diseases vary among different categories of DF (e.g. source/solubility) (Baer et al., 1997; Isken et al., 2010; Threapleton et al., 2013; Wu et al., 2015) and there is interest in investigating DF’s physiochemical properties and correlated physiological effects. The physiological functionalities of DF may be attributed to their physical and chemical properties, such as solubility, viscosity, and fermentability by colonic microbiota (Guillon & Champ, 2000; Topping, 2013).

DF solubility indicates the ability of the DF to be well-dispersed in solution and depends on their molecular structure; when there are more irregularities, such as multi-type linkages between monomers, branched structures, and existence of charged groups, the DF molecules are less able to form ordered crystalline structures, allowing the molecules to be dispersed in water. Furthermore, there could be physical interactions between polysaccharide molecules, which cause molecular entanglement, thereby inducing viscosity. Some common types of soluble DF include pectin, gums (e.g. guar gum), β-glucan, and psyllium, which natural food sources include some vegetables and fruits, oats, barley, and dried beans (Theuwissen & Mensink, 2008). When
ingested, some water-soluble DF such as pectin and gums are able to thicken gastric and intestinal contents, which has been associated with beneficial physiological responses (Dikeman & Fahey, 2006). Firstly, postprandial glucose response may be delayed and/or attenuated, potentially by slowing down gastric emptying and extending the small intestinal transit time through forming a gel matrix (Dikeman & Fahey, 2006). DF can thicken GIT contents and alter resistance of digesta to contractile movements in the GIT, thereby decreasing contact between food and digestive enzymes and diffusion of nutrients for absorption (Dikeman & Fahey, 2006).

Secondly, the consumption of foods rich in soluble viscous DF has been acknowledged to improve lipid profiles, especially in terms of lowering cholesterol (Gunness & Gidley, 2010). In addition to slowing down lipid digestion and absorption (further discussion in section 1.3.4) in a manner similar to reducing postprandial glucose response, three other potential mechanisms are proposed: 1) soluble viscous DF may form a local matrix entrapping bile salt micelles or bind with bile salts, inducing excess fecal bile salt excretion and preventing their reabsorption into enterohepatic circulation; 2) the low glycemic response caused by soluble viscous DF consumption leads to attenuated insulin levels, resulting in the inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-Co AR), an enzyme necessary for hepatic cholesterol synthesis; 3) short chain fatty acids (SCFA), i.e. the products of DF fermentation by colonic microbiota, could inhibit hepatic cholesterol metabolism by reducing activity of HMG-Co AR (Gunness & Gidley, 2010).

Additionally, soluble viscous DF improve laxation, as they retain more moisture in the large intestine, resulting in softer stools (Dikeman & Fahey, 2006). Although insoluble DF may not have the same abovementioned functions as soluble viscous DF, they exert additional physiological effects such as increasing stool bulk and promoting regularity (Anderson et al.,
Furthermore, a recent in vitro study revealed that cellulose, a common insoluble DF in diet, may inhibit the activity of α-amylase against starch by non-specific binding; therefore, cellulose in the diet can potentially attenuate starch hydrolysis and lead to decreased postprandial glycemia and insulinemia (Dhital, Gidley, & Warren, 2015).

Another important property related to DF functionality is fermentability, i.e. the ability of DF to be digested by colonic microbiota. Butyrate, one of the major SCFA produced from colonic fermentation, is the preferred energy source for colonic epithelial cells; it promotes normal cell differentiation and proliferation and reduces chronic inflammation and cancer cell migration and invasion, thereby reducing colorectal cancer risk (Slavin, 2013; Zeng, Lazarova, & Bordonaro, 2014). SCFA also participate in regulating the absorption of water, sodium and other minerals, and maintaining the balance between the growth of potential pathogens (e.g. Salmonella) and beneficial bacteria (e.g. Bifidobacteria and Lactobacilli) (Slavin, 2013; Sun & O’Riordan, 2013). DF fermentability, including the amounts, ratios, and rate of production of SCFA, depends on the accessibility to microorganisms, which can be influenced by the chemical structure and physical properties of DF (Slavin, 2013). Particle size, porosity and surface area of DF molecules can influence DF fermentability, but they vary during GI transit such that their properties before digestion are not necessarily related to subsequent behaviors in the GIT (Oakenfull, 2001). In contrast, solubility plays the most important role in DF fermentability. Generally, soluble DF such as pectin and guar gum are very accessible and fermented rapidly in the proximal colon, while insoluble DF are less available for fermentation (Topping, 1991). Overall, soluble DF appear to have more remarkable influences on the processes of lipid digestion compared to insoluble DF.
1.3.4. Soluble DF’s effects on lipid digestion – evidence from in vitro digestion studies

Simulated in vitro digestion models have been applied to study the potential mechanisms by which soluble DF could potentially affect lipid digestion. In terms of the effects on lipase activity, 10 g/L different soluble DF (pectin, carrageenan, or gum arabic) was found to have no effect on the lipolysis of a 33 wt% tributyrin sample, measured by titration (Hendrick, Tadokoro, Emenhiser, Nienaber, & Fennema, 1992). Of note, those lipolysis experiments were conducted for 30 minutes only, while digestion in the human body would persist for longer. Another study agreed with the finding, however, i.e. the addition of 0.3 wt% soluble DF (guar gum, apple pectin, and gum arabic) solution did not change the initial velocity of lipolysis of an emulsion of triolein, lecithin and cholesterol (Pasquier et al., 1996). Interestingly, in the same study, 0.3 wt% guar gum did lower the overall extent of lipolysis after 2 hours of duodenal digestion, indicating the potential for inhibiting lipolysis over time (Pasquier et al., 1996). Similarly, the inclusion of 0.5 wt% citrus pectin decreased FFA release from 9.5% fat beef patties after 2 hours of duodenal digestion (Hur, Lim, Park, & Joo, 2009). In contrast, other studies have found no effects of soluble DF on in vitro simulated lipolysis (Pasquier et al., 1996; Tokle, Lesmes, Decker, & McClements, 2012), potentially attributable to discrepancies in sample composition and/or experimental procedures, as discussed in section 1.2.2.

The interactions between soluble DF and surfactants on lipid droplets and/or other digestive components depend heavily on molecular and physicochemical properties and such interactions can also induce structural changes in lipid droplet structure and affect lipolysis (Dickinson, 2003). Zhang et al. (2015) demonstrated that the addition of low methoxyl pectin (LMP) improved lipid digestion in caseinate-stabilized emulsions, by adsorbing onto droplet surface and suppressing the droplet flocculation, while it limited the initial rate of lipolysis of
Tween 80- and lactoferrin-stabilized droplets, potentially due to calcium-binding or gel formation effects (Zhang et al., 2015). In contrast, Qin et al. (2016) investigated different DF’s effect on β-lactoglobulin-stabilized emulsions; it was found that alginate inhibited lipid hydrolysis by binding calcium ions and promoting depletion flocculation, while locust bean gum did not appear to interact strongly with any charged species in the GIT and therefore, had no significant impact on lipolysis (Qin, Yang, Gao, Yao, & McClements, 2016). Similarly, another study applied either alginate or high methoxyl pectin (HMP) in an emulsion digestion in the presence of calcium, and found that the calcium-binding alginate strongly depressed the emulsion digestion, while HMP did not bind calcium and showed no inhibitory effect (Hu, Li, Decker, & McClements, 2010). This highlights the complex interactions that might occur and impact DF’s role in lipid digestion.

The abovementioned effects of soluble DF mainly involve the following mechanisms: 1) electrostatic interactions (e.g. bridging flocculation and steric stabilization), which depend on the nature of the DF molecules and the solution conditions, including pH and ionic strength; 2) depletion flocculation induced by the presence of excessive non-adsorbing DF molecules; and 3) their interactions with other molecular species involved in lipolysis (e.g. calcium, bile salts and fatty acids) (Dickinson, 2003; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014; Hu et al., 2010; Qin et al., 2016; Zhang et al., 2015). Because of sampling ease with in vitro digestion models, researchers are able to study the structural changes of lipid droplets during digestion, mainly by surface charge measurements, particle size analysis, and microscopy. When soluble DF polymers carry the opposite charge of surfactants of the lipid droplets, they adsorb onto the droplet surface due to the electrostatic attraction between them (Shi, 2002). This may cause 1) steric stabilization, if the repulsion caused by the adsorbed
polymer layers between two droplets is strong enough, or 2) bridging flocculation, if the affinity between the polymers overcomes the repulsion or if one long polymer adsorbs onto two droplets when the polymer concentration in the system is low (Shi, 2002). Due to the polymer adsorption, the surface charge of lipid droplets is changed, and the droplet destabilization can be observed in confocal microscopy. However, sometimes the flocculation may be too weak to withstand the dilution and stirring during the sampling process for particle size analysis therefore, it is not reflected as increased droplet sizes (Espinal-Ruiz et al., 2014; Qin et al., 2016). On the other hand, when soluble DF polymers carry the same charge as surfactants, or when the surfactants are neutral, the non-adsorbed polymers (beyond a certain critical polymer concentration) could induce depletion flocculation by affecting the osmotic pressure gradient of the solution, which allows pairs of particles’ surfaces to approach each other, deplete polymers between the gap, and flocculate under the influence of an attractive interparticle force (Dickinson, 2003). Of note, in addition to the concentration of polymers in solution, there are other factors affecting the magnitude of depletion flocculation, including polymer molecular weight and sphericity (Dickinson, 2003; Espinal-Ruiz et al., 2014). Thus, depletion flocculation could be relatively weak and reversible such that it is not observed by particle size analysis (Espinal-Ruiz et al., 2014; Qin et al., 2016; Zhang et al., 2015).

Digestate viscosity is another attribute that has been investigated and related to soluble DF in relation to possible impacts on lipid digestion. It is postulated that, digestive events, such as the adsorption of lipases onto lipid droplets and the diffusion of digested lipids, could be impacted by a viscous medium (Dikeman & Fahey, 2006). Using an in vitro model, soluble DF, including guar gum, psyllium and oat bran, induced viscous characteristics throughout small intestinal content simulations (Dikeman, Murphy, & Fahey, 2006). Furthermore, when soluble
DF were added to a lipid mixture before emulsification, the extent of emulsification, droplet size, and the overall droplet surface area were strongly correlated to the medium viscosity in the range 0-20 mPa·s, and that high- and medium-viscosity guar gums significantly reduced the extent of TAG in vitro hydrolysis, while low viscous DF did not exert any inhibitory effects (Pasquier et al., 1996; Pasquier et al., 1996).

Overall, there are several potential mechanisms by which soluble DF can influence lipid digestion, dependent on their different structural and physicochemical properties. In the next section, the discussion will be mainly focused on a specific soluble DF, pectin, which is commonly occurring naturally in foods and as a food additive and which has specific relevance to this thesis.

1.4. **Pectin and the effects of its consumption on lipid metabolism**

1.4.1. **Introduction to pectin**

Pectin is a group of polymers commonly found in the middle lamellae of plant cell walls. It consists of a 1,4-linked α-D-galactosyluronic backbone and neutral sugar side chains, and is overall negatively charged (Thakur, Singh, Handa, & Rao, 2009; Yapo, 2011) (Figure 1.4).

There are many factors affecting pectin’s physicochemical and functional properties. For example, its water solubility depends on the degree of polymerization (the number of monomeric units in a polymer, which contributes to the molecular weight) and the number and distribution of methoxyl groups (degree of methylation, DM) on the galacturonic acid residue, as shown in Figure 1.4 (Thakur et al., 2009). The gelling properties of pectin depend heavily on DM, i.e. HMP (DM = 50 – 80%) forms gels when the pH is below 3.6 and a cosolute (e.g. sucrose > 55 wt%) is present, while LMP (DM = 25 – 50%) forms gels in the presence of calcium ions, which
bridge the carboxyl groups between pectin molecules (Thakur et al., 2009). Pectin from natural sources (e.g. apple pomace and citrus peels) are usually HMP and can be processed with pectin methylesterase to yield LMP (Thakur et al., 2009). Various other factors, both intrinsic and extrinsic, affect pectin solubility and gelation, including charge distribution along the backbone, average molecular weight, ionic strength, pH, and temperature (Thakur et al., 2009). Therefore, when comparing studies involving pectin, these factors should be considered.

Figure 1.4. A repeating segment of the pectin molecule, with a methylated carboxyl group on the second galacturonic acid residue (Thakur et al., 2009).

1.4.2. Pectin consumption and dietary lipid metabolism

There is extensive evidence from animal and human studies about cholesterol-lowering effects of regular consumption of pectin or a pectin-rich diet (Aprikian et al., 2002; Fillery-travis, Gee, Waldron, Robins, & Johnson, 1997; Judd & Truswell, 1985; Marounek, Volek, Synytsya, & Čopíková, 2007; Sánchez et al., 2008; Wilson, Wilson, & Eaton, 1984). For example, in a meta-analysis by Brown et al. (1999), it was estimated that 1 g/day pectin consumption could
lead to $-0.07$ and $-0.05$ mmol/L changes in total and LDL cholesterol concentrations, respectively (Brown, Rosner, Willett, & Sacks, 1999). In addition, several postprandial studies reported other beneficial health-related outcomes after pectin consumption, such as delaying gastric emptying (Di Lorenzo, Williams, Hajnal, & Valenzuela, 1988; Sandhu, El Samahi, Mena, Dooley, & Valenzuela, 1987; Schwartz, Levine, Singh, Scheidecker, & Track, 1982) and increasing satiety (Di Lorenzo et al., 1988). In contrast, less attention has been paid to pectin’s effects on postprandial plasma TAG level, which is a significant risk factor for cardiovascular health (as discussed in section 1.2.4), and there is more discrepancy in the results that do exist. Chronically, pectin consumption was shown to decrease plasma TAG in hamsters (Trautwein, Kunath-Rau, & Erbersdobler, 1998) and rats (Lærke, Meyer, Kaack, & Larsen, 2007), but not in human participants with either normal (Frape & Jones, 1995) or abnormal glucose metabolism (Schwab, Louheranta, Törrönen, & Uusitupa, 2006). There are even scarcer researches about the acute effects of pectin on postprandial lipemia. One study found that feeding pigs a simulated human meal containing 60 g/kg of beet pulp (rich in pectin, 13.7 – 24.4% soluble DF) delayed the postprandial TAG peak (Leclere, Lairon, Champ, & Cherbut, 1993), while another showed that sugar beet fibre (rich in pectin, unspecified concentration) consumption with a high-fat meal did not change the postprandial circulation of TAG in healthy adults (Morgan, Tredger, Shavila, Travis, & Wright, 1993). In that human study, participants were given the test meal (52 g double cream, 17 g plain chocolate and 1.5 g paracetamol) with or without 10 g sugar beet fibre; blood samples were routinely analyzed up to 210 min for serum TAG and plasma paracetamol, i.e. assessments of postprandial lipemia and gastric emptying, respectively (Morgan et al., 1993). Given that chocolate is a good resource of polyphenols, which have been shown to positively influence postprandial lipemia and oxidative stress (Annuzzi et al., 2014), the lipemia-
attenuating functions of DF may have been affected. Clinical trials should have more detailed attention to test meal composition, in order to acknowledge and control for potential influences from other food components. Additionally, *in vitro* studies have investigated the effects of pectin on different gastrointestinal processes at the molecular level and since these results and physicochemical properties can vary significantly based on DM, molecular weight and interactions with other constituents, these details need to be stated and considered, whenever possible.

Pectin has been shown to delay gastric emptying in healthy and obese individuals, potentially contributing to satiety and prolonging the time required for lipid digestion (Di Lorenzo et al., 1988; Flourie, Vidon, Chayvialle, Palma, & Bernier, 1985; Schwartz et al., 1982). A recent study attempted to relate digestate *in vitro* viscosity induced by both HMP and LMP to *in vivo* human satiety (measured by subjective ratings). However, a strong correlation was not found, potentially because digestate viscosity is not the only factor contributing to fibre-induced satiety (Logan, Wright, & Goff, 2015). Other *in vitro* studies have demonstrated that a relatively high concentration (0.6 – 0.8 %) of pectin reduced fat emulsification in acidic gastric and duodenal mediums, i.e. larger emulsion droplets were formed in the presence of pectin (Pasquier et al., 1996; Pasquier et al., 1996). However, this increased gastric droplet size was not observed in an animal study and, again, it was postulated that continuous phase viscosity is not the primary determinant of droplet size, as long as it is not so high as to prevent isotropic turbulence in the stomach (Fillery-travis et al., 1997). Unfortunately, no viscosity data was presented in that study, so it is unknown if test meal viscosity in the animal study was comparable to the *in vitro* studies.

*In vitro* digestion experiments have shown that the presence of HMP (Beysseriat, Decker, & McClements, 2006) and LMP (Tokle et al., 2012) in emulsion-based meals can impact
emulsion stability during digestion differently, based on the interfacial composition of the emulsion droplets and varying pH in the GIT (Beysseriat et al., 2006; Tokle et al., 2012). Adding HMP in a Tween-80 stabilized emulsion led to aggregation throughout digestion, and depletion flocculation was postulated because both Tween-80 and pectin are anionic at the pH of different digestion compartments (2 – 7.5) (Beysseriat et al., 2006). In contrast, both HMP and LMP-containing emulsions stabilized with lactoferrin exhibited gastric stability but destabilized in the intestine. This was attributed to lactoferrin being cationic in the acidic pH range such that the anionic pectin may have adsorbed to the interface, formed a second coating layer on the emulsion droplets and inducing steric repulsion from the side branches on the pectin molecules (Tokle et al., 2012). However, in the intestinal phase, the lactoferrin-coated droplet surface exhibited a negative charge such that pectin did not adsorb (Tokle et al., 2012). A recent study also showed that, during in vitro digestion, pectin with different DM formed gel-like structures entrapping oil droplets at different stage of digestion; this gel-like structure was firstly observed with LMP (DM = 14%) before digestion, during the stomach phase, and then with medium-methoxylated pectin (DM = 66%) in the duodenal phase, while HMP (DM = 99%) had minimal effects on digestate viscosity (Verrijssen et al., 2014). The formation of gel-like structures was related to the digestive fluid ionic strength and pH (Verrijssen et al., 2014). Thus, the design of pectin-containing emulsions should be done carefully and with consideration of GIT pH at different stages, emulsifier charge, and the potential interaction between pectin molecules and interfacially active molecules.

The effect of pectin on lipase activity in the small intestine has also been investigated. Two studies using a triolein-phospholipid suspension as the substrate demonstrated that 0.25 – 5 mg/mL citrus pectin, especially the high molecular weight section containing galacturonic acid,
exhibited strong inhibitory effects on lipolysis (Edashige, Murakami, & Tsujita, 2008; Tsujita et al., 2003). Furthermore, in a dose-dependent manner, pectin reduced the amount of lipase in the fat layer of an emulsion, which may lead to fewer interactions between lipase and the substrate (Tsujita et al., 2003). It was postulated that pectin interacted with the emulsified lipids, as opposed to interacting directly with the lipase (Tsujita et al., 2003). Indeed, in another study where lipase activity was studied in model digestion solutions (4-nitrophenyl palmitate as the substrate), pectic polysaccharides resulted in non-competitive lipase inhibition (Espinal-Ruiz et al., 2014).

The mechanisms by which pectin influences lipase activity have been investigated. One study found that the presence of 0.8% (w/v) pectin reduced the efficiency of emulsifying triolein with phospholipids and increased the emulsion droplet diameters and the duodenal digestate viscosity, i.e. factors which could influence lipid digestibility in the duodenal phase (Pasquier et al., 1996). In addition, food composition can play an important role in pectin’s effect on the overall lipolysis. Lower in vitro lipolysis was observed with 0.5% HMP-added beef patties (Hur et al., 2009), and less FFA were released from egg yolk encapsulated with 1% pectin (Hur, Kim, Choi, & Lee, 2013). However, the addition of 0.5% pectin to a lactoferrin-stabilized emulsion did not change the extent of lipolysis (Tokle et al., 2012). The discrepant results may be explained by the interactions between pectin and other food components which can be drastically different depending on their structural and physicochemical properties.

TAG digestion products, especially long-chain fatty acids and monoacylglycerols need to be solubilized in mixed micelles with bile salts for absorption. The processes involved could be impeded by the presence of pectin. For example, pectin binds to some individual bile acids or salts with a concentration of 0.1-2.2 mM (Cheewatanakornkool et al., 2012; Dongowski, 1995;
Falk & Nagyvary, 1982). Although these experiments were not conducted with a mixture of representative bile acids or bile salts under simulated intestinal condition and the concentration of bile salts applied in the standardized in vitro digestion model is 10 mM (Minekus et al., 2014), i.e. much higher than those studied, this is evidence of the potential interactions. Indeed, Hillman et al. (1986) found that 4 weeks of administration of HMP altered the bile salt metabolism and biliary lipid composition (Hillman, Peters, Fisher, & Pomare, 1986), which may be partially explained by the bile acid-binding theory.

Before digested and solubilized lipids can be absorbed, the mixed micelles have to pass through the unstirred water layer along the intestinal lining. Using an electrical technique, it was shown that pectin (unspecified type) thickens the unstirred water layer in rabbits (unspecified pectin) (Gerencser, Cerda, Burgin, Baig, & Guild, 1984), rats (citrus pectin with unknown DM) (Fuse, Bamba, & Hosoda, 1989), and humans (apple-derived HMP (Flourie, Vidon, Florent, & Bernier, 1984) and citrus pectin with unknown DM (Fuse et al., 1989)). The human study utilized an electrical technique, which measures the transmural potential difference between an electrode situated in the intestinal lumen and a subcutaneous reference electrode; it also requires an intramuscular injection of propantheline bromide to abolish the fluctuations in potential differences associated with intestinal motility (Flourie et al., 1984). Of note, because it is difficult to measure how many micelles successfully penetrate the water layer and the mucosal cell membrane in humans, it can only be postulated that thickened water layer may lead to difficulties for micelle transfer and subsequent absorption (Fuse et al., 1989).

1.4.3. Apple as a complex food matrix rich in pectin

Apples are known to be a good source of pectin. For example, gala apples can provide ~5.7 mg/g fresh weight pectin (Gheyas, Blankenship, Young, & McFeeters, 1997). Additionally,
apple is also rich in some insoluble DF such as cellulose and xyloglucans (Colin-Henrion, Mehinagic, Renard, Richomme, & Jourjon, 2009). Since apples are a leading produce crop in Ontario, they constitute a readily accessible food item for the customers to consume in order to increase their consumption of DF. In addition to DF, apples contains other anti-lipolytic components, including polyphenols; 10 μg/mL apple polyphenols have been shown to inhibit ~65% of pancreatic lipase activity in vitro (Sugiyama et al., 2007). Therefore, it would be interesting to investigate the effects of consuming apples and apple-derived products on lipid metabolism. There is limited work published in this area. Ravn-Haren et al. (2013) demonstrated that 4-week consumption of whole apples (550 g/day) or apple pomace (22 g/day) decreased fasting LDL cholesterol concentrations, compared to when DF-free apple juice (500 mL/day) was consumed. Hence, they concluded that the DF component is necessary for apple’s cholesterol-lowering effects (Ravn-Haren et al., 2013). Similarly, 20% lyophilized apple supplementation lowered fasting LDL cholesterol by 70% and also reduced TAG accumulation in the hearts and livers of obese rats (Aprikian et al., 2002). The same research group found that these lipid-lowering effects were more significant when apple pectin and a polyphenol-rich apple concentrate were fed together, versus separately, in rats (Aprikian et al., 2003). This further illustrates the potential benefit of consuming whole apples as a complex food matrix. It agrees with a recent literature review suggesting a synergistic relationship between fibre and polyphenols in whole apples (Bondonno, Bondonno, Ward, Hodgson, & Croft, 2017); it was speculated that the synergistic effects were mediated by gut microbiota (Bondonno et al., 2017) and hence are more relevant to the chronic consumption of apples. Very critical, to date, there have been no studies published on the acute effects of apple consumption on postprandial
lipemia in humans. This gap is particularly important considered that postprandial lipemia is a risk factor for human health (as discussed in 1.2.4).

1.5. Oral fat tolerance testing (OFTT)

An OFTT is a test meal providing a high amount of fat and where the rise in postprandial circulating lipids is measured. The fat can be delivered in different forms, each with advantages and disadvantages. These include simple beverages like milk shakes (simple and accommodate large amount of fat) or more complex liquid-solid meals (more akin to typical eating behaviors and feelings, but complicated food matrix effects) (Lairon, Lopez-Miranda, & Williams, 2007). OFTT has been widely applied in clinical trials to study postprandial lipid metabolism, such as detecting the underlying defects of lipid metabolism and comparing the postprandial lipid responses to specific foods or nutrient mixtures (Lairon et al., 2007). For example, it was found that an OFTT consisted of solely emulsified lipids induced accelerated postprandial TAG response and increased concentration of inflammatory biomarkers in subjects with elevated fasting TAG level, while the polyunsaturated/saturated fatty acid ratios in the OFTT did not have any influence (Dekker et al., 2009). Many of the previously mentioned studies investigating DF’s effects on lipid metabolism utilized OFTT in their crossover intervention trials, i.e., participants consumed two meals on separate days, including an experimental test meal containing studied components and a control test meal (same composition but without studied components), and the effects of the two meals were compared (Lairon et al., 2007). Often, to account for variability in participant body weight (i.e. with large range of body weights and therefore blood volumes), the fat content in the OFTT is often adjusted based on the body weight (Lairon et al., 2007); for example, 1 g fat/kg body weight is commonly provided (Cohen, Noakes, & Benade, 1988; Dekker et al., 2009; Lopez-Miranda et al., 1997).
Dairy products, especially heavy whipping cream, have been commonly used in clinical research as the OFTT fat source (Bahceci, Aydemir, & Tuzcu, 2007; Irvin et al., 2014; Patsch, Karlin, Scott, Smith, & Gotto, 1983; Tiret et al., 2000), including studies investigating the effects of DF on postprandial lipemia (Khossousi, Binns, Dhaliwal, & Pal, 2008; Kondo et al., 2004). Nevertheless, to the author’s knowledge, there has been insufficient consideration regarding the potential interactions between the test ingredients (e.g. DF) and dairy constituents other than fat globules, which could influence the effects of the studied components on postprandial lipemia. For example, dairy products are rich in milk fat globule membrane proteins serving as surfactants, and some DF could have interactions with the surface-stabilizing proteins and lead to destabilization of the milk fat globules and reduced lipid digestion (e.g. chitosan limited lipolysis from a β-lactoglobulin stabilized emulsion by electrostatic attraction (Qin et al., 2016)); such effects may not be observed in the same DF with other fat meals (e.g. unemulsified oil). When presenting a human trial involving OFTT, it is important to acknowledge the composition of the test meal and take potential interactions between constituents into consideration.

1.5.1. Dairy products as food emulsions

To better acknowledge the impacts of dairy products as food matrices when explaining the effects of studied components, it is necessary to understand the physical and chemical properties of dairy components. Fluid dairy products are considered oil-in-water emulsions with the milk fat globules dispersed in a continuous phase (Walstra, 1999). However, the fat globules in dairy are more complicated than common emulsion droplets due to the complexity of the globule surface membrane; it is not an adsorbed layer of one single substance, but rather of many components, including protein, phospholipids and various other minor lipids (Walstra, 1999). The surface membrane functions to prevent the fat globules from coalescence and keep them
dispersed. Milk fat can be separated from whole milk by a flow-through centrifugation, which yields skim milk and cream and a desired fat content (e.g. 2% skimmed milk and 35% whipping cream) can be achieved by mixing skim milk and cream at certain ratios (Walstra, 1999). In addition to milk fat globules, milk also contains water, carbohydrates, casein micelles (small particles containing water and casein protein binding with cations), whey proteins (e.g. β-lactoglobulin and α-lactalbumin), lipoprotein particles, and trace amounts of leukocytes and enzymes (Walstra, 1999). The average concentration of protein in milk is 33 g/L, with casein and whey proteins account for 78.8% and 19.4% of the total protein respectively, and the rest being miscellaneous (e.g. enzymes and glycoproteins) (Goff, 2017). The functionality of milk proteins is critical to the stability of milk products. For example, when milk is homogenized, surface-active proteins adsorb onto the oil-water interface due to the reduced globule size and increased interfacial area, and further stabilize the fat globules (Walstra, 1999). The stability of milk proteins is influenced by many factors, such as salt content (mainly calcium), temperature, dehydration (e.g. ethanol leads to aggregation of the casein micelles), and pH (Goff, 2017).

Caseins are hydrophobic and are usually bound with calcium phosphate in the micelles; however, with the decreasing pH, calcium phosphate disassociates and caseins eventually precipitate at pH 4.6 (Goff, 2017). The proteins appearing in the supernatant of milk after precipitation at pH 4.6 are whey proteins, which are less hydrophobic comparing to caseins; in terms of the effects of pH, β-lactoglobulin is dissociated into monomers at pH < 3.4 and α-lactalbumin releases the bound calcium at pH < 4.0 (Goff, 2017). The milk proteins’ responses to pH change could have implications on the stability of dairy products in an acidic environment. Furthermore, given the ability of milk proteins to adsorb readily to the oil-water interface and form a stabilizing surface layer (Singh, Ye, & Horne, 2009), they have been used as emulsifiers in a few recent \textit{in vitro}
digestion studies investigating DF’s effects on emulsion stability and digestibility (Qin et al., 2016; Tokle et al., 2012; Zhang et al., 2015). It was found that, cationic DF promoted bridging flocculation of anionic β-lactoglobulin-coated droplets before digestion (Qin et al., 2016), while anionic pectin molecules bound to cationic caseinate-coated droplets in the acidic gastric environment (Zhang et al., 2015). Based on these in vitro findings, human trials unravelling such interactions at a physiological level are warranted.

1.6. Conclusions

Many studies, at multiple levels, have investigated the role DF (e.g. pectin) may play in metabolic response, including lipid digestion and absorption. However, discrepancies exist and, although the viscous properties of pectin are considered a main contributor to potential health benefits (Lattimer & Haub, 2010), other characteristics likely contribute to its effects on lipid digestion and absorption, including interactions with digestive molecules and other food ingredients within the GIT. Apple and apple products, as DF-rich foods commonly consumed in Ontario, have potential to beneficially impact postprandial TAG levels, i.e. a risk factor for CVD. Hence further investigations are needed to clarify the effects of apples and apple constituents on postprandial lipemia and the digestive mechanisms involved, as well as the correlations between in vitro (static and dynamic) and human levels of investigation.
CHAPTER 2. Rationale, Objective and Hypotheses

The problem addressed at the outset of this thesis is the lack of understanding about how pectin, as a dietary fibre, and apples, as a food matrix rich in pectin, can influence postprandial lipemic response. The present research aims to study how apple pectin (as a dietary fibre) and whole apples (as pectin-containing food matrices) affect the digestibility and bioaccessibility/absorption of emulsified lipids, to support the design of food products and nutritional guidelines aimed at reducing disease risk. Using standardized static and dynamic in vitro digestion models, we determined how variable gastric pH, and the presence of pectin and pectin-containing food matrices, alters the digestion of emulsified lipids. Also, the effects of whole apple consumption on postprandial lipid metabolism were investigated in a randomized crossover acute meal human trial. The results obtained from the in vitro methods and the human trial were compared to seek potential correlation and to explore the suitability of in vitro methods to address research questions about postprandial lipemia when digestive interactions between meal constituents are expected to occur in the GIT, thereby influencing metabolism.

Specific Objectives and Hypotheses:

Study 1: To investigate the effects of pectin on the microstructure (i.e. stability in the GIT) of an oil-in-water emulsion rich in DHA and lipid digestibility and bioaccessibility of DHA, under simulated digestive conditions in a standardized static model. It was hypothesized that pectin would destabilize the emulsion in the simulated GIT and hence reduce lipid digestibility and DHA bioaccessibility.

Study 2: To understand how consuming whole apples, as a widely consumed food item rich in pectin, impacts gastric emptying and postprandial lipemic responses after an oral fat
tolerance test (OFTT) in otherwise healthy overweight and obese population, who tends to have impaired lipid metabolism. It was hypothesized that the consumption of apples would slow down gastric emptying and delay/limit the extent of postprandial lipemia after ingestion of the OFTT by participants.

Study 3: To study how a complex food matrix like whole apples influence lipid microstructure, digestibility and bioaccessibility in in vitro simulated digestions using two different models (static and dynamic), and to compare the practicability of those two models for lipid research. It was hypothesized that whole apples would decrease lipid droplet stability, digestibility and bioaccessibility in vitro, and that the dynamic digestion model would be the most appropriate model for predicting postprandial lipemic response.
CHAPTER 3. Pectin and gastric pH interactively affect DHA-rich emulsion in vitro digestion microstructure, digestibility and bioaccessibility


This chapter is presented with minimal edits to the version published in Food Hydrocolloids.
3.1. Abstract

Lipid digestibility and bioaccessibility (i.e. solubilization) may be impacted by various factors. For example, dietary fibres may interact with lipids and other digestive molecules during gastrointestinal digestion, and gastric pH may impact lipid droplet microstructure. Thus, these parameters may have implications for the release and uptake of bioactive fatty acids such as DHA. This study investigated how apple pectin (0.00, 5.68, or 100.00 mg/5.0 g emulsion), combined with variable gastric pH (2.0, 3.0 or 4.8), impacted the in vitro digestion of a DHA-rich algal oil lecithin-stabilized emulsion (10:1.2:88.8 oil:lecithin:water, D_{3.2} = 0.137 ± 0.001 \mu m). Low gastric pH (2.0 and 3.0) induced severe emulsion destabilization. However, the addition of a low-level of pectin (5.68 mg/5.0 g emulsion) reduced the destabilization at pH 3.0. Small lipid droplets, maintained either by this low-level of pectin at pH 3.0 or by a higher gastric pH (4.8), were associated with greater early (p<0.05), but not eventual (p>0.05) duodenal lipolysis (pH 7.0) and higher DHA bioaccessibility (>69%, p<0.05). Samples containing 100.00 mg pectin/5.0 g emulsion had the lowest overall lipolysis and DHA bioaccessibility across all pH values (p<0.05). Therefore, pectin content and gastric pH interactively impacted emulsion digestion. The presence of applesauce, matched for pectin concentration, further destabilized the emulsion and limited lipid digestibility and DHA bioaccessibility, compared to the pectin-only samples. Overall, a stable emulsion microstructure during gastric digestion promoted in vitro lipid digestibility and DHA bioaccessibility.

Key Words

Apple pectin; Emulsification; Food matrix; In vitro digestibility; DHA bioaccessibility
3.2. Introduction

Lipids play essential, but complicated, roles in health and disease. Understanding the factors that impact lipid digestion and absorption can offer insights into ways to modify lipemia, with implications for cardiovascular disease risk. The digestion of dietary triacylglycerols (TAG) in the gastrointestinal (GI) tract is a critical process determining their availability for absorption, with the proportion of lipids solubilized within the aqueous phase of the digestate referred to as ‘bioaccessible’ (Hedrén, Mulokozi, & Svanberg, 2002) and recognized as a critical precursor to bioavailability (Gregory, Quinlivan, & Davis, 2005). Various factors, including lipid intramolecular and supramolecular structure (Michalski et al., 2013), impact TAG digestion. For example, long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be structurally resistant to lipolysis when located in the sn-1 and sn-3 positions of a TAG molecule (Bottino et al., 1967; Brockerhoff, Hoyle, & Huang, 1966). In terms of supramolecular structure, emulsification can aid in digestion by minimizing the requirement for emulsification within the GI tract, and increasing the oil-water interfacial area for lipase adsorption (Michalski et al., 2013; Parada & Aguilera, 2007; Wilde & Chu, 2011). Therefore, pre-emulsification can be a strategy to enhance the absorption of TAG containing long-chain polyunsaturated fatty acids, which tend to be lipolysis-resistant DHA and EPA (Walker, Decker, & McClements, 2015).

Dietary ingredients may also impact lipid digestive processes. For example, viscous fibres have the potential to alter the microstructure of emulsions and therefore impact lipid digestion. This includes by inducing emulsion droplet aggregation, thereby reducing the interfacial area for lipases, thickening the intestinal contents, thereby limiting contact between lipases and lipid droplets, and binding calcium ions and/or bile salts which act to reduce the
presence of accumulated FFA at lipid droplet surfaces (Dikeman & Fahey, 2006; Zhang et al., 2015). Such interactions and their impacts on lipid digestion and bioavailability require further investigation, especially with the intense interest in development of so-called functional foods and dietary recommendations related to PUFA and dietary fibre consumption. The apple-derived soluble fibre pectin is one of the most widely utilized soluble fibres by the food industry (Colin-Henrion et al., 2009). Although soluble fibre consumption has widely recognized health benefits, the impact of pectin and other dietary fibres on the bioavailability and metabolism of other nutrients, including lipids, is not entirely clear. Some animal studies have shown attenuated postprandial lipemia (Lærke et al., 2007; Leclere et al., 1993; Suzuki & Kajuu, 1983) or fasting blood lipid levels (Trautwein et al., 1998) with pectin consumption, although such lipid-lowering effects in humans are not as widely studied or observed (Brouns et al., 2012; Morgan et al., 1993). Variable study results could be attributed to the different experiment conditions, and the fact that physiological properties of pectin can be affected by parameters such as concentration (Flourie et al., 1985; Verrijssen et al., 2014), degree of methylation (Brouns et al., 2012; Dongowski, 1995; Trautwein et al., 1998), degree of amidation (Hagesaether & Sande, 2007), and the presence of other molecules such as emulsifiers in the digestate (Zhang et al., 2015).

Some in vitro studies have also shown that pectin has the potential to impact lipid digestion processes (Beysseriat et al., 2006; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, & Narváez-Cuenca, 2014; Verrijssen, Verkempinck, Christiaens, Van Loey, & Hendrickx, 2015; Zhang et al., 2015); however, due to the variability in experimental conditions and types of pectin applied in the studies, more research is required to verify the correlation between the physiological mechanisms and the effects of pectin on lipid digestion.
Indeed, interactions between dietary lipids and fibres should be considered when formulating foods or making dietary and supplement recommendations. Interactions between ingested food molecules and digestive parameters are additionally important to consider in terms of possible impacts on GI function and nutrient absorption. For example, gastric pH can vary depending on stomach contents. While the pH of an empty stomach may be below 2.0, the ingestion of food can buffer the pH to as high as 6.7, with the pH gradually returning to fasted state levels (Dressman et al., 1990; Minekus et al., 2014). We recently reported that a soy lecithin-stabilized emulsion was extensively destabilized at an in vitro gastric pH of 1.6, and that these changes resulted in a significantly lower DHA in vitro bioaccessibility (Lin, Wang, Li, & Wright, 2014). Therefore, the purpose of this research was to investigate how the presence of apple pectin (5.68 or 100.00 mg in 5.0 g emulsion), as purified powder or contained in applesauce, and gastric acidity (pH 2.0, 3.0 or 4.8) influenced the in vitro GI fate of an algal oil emulsion stabilized by soy lecithin, in order to support the design of emulsions for enhanced bioavailability. It was hypothesized that apple pectin and gastric pH would interactively change the emulsion microstructure and, in turn, influence the subsequent in vitro lipolysis and DHA bioaccessibility.

3.3. Material and Methods

3.3.1. Materials

All chemicals and reagents were purchased from Sigma Chemical Co. (MO, USA), including pectin from apple (76282), D-galacturonic acid monohydrate (48280), porcine bile extract (B8631), pancreatin (from porcine pancreas, P1750), pancreatic lipase (from porcine pancreas, L3126), and Nile Red (72485). Life’s DHA S35-O300 algal oil was supplied by DSM (MD, USA), and liquid soy lecithin was from NOW Foods (IL, USA). Applesauce (Mott’s
Fruitsations unsweetened apple sauce, Canada Dry Mott’s Inc, Mississauga, Ontario, Canada) was purchased from a local supermarket.

3.3.2. Characterization of Applesauce and Pectin

The total solids content of the applesauce was determined by drying to constant weight using a 105°C oven method (Sluiter et al., 2008). The alcohol insoluble solids (AIS) fraction of the applesauce was also determined, as described by Le Bourvellec et al. (2011). Briefly, the applesauce was extracted by ethanol at room temperature, then filtered and washed with acetone solutions until the supernatant was colorless. The residue was then dried at 40 °C for 24 h and weighed as AIS. The soluble pectin content of the applesauce was determined by colorimetric assay (Melton & Smith, 2001). The polysaccharide in the AIS was first hydrolyzed by sulphuric acid to galacturonic acid, which react with m-hydroxydiphenyl and absorption at 525 nm was measured using a UV–vis spectrophotometer (Hewlett Packard 8451A Diode Array Spectrophotometer) and with a standard curve based on D-galacturonic acid (0 – 80 mg/mL). Accordingly, the pectin content of the applesauce was determined to be 5.68 ± 0.50 mg/g. Total polyphenols in the applesauce were determined by an adapted Folin–Ciocalteu method (Almeida et al., 2011). Briefly, applesauce was extracted by acidified methanol for 2 h at 85 °C to eliminate vitamin C. It should be noted that apple polyphenols have been shown to be stable during heat treatments between 70 to 90 °C (Chen et al., 2014), therefore the loss of polyphenols due to this 85 °C treatment was not expected to be significant. After cooling, the sample was centrifuged at 2,500 xg for 15 min, and the aliquot diluted and then mixed with the Folin–Ciocalteu reagent and saturated sodium carbonate solution. The absorbance at 750 nm was determined after standing at room temperature for 30 min. Polyphenol concentration was calculated based on a gallic acid (0 – 0.5 mg/mL) calibration curve. Accordingly, the total
polyphenols per gram (wet weight basis) of the applesauce was 0.778 ± 0.016 milligram of gallic acid equivalents (GAE). The protein content in the purified apple pectin was determined using the Bradford method as described (Chee & Williams, 2008) and found to be 0.74 ± 0.03 wt% protein. All the above determinations were made in triplicate.

3.3.3. Preparation of Soy Lecithin-stabilized Emulsions

A soy lecithin-stabilized emulsion (10.0 wt% algal oil, 1.2 wt% soy lecithin and 88.8 wt% Milli-Q water) was prepared as previously described (Lin et al., 2014). Soy lecithin was first dispersed in the oil phase and the mixture pre-homogenized for 30-60 seconds with water using a handheld mixer at speed 2 (10,000 rpm) (Ultra-Turrax, IKA T18 Basic, Germany) and then passed at 15,000 psi (103.42 MPa) through a microfluidizer (M110-EH Microfluidizer Processor, Microfluidics, MA, USA). The freshly prepared emulsion was collected in amber glass jars and stored under nitrogen at 4 °C for up to one week.

3.3.4. Particle Size and ζ-potential Measurements

Mean droplet diameters (D_{3,2}: the surface area-weighted mean, D_{4,3}: the volume-weighted mean) before and after gastric digestion were determined by laser diffraction (Mastersizer S, Malvern Southborough, MA, USA) using a refractive index of 1.488 for the algal oil. Samples analyzed during the in vitro digestion experiments were drawn 0.5 cm from the bottom of vials. Control solutions of purified pectin (0, 0.568 and 10 mg/mL, equal to those in the gastric digestate) and applesauce (0.1 g/mL) were also subjected to analysis, although those based on purified pectin were too diluted to reach the necessary opacity for the light scattering measurement.
\( \zeta \)-potential was measured using a particle electrophoresis instrument (NanoZS, Malvern Instruments Ltd, UK). Prior to analysis, in order to minimize multiple scattering effects, meal samples and gastric digestates were dispersed in 5 mM sodium phosphate buffer solutions at the pH of samples to achieve an oil concentration of 0.01 wt%.

3.3.5. In vitro Digestion Experiments

Firstly, 0.00, 5.68, or 100.00 mg purified apple pectin was added to 5.0 g of the aforementioned emulsion to study the effects of pectin content on the emulsion digestion. In another set of samples, 1.0 g applesauce (containing 5.68 mg pectin) was added to the emulsion, instead of pectin, to compare the effects of the food matrix versus isolated pectin. The pectin in applesauce has a high degree of methylation (Le Bourvellec et al., 2011), similar to the purified apple pectin used in this study. To achieve the highest pectin concentration (100.00 mg/5.0 g emulsion), an additional applesauce-containing meal sample was prepared with 5.0 g emulsion, 1.0 g applesauce and 94.32 mg purified pectin. The five meal samples studied are listed in Table 3.1.

Meal samples were subjected to in vitro digestion experiments simulating gastric and duodenal conditions using a method slightly modified from Minekus et al. (Minekus et al., 2014). Simulated digestion fluids were prepared as described (Minekus et al., 2014) with the exception that additional phospholipids (76 mg) were included in the 10 mL simulated duodenal fluid (SDF) (Mansbach II, Tso, & Kuksis, 2001). The digestions were performed in amber glass jars placed in a 37 °C 250 rpm shaking water bath (New Brunswick Scientific Co. Inc., NJ, USA). Meal samples warmed at 37 °C for 15 min were then mixed with 5 mL simulated gastric fluids (SGF). The mixture contained 2,000 U/mL pepsin and 12.6 mg/mL pyrogallol (as an antioxidant) and was incubated for 2 h at pH 2.0, 3.0 or 4.8, i.e. representing a range of gastric
pH values observed depending on food buffering capacity (Minekus et al., 2014; Simonian, Vo, Doma, Fisher, & Parkman, 2005). Ten mL of SDF were then added to start the duodenal phase of digestion. The final digestion mixture contained 550 U/ml pancreatic lipase, 10 mg/mL bile extract (~10 mM bile salts), and 3.8 mg/mL (~8 mM) phospholipids. The duodenal digestate was adjusted to pH 7.0 by addition of 1 N sodium hydroxide and the digestion was carried out for 2 h. For the particle size measurements, sample and digestive fluid volumes were reduced to 1/5 of those stated above. To assess the potential for PUFA oxidation during digestion, the concentration of thiobarbituric acid reactive substances (TBARS) were determined in the final digestate samples using a colorimetric assay (Larsson, Cavonius, Alminger, & Undeland, 2012). TBARS concentrations in the digestate samples were not significantly different from DHA-lipid-free controls containing only water, emulsifier, digestive fluids, and applesauce and/or pectin (p>0.05, data not shown), indicating a high degree of n-3 PUFA preservation during the digestion experiments.

Table 3.1. Composition of meal samples (E, EP, EPP, EA, and EPP) studied and the corresponding duodenal digestate pectin concentration.

<table>
<thead>
<tr>
<th>Emulsion (5.0 g)</th>
<th>Purified Pectin (mg)</th>
<th>1.0 g Applesauce (5.68 mg Pectin)</th>
<th>Duodenal Digestate Pectin Content (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.68</td>
<td>94.32</td>
<td>100.00</td>
</tr>
<tr>
<td>E</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EP</td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td>EPP</td>
<td>√</td>
<td>-</td>
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<tr>
<td>EA</td>
<td>√</td>
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<tr>
<td>EAP</td>
<td>√</td>
<td>-</td>
<td>√</td>
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</tbody>
</table>

3.3.6. Measurement of Digestate Viscosity

Digestate viscosity during the gastric and duodenal phases was measured using a stress-controlled rheometer (AR 2000, TA Instruments, New Castle, DE, USA) equipped with a
concentric cylinder (double wall Couette cell) geometry at 37 °C. A constant shear of 10 torque (N•m) was applied for 10 s to eliminate any loading history and apparent viscosity determined by increasing the shear rate logarithmically from 10 to 120 s⁻¹ at 10 points for 60 s each. All analyses were performed in triplicate.

3.3.7. Determination of FFA Release during in vitro Digestion

0.1 mL aliquots of duodenal digestate were collected at 2, 5, 10, 15, 30, 60, 90, and 120 min for determination of FFA using a non-esterified fatty acid (NEFA) kit (Wako Diagnostics, VA, USA) as per the manufacturer’s instructions (Nik, Corredig, & Wright, 2010). FFA from 100 uL of sample were extracted into hexane (1000 uL) under acidic conditions (100 uL 1 M HCl) and the mixture vortexed for 10 s before centrifugation at 16,873 g for 30 min (5418 Laboratory Centrifuge, Eppendorf Hamburg, Germany). The hexane extract was then diluted to 3 mL and 5 uL was added to 225 uL of Reagent A in a 96-well plate (in duplicate) and the plate incubated at 37 °C for 10 min. 75 uL of Reagent B was then added with an additional 15 min incubation at 37 °C. FFA were determined using a UV-VIS micro-plate spectrophotometer (Spectramax plus, Molecular Devices Corporation, CA, USA) by measuring the absorbance at λ_max of 550 nm and a standard curve prepared using oleic acid ranging from 0.1 to 1.97 mM. The percentage of lipid hydrolysis was calculated based on the moles of FFA present at each time point with respect to the total moles of FA present initially, correcting for background fluid contributions.

3.3.8. Isolation of Aqueous Phase Containing Bile Salt Micelles

Digestate samples at the end of digestion were immediately centrifuged at 144,000 g at 7 °C for 1 h using a Sorvall WX Ultra 80 Ultracentrifuge (Mandel Scientific, ON, Canada) to
separate any undigested oil from the aqueous phase and pellet. The aqueous fraction was collected with a fine tip disposable pipette, quantified using a graduated cylinder, and stored in glass vials under nitrogen at -80 °C until lipid extraction and analysis.

3.3.9. Analysis of DHA Bioaccessibility by Gas Chromatography

Lipid extractions were performed based on the Folch method, as described (Martin, Nieto-Fuentes, Señoráns, Reglero, & Soler-Rivas, 2010) with slight modifications. Afterwards, the samples were saponified, methylated and FAME quantified, as previously described (Merino et al., 2011). FAMEs were separated by gas chromatography using an Agilent 7890A Network GC System (Agilent Technologies, CA, USA) with a Supelco SP 2560 fused-silica capillary column (100m × 0.25mm i.d., 0.2 μm film thickness; Sigma-Aldrich, St Louis, MO). The internal standard (C17:0) method was used to determine the concentration of DHA in the digestate aqueous phase samples. DHA bioaccessibility was defined as the weight percentage of DHA from the original algal oil that was incorporated into the aqueous phase by the end of the digestion.

3.3.10. Confocal Microscopy

Confocal laser microscopy imaging was used to investigate differences in emulsion droplet microstructure before and after in vitro digestion. Undiluted emulsion and digestate samples were placed on concave glass slides, covered and sealed tight to prevent sample dryness. Nile Red (added to the algal oil prior to emulsification at 0.01% (w/w) per gram) was excited at 543 nm. The resulting images (1024×1024 pixels) were obtained using a 63 x oil immersion objective lens.
3.3.11. Statistical Analyses

All measurements were completed in triplicate, unless otherwise stated. Results are expressed as mean ± standard deviation. ANOVA (with Tukey’s post hoc testing) and Pearson correlation analyses were performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, San Diego California USA, www.graphpad.com) with a significance level of p<0.05.

3.4. Results and Discussion

3.4.1 Physicochemical Properties of the Meal and Gastric Digestate Samples

The D_{3,2} and D_{4,3} of 10% algal oil emulsion droplets were 0.137 ± 0.001 and 0.215 ± 0.001 μm, respectively (pH = 5.1). The emulsion had a monomodal size distribution with the peak centered near 0.1 μm (Figure 3.1). All digestions and analyses were done using emulsions within 1 week of preparation, over which period of time particle size and ζ-potential did not significantly change (p>0.05, data not shown). The ζ-potential measurement (−72.0 ± 0.7 mV) demonstrated that the emulsion stabilized by soy lecithin was strongly negatively charged, agreeing with a previous study on soy lecithin-stabilized emulsion (Lin et al., 2014; Mantovani, Cavallieri, Netto, & Cunha, 2013). The negative charge is attributed to the anionic components in soy lecithin molecules, such as phosphatidylinositol and phosphatidic acids (Rydhag & Wilton, 1981; Xu, Nakajima, Liu, & Shiina, 2010).

The addition of 5.68 mg purified pectin (i.e. EP) slightly increased the particle size (D_{3,2} = 0.141 ± 0.002 μm and D_{4,3} = 0.251 ± 0.005 μm; p<0.05) and slightly decreased the ζ-potential (−69.1 ± 1.3 mV, p<0.05). Greater increases in droplet size were observed with the addition of 100.00 mg pectin (i.e. EPP, D_{3,2} = 1.872 ± 0.027 and D_{4,3} = 2.304 ± 0.035 μm) and the negative
charge on the lipid droplets was significantly reduced ($-35.1 \pm 1.1$ mV, p<0.05). According to confocal microscopy (Figure 3.2), droplet aggregation occurred in both EP and EPP (Figure 3.2). However, the small particle size increase with EP suggests the aggregation in this sample was reversible with the shear applied during the laser diffraction measurements. The decrease in charge of the emulsion droplets in the presence of 100.00 mg (2.0 wt%) pectin may be attributed to depletion flocculation induced by the presence of excessive anionic pectin molecules, which was observed with above 0.2 wt% pectin in a Tween-80-stabilized emulsion (Beysseriat et al. 2006).

The meal samples were exposed to three different gastric pH conditions prior to simulated duodenal digestion at pH 7.0. After gastric digestion at pH 2.0, large increases in emulsion droplet size were observed for all samples (Figures 3.1D and 3.2) and the surface charge was significantly reduced (Figure 3.3). Increased droplet size was also observed at gastric pH 3.0, although $D_{3,2}$ for EP was less impacted compared to E and EPP (Figures 3.1E and 3.2). Based on the main peak for EP, most of the droplets at pH 3.0 were much smaller than at pH 2.0, although there was still a small peak at ~200 μm (Figure 3.1E), indicating the presence of some large particles. Corresponding to the small droplet size, EP also had the highest surface charge among the three meal samples (Figure 3.3). With the highest gastric pH investigated (i.e. 4.8), the lipid droplets in E and EP remained relatively small compared to those at lower gastric pH values (Figure 3.2). Similarly, the surface charge of those meal samples was higher than that at lower gastric pH values (Figure 3.3). The main peaks for E and EP were both left skewed and located near 1 μm, although the small peak near 200 μm was still observed for EP (Figure 3.1F). In terms of EPP at pH 4.8, the main peak was located further right compared to E and EP, i.e. near 2 μm, indicating the formation of large particles with the higher amount of pectin present.
The confocal image of EPP at pH 4.8 shows the flocculation and coalescence of small lipid droplets (Figure 3.2), supporting the observations based on size distribution and $D_{3,2}$ and $D_{4,3}$ (i.e. $2.629 \pm 0.567$ and $13.398 \pm 3.253 \, \mu m$, respectively). This could be related to the limited surface charge of EPP compared to the other samples (Figure 3.3).

Factors affecting emulsion microstructure may include the following. Firstly, an acidic gastric pH was found to destabilize the lipid droplets emulsified by soy lecithin. Without the addition of any pectin, the $D_{3,2}$ and $D_{4,3}$ of the emulsion increased to $10.300 \pm 0.902$ and $14.252 \pm 1.535 \, \mu m$, respectively ($p<0.05$) when the pH was adjusted to 2.0 (from 5.1). At pH 3.0, the droplet size was relatively less impacted ($D_{3,2} = 2.319 \pm 0.012 \, \mu m$ and $D_{4,3} = 3.952 \pm 0.022 \, \mu m$). At pH 4.8, the particle size was relatively very minimally impacted (i.e. $D_{3,2}$ of $0.196 \pm 0.000$ and $D_{4,3}$ of $0.322 \pm 0.001 \, \mu m$, $p<0.05$). This observation that particle size of a soy lecithin-stabilized emulsion increases with decreasing pH agrees with previous reports (Comas, Wagner, & Tomás, 2006; Lin et al., 2014), and is likely related to shielding of the emulsifier’s more negatively charged components, such as phosphatidylinositol and phosphatidic acids, by hydrogen ions at pH values closer to their pKa (~1.5) (Mantovani et al., 2013).

In addition, the physiochemical properties of pectin could impact the stability of the emulsion system. Pectin is an anionic polysaccharide. Thus, at neutral pH, it can lead to the depletion of negatively charged emulsifier molecules on the surface of emulsion droplets and result in flocculation (Roudsari, Nakamura, Smith, & Corredig, 2006). This may explain the reversible flocculation observed when pectin was added to emulsion before digestion (Figure 3.2). Other than destabilizing soy lecithin-covered emulsion droplets, the extreme acidity (pH 2.0) during gastric digestion would also protonate free carboxyl groups, resulting in limited negative charge on the pectin molecules. Thus, no electrostatic interactions between pectin
molecules and soy lecithin are expected at pH 2.0. At a pH of 3.0, the meal samples were also destabilized, except EP, which showed a bridging network of relatively small lipid droplets, according to Figure 3.2.

The pectin used in this study contained 0.74 ± 0.03 wt% protein which may contribute to its emulsifying ability (Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015) and impact electrostatic interactions. To investigate the charge of the pectin at pH 3.0, an algal oil-in-water emulsion was prepared using 0.1 wt% pectin (same pectin content as EP) with no additional emulsifier. After adjusting the pH to 3.0, this emulsion was found to be negatively charged (−43.3 ± 0.5 mV), indicating that the pectin overall carries a negative charge at this pH. Thus, the negatively charged pectin may not have electrostatic interactions with the anionic components of soy lecithin molecules. However, there are cationic functional groups on soy lecithin, such as choline and ethanolamine (Rosenberg, 1990), and electrostatic interactions between these cationic groups and anionic pectin molecules may allow pectin interfacial adsorption. Despite this, at low pectin concentration individual pectin molecules may adsorb onto multiple lecithin-stabilized emulsion droplets, leading to bridging flocculation (Figure 3.2). This was observed in another study with a β-lactoglobulin-stabilized emulsion in the presence of a low concentration of pectin (Cho & McClements, 2009). In terms of EPP, the instability of this emulsion in the presence of the highest pectin concentration could be attributed to depletion flocculation induced by non-adsorbed pectin in the aqueous phase of emulsion (Cho & McClements, 2009).
Figure 3.1. Size distribution of the emulsion exposed to different acidic pH conditions (A), meal samples EP, EPP, EA, EAP, applesauce and pectin before digestion (B and C), and meal samples EP, EPP, EA, and EAP after 2 h of gastric digestion at pH 2.0 (D), 3.0 (E) and 4.8 (F).
Figure 3.2. Microstructure of the meal samples before and after 2 h of gastric digestion at pH 2.0, 3.0, and 4.8. G0 = before gastric digestion; G120 = after 2 h (120 min) of gastric digestion; E = 5.0 g emulsion only; EP = 5.0 g emulsion + 5.68 mg pectin; EPP = 5.0 g emulsion + 100.00 mg pectin; EA = 5.0 g emulsion + 1.0 g applesauce; EAP = 5.0 g emulsion + 1.0 g applesauce + 94.32 mg pectin. Scale bars represent 50 μm.
Figure 3.3. ζ-potential of digestate of different meal samples after gastric digestion. Data are the mean ± SD. Within each figure, different uppercase letters (A, B, C) indicate significant differences between meal sample type and different lowercase letters (a, b, c) indicate significant differences between gastric pH values (p<0.05).

Table 3.2. Mean droplet diameters (μm) of meal samples before and after gastric digestion at pH 2.0, 3.0 or 4.8.

<table>
<thead>
<tr>
<th>Meal Samples</th>
<th>Before Digestion¹</th>
<th>After Gastric Digestion²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastric pH = 2.0</td>
<td>Gastric pH = 3.0</td>
</tr>
<tr>
<td>E - D₃,₂</td>
<td>0.137 ± 0.001</td>
<td>34.216 ± 1.491</td>
</tr>
<tr>
<td>E - D₄,₃</td>
<td>0.215 ± 0.000</td>
<td>38.161 ± 2.352</td>
</tr>
<tr>
<td>EP - D₃,₂</td>
<td>0.141 ± 0.002</td>
<td>23.516 ± 1.157</td>
</tr>
<tr>
<td>EP - D₄,₃</td>
<td>0.251 ± 0.005</td>
<td>37.131 ± 3.917</td>
</tr>
<tr>
<td>EPP - D₃,₂</td>
<td>1.872 ± 0.027</td>
<td>18.540 ± 2.523</td>
</tr>
<tr>
<td>EPP - D₄,₃</td>
<td>2.304 ± 0.035</td>
<td>39.845 ± 2.511</td>
</tr>
<tr>
<td>EA - D₃,₂</td>
<td>0.901 ± 0.079</td>
<td>74.180 ± 3.052</td>
</tr>
<tr>
<td>EA - D₄,₃</td>
<td>267.337 ± 54.691</td>
<td>188.243 ± 4.305</td>
</tr>
<tr>
<td>EAP - D₃,₂</td>
<td>7.978 ± 0.367</td>
<td>85.608 ± 5.247</td>
</tr>
<tr>
<td>EAP - D₄,₃</td>
<td>469.108 ± 66.966</td>
<td>409.911 ± 49.598</td>
</tr>
</tbody>
</table>

¹ Samples were taken after addition of pectin and/or applesauce and being mixed without any digestive fluid for 15 min in the 37°C 250 rpm shaking water bath.

² Samples were taken after 120 min of gastric digestion in the 37°C 250 rpm shaking water bath.
3.4.2. Viscosity of Gastric and Duodenal Digestates

The digestate viscosity of each meal sample determined after 1 h of gastric digestion at pH 2.0, 3.0 or 4.8 is shown in Figure 3.4. Accordingly, at all gastric pH levels, the viscosity of the E and EP digestates was low and independent of shear rate (p>0.05, Figure 3.4A, B, and C). The viscosity of the EPP gastric digestates was slightly higher than those of E and EP, but still less than 0.1 Pa·s at all shear rates. At pH 2.0 and 3.0, but not at pH 4.8, the viscosity of EPP digestate decreased with increasing shear rate (Figure 3.4A, B, and C). The viscosity of meal samples after 5 min duodenal digestion was also measured (Figure 3.4D, E, and F). Shear-thinning behavior was only observed in EP and EPP at gastric pH 3.0, while all other treatments showed Newtonian type behaviour regardless of pH (p>0.05). The pseudoplastic behaviour of EP at gastric pH 4.8 and EPP at all gastric pH values studied may be attributed to the entanglement of pectin molecules formed at low shear rates (Fabek, 2011) and/or weak flocs formed by depletion flocculation (Surh, Decker, & McClements, 2006) being interrupted at the higher shear rates. Comparing at a shear rate of 56.23 s⁻¹, i.e. within a range (10 - 100 s⁻¹) with physiological relevance for in vitro studies (Fabek, Messerschmidt, Brulport, & Goff, 2014), the EPP gastric digestate exhibited the highest apparent viscosity values at all gastric pH values (p<0.05), whereas E and EP consistently had similarly low apparent viscosity (p>0.05, Figure 3.5). Similar trends were observed when comparing apparent viscosities at a shear rate of 100 s⁻¹ (data not shown).

At the start of duodenal digestion, the digestate of each meal sample was adjusted to the same pH (i.e. 7.0). As expected, the duodenal digestates were less viscous than the gastric samples (Figures 4 and 5) because of dilution (Espinal-Ruiz et al., 2014; Fabek et al., 2014; Verrijssen et al., 2014). Despite the low values, the trends in duodenal apparent viscosity were
similar as those observed for the gastric phase (Figure 3.5). Therefore, the presence of pectin at the highest concentration studied was associated with higher digestate viscosity, which may restrict the movability of droplets and so further impact the digestion process (Ngouémazong et al., 2015; Zhang et al., 2015).

Figure 3.4. Flow behavior of gastric (A, B, C) and duodenal (D, E, F) digestates of meal samples at gastric pH 2.0 (A and D), 3.0 (B and E), and 4.8 (C and F).

Figure 3.5. Apparent viscosity (Pa·s) at 56.23 s⁻¹ of digestates for meal samples E, EP, EPP, EA, and EAP after 1 h gastric (A) and 5 min duodenal (B) digestion. Data are mean ± SD. Within A or B, different uppercase letters (A, B, C) indicate significant differences between sample type and different lowercase letters (a, b, c) indicate significant differences between gastric pH levels (p<0.05).
3.4.3. *In vitro Digestibility of the Algal Oil Emulsion*

Following gastric digestion, the meal samples were exposed to simulated duodenal digestive conditions at pH 7.0 for 2 h. The effects of sample composition on duodenal lipolysis will be discussed first. With a gastric pH of 2.0, there were no significant differences in the early lipolysis observed between E, EP and EPP (i.e. within the first 5 min of duodenal digestion) (p>0.05, Figure 3.6A). By 2 h, the lipids in E and EP were hydrolyzed to a similar extent (p>0.05, Figure 3.6B) and EPP showed significantly less lipolysis (p<0.05, Figure 3.6B).

Following gastric pH of 3.0, the early lipolysis of EP was higher than E (p<0.05) and was the highest in this group after 2 h (p<0.05, Figure 3.6). As with gastric pH 2.0, EPP achieved the lowest 2 h lipolysis (p<0.05, Figure 3.6). At gastric pH 4.8, E had the highest 5 min lipolysis, followed by EP and then EPP (p<0.05, Figure 3.6A). However, the difference between E and EP disappeared by 2 h (p>0.05, Figure 3.6B).

Within meal type, gastric pH impacted duodenal lipolysis. For example, higher gastric pH was generally associated with higher 5 min and 2 h lipolysis (p<0.05, Figure 3.6). Of all the samples and conditions investigated, the emulsion (E) treated with gastric pH 4.8 achieved the highest 5 min and 2 h lipolysis (p<0.05), i.e. 22.64 ± 0.81 and 51.34 ± 2.53 %, respectively. EPP consistently had the lowest 2 h lipolysis compared (p<0.05, Figure 3.6). Therefore, both gastric pH and the inclusion of pectin impacted emulsion lipid digestibility. This seems to relate to differences in microstructure, in that E and EP tended to have smaller emulsion droplets at higher gastric pH (3.0 and 4.8). The early lipolysis achieved was negatively correlated with the D₃,₂ of lipid droplets at the beginning of duodenal digestion, i.e. the smaller the D₃,₂ (indicating an intact emulsion microstructure), the greater the early lipolysis (p<0.05, R² = 0.4377). This is consistent with previous reports that smaller emulsion droplets have relatively higher oil-water interfacial
area available for the pancreatic lipase activity and facilitate lipolysis (Golding et al., 2011; Lin et al., 2014; McClements, Decker, & Weiss, 2007). However, in this study, the ultimate lipolysis achieved (2 h) was not favored by an intact emulsion structure (small droplets) before the start of duodenal digestion (p>0.05). Of note, in the current study, FFA released were not removed during digestion. Their interfacial accumulation may have impeded lipolysis (McClements & Li, 2010) and eliminated any advantage of smaller emulsion droplets at the beginning of the duodenal stage, albeit the presence of bile salts regulates triacylglycerol hydrolysis by solubilizing FFA to reduce the interfacial accumulation (McClements & Li, 2010).

Interestingly, the 2 h lipolysis was correlated with the apparent viscosity of gastric and duodenal digestates at 56.23 s^{-1}, i.e. the higher the viscosity, the lower the lipolysis (p<0.05). Indeed, higher digestate viscosity may restrict contact between lipid droplets and digestive components such as lipases and bile salts (Dikeman & Fahey, 2006). However, this correlation (i.e. between lipolysis and gastric/duodenal stage apparent viscosity) was not observed at the first 5 min of duodenal digestion (p>0.05). Overall, these results suggest that an intact emulsion structure at the beginning of duodenal digestion is important for promoting initial lipolysis, while the digestate viscosity is critical to the overall lipolysis. Other studies have also concluded that pectin can have suppressive effects on in vitro lipolysis. An enzyme kinetics study has demonstrated that pectic polysaccharides are non-competitive inhibitors of digestive enzymes, especially for lipase (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, & Narváez-Cuenca, 2014). As little as 0.18% pectin was able to decrease the lipolysis of a Tween-80 stabilized emulsion, which was further reduced at higher pectin concentrations (up to 0.90%) (Espinal-Ruiz et al., 2014). The inclusion of 1.19 mg/mL pectin in a simulated duodenal digestate was also found to suppress lipolysis from egg yolk and trans fatty acid enriched fats (Hur, Kim, Chun,
Lee, & Keum, 2013; Hur, Kim, Choi, & Lee, 2013). Similarly, in this study, EPP (100.00 mg pectin/20 mL final digestate = 5.00 mg/mL) had the lowest lipolysis, regardless of gastric pH. Nevertheless, it should be noted that pectin’s effects on lipid digestion depends on many factors, including the concentration of pectin and the interfacial composition of lipid droplets. It was found that 0.5 wt% pectin reduced >35% of the lipolysis of nonionic Tween-80- and lactoferrin-stabilized emulsions, potentially by bridging or depletion mechanisms, while 0.025 wt% pectin doubled the lipolysis in caseinate-stabilized emulsions by adsorbing to the surfaces of cationic casein-coated droplets and promoting electrostatic and steric repulsion (Zhang et al., 2015). This highlights the importance of cautious emulsion formulation to achieve the desired effects of pectin, either to inhibit or promote lipid digestion.

Figure 3.6. Duodenal lipolysis (% FFA release, maximum = 66.7%) of meal samples treated with different gastric pH values. Lipolysis at 5 min (start of duodenal digestion) (A) and 2 h (end of duodenal digestion) (B). Data are the mean ± SD. Different uppercase letters (A, B, C, D) indicate significant differences between samples (E, AP, EP, EAP, EPP). Different lowercase letters (a, b, c) indicate significant differences between gastric pH levels (p<0.05).
DHA bioaccessibility at the end of duodenal digestion is compared in Figure 3.7. Similar as for lipolysis, the DHA bioaccessibility results for EPP were the lowest at every gastric pH investigated (p<0.05). In contrast, E treated with gastric pH 4.8 had the highest DHA bioaccessibility (87.67 ± 2.36%) among all samples, followed by EP after gastric pH 4.8 (75.84 ± 2.91%). Interestingly, a higher DHA bioaccessibility was observed for EP compared with the other samples with gastric pH 3.0 (69.87 ± 3.28%) (p<0.05). Thus, at this pH, the small amount of pectin present was associated with increases in emulsion DHA bioaccessibility, even though the emulsion microstructure was compromised by gastric pH 3.0 (as evidenced by E at pH 3.0 in Figures 3.1 and 3.2). Other than the aforementioned three samples (E at pH 4.8 and E & EP at pH 3.0), none of the samples exceeded a DHA bioaccessibility of 30%. This may be attributed to the fact that micellar solubilization of long-chain PUFA can be less efficient than short- or medium-chain fatty acids, as their chain length leads to lower solubility and adversely impacts their partitioning between oil and aqueous phases (Carlier, Bernard, & Caselli, 1991; Sek, Porter, Kaukonen, & Charman, 2002).

Overall, a positive correlation was observed between emulsion lipolysis and DHA bioaccessibility (p<0.05, R² = 0.8211), despite the fact there were large differences in gastric structural stability based on meal sample type and pH differences. However, correlations were not observed comparing across different gastric pH values or pectin concentrations (p>0.05), with the exception that a positive correlation between lipolysis and DHA bioaccessibility was observed for samples treated at gastric pH of 4.8 (p<0.05, R² = 0.9528). A negative correlation was observed between the gastric digestate apparent viscosity at 56.23 s⁻¹ and meal sample DHA bioaccessibility (p<0.05, R² = 0.3426), although the duodenal digestate apparent viscosity at
56.23 s\(^{-1}\) was not correlated with DHA bioaccessibility (p>0.05). No correlation was found either between particle size (D\(_{3,2}\) or D\(_{4,3}\)) before duodenal digestion and DHA bioaccessibility. Instead, based on the confocal microscopy (Figure 3.2), EP had smaller droplets than E and EPP at gastric pH of 3.0 and this may be related to its relatively high DHA bioaccessibility. The smaller droplets of EP could provide larger surface area for pancreatic lipase absorption, facilitating lipolysis (Figure 3.5). Nevertheless, the extent of lipolysis in EP was not as outstanding as its DHA bioaccessibility. Thus, the low concentration of pectin present may have also influenced the transfer of DHA to the aqueous phase bile salt micelles and vesicles, although further investigations are required.

Overall, in the current study, the bioaccessibility of DHA, as one of the products of lipid digestion, is influenced by pectin’s presence. However, when 2% pectin was present in a beta-carotene-rich emulsion stabilized with phosphatidylcholine (the main component of the soy lecithin used in the current study), the incorporation of digested lipids into micelles was not significantly different from the sample without pectin (Verrijssen et al., 2015). This may be due to the limited incorporation of lipids in the micelles (1/4th of the digestive products) in that study (Verrijssen et al., 2015), while the potential difference in the transfer efficiency of different fatty acids was not considered. Despite that, beta-carotene bioaccessibility was reduced by the presence of high and medium methoxyl pectin, which may be attributed to the ability of such pectin forming gel-like clusters with phosphatidylcholine, entrapping oil droplets and leading to a slower transport; the presence of low methoxyl pectin or the absence of phosphatidylcholine did not result in the same effect (Verrijssen et al., 2015). It is generally understood that dietary fibre can interfere with micelle formation by the entrapment of oil droplets and bile salts (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). However, again, the current study
and the beta-carotene study discussed above confirmed the complexity of pectin’s interaction with other GI components; it depends on factors such as the type and concentration of pectin, and the interfacial composition of the lipid droplets. These factors should be taken into consideration when evaluating pectin’s effect on lipid digestion and bioaccessibility.

Figure 3.7. DHA bioaccessibility for meal samples treated with gastric pH 2.0, 3.0 or 4.8. Data are mean ± SD of triplicate lipid extractions from digestions performed in triplicate. Different uppercase letters (A, B, C, D) indicate significant differences between samples. Different lowercase letters (a, b, c) indicate significant differences between gastric pH conditions.

3.4.5. Digestion of Emulsion with Applesauce

The overall aim of this work was to understand ingredient interactions as they apply to lipid digestibility and bioaccessibility. Therefore, digestions of the algal oil emulsion in the presence of applesauce, i.e. a complex food matrix rich in pectin, were also performed. This was done at concentrations of pectin matching the EP and EPP meal samples. This involved adding 1.0 g applesauce (containing 5.68 mg pectin) to 5.0 g of the emulsion in the case of EA and supplementing with additional 94.32 mg purified apple pectin in the case of EAP.
The $D_{3,2}$ and $D_{4,3}$ of EA were $0.901 \pm 0.079 \ \mu m$ and $267.337 \pm 54.691 \ \mu m$, respectively, i.e. significantly higher than those of E and EP (p<0.05, Figure 3.1C) owing to the presence of applesauce which, alone, had $D_{3,2}$ and $D_{4,3}$ values of $365.339 \pm 6.675$ and $716.573 \pm 12.611 \ \mu m$, respectively. The addition of applesauce slightly decreased the negative charge of the original emulsion droplets ($-63.7 \pm 1.1 \ mV$, p<0.05, Figure 3.3). This could be attributed to the acidity of applesauce (pH of EA = 3.8 versus pH of E = 5.1), which may reduce the emulsion-stabilizing ability of soy lecithin, as discussed previously. For EAP the $\zeta$-potential was reduced significantly to $-36.7 \pm 0.8 \ mV$ (p<0.05, Figure 3.3) and further increases in droplet size were observed, i.e. to $7.978 \pm 0.367$ and $469.108 \pm 66.966 \ \mu m$, for $D_{3,2}$ and $D_{4,3}$ respectively (p<0.05, Figure 3.1C). Although the large particles present in the applesauce contribute to a high degree of polydispersity in the laser diffraction analysis, the confocal microscopy confirms that the lipid droplets of meal sample before digestion was similar in size (Figure 3.2). Similarly, after gastric digestion at pH 4.8, EA and EAP had more significant increases in droplet size than EP and EPP (Figure 3.2), likely contributed by large non-lipid particles from the applesauce, although potentially also reflective of lipid droplet destabilization caused by the acidity of applesauce and/or the presence of pectin, as observed in Figure 3.2.

In terms of viscosity, the EA and EAP gastric digestates were higher than EP and EPP (p<0.05) and exhibited obvious shear-thinning behavior at all pH values. Similar observations were found for all EA duodenal digestates, but not EAP for which the duodenal digestate viscosity was independent of shear rate following gastric pH 3.0 and 4.8 (p>0.05, Figure 3.3). The apparent viscosity of the EAP digestate at both the gastric and duodenal phases was the highest of all gastric pH treatments, potentially because of the presence of both applesauce and the highest concentration of pectin (Figure 3.4). The EA digestates had significantly higher
apparent viscosity values than EP (p<0.05) and similar to EPP (p>0.05), except during gastric digestion at a pH of 2.0 (EA < EPP, p<0.05, Figure 3.4A). The total solids in applesauce (10.45 ± 0.03 wt%) may contribute to the higher viscosities of applesauce-containing samples.

In terms of lipolysis, EA was similar to EP and EAP was similar to EPP, despite the fact that the applesauce-containing samples had lower early stage lipolysis when gastric pH was 3.0 and 4.8 (p<0.05, Figure 3.5A), and lower overall lipolysis with gastric pH 4.8 (p<0.05, Figure 3.5B). Therefore, when the emulsion was treated with a relatively higher gastric pH and was able to maintain a stable microstructure (with the help of a low level pectin at pH 3.0), the presence of applesauce limited the lipolysis, potentially because of emulsion-destabilizing effects (pH 3.0 and 4.8 in Figure 3.2). Applesauce is a complex food matrix which includes potentially anti-lipolytic components such as polyphenols (Sugiyama et al., 2007). For example, 10 μg/mL apple polyphenols have been shown to inhibit ~65% of pancreatic lipase activity in vitro (Sugiyama et al., 2007). The total polyphenol content of the applesauce used in this study was 0.778 ± 0.016 mg GAE/g wet weight basis, which led to a polyphenol concentration of 39 μg/mL GAE in duodenal digestates of EA and EAP. Thus, polyphenols were present in the applesauce at levels which may have contributed to lipid digestion. However, the lack of difference between meal samples with or without applesauce when treated with low gastric pH indicates that emulsion destabilization overlapped with any lipase-inhibiting effects of applesauce as a complicated food matrix, and the lipolysis was not limited further by the presence of polyphenols or additional pectin.

3.5. Conclusions

This research demonstrated that pectin content and gastric pH had interactive impacts on
emulsion digestion. The possibility exists for other digestive conditions to play a role in these processes and also to be influenced by the chemical and physical properties of ingested foods. A low level of pectin (5.68 mg/5.0 g emulsion) protected the emulsion from severe acidic destabilization at gastric pH 3.0, but not 2.0, and contributed to a higher duodenal DHA bioaccessibility. In contrast, at 100.00 mg pectin/5.0 g emulsion, even a relatively high gastric pH (i.e. pH 4.8) could not maintain the emulsion microstructure to allow efficient duodenal digestion and DHA transfer. The experiments with applesauce revealed more extensive destabilization and lower digestibility and DHA bioaccessibility at equivalent levels of pectin, highlighting the complex interactions between molecules from whole food matrices. Lipid digestibility and DHA bioaccessibility were closely related to the stabilization of emulsion droplets during gastric digestion. This contributes to an understanding of how in vitro digestion methods (i.e. gastric pH) can impact results. It can also be useful for the design of DHA-rich lipid based products by suggesting that the availability of DHA can be compromised, by the addition of apple pectin and/or consumption with products such as applesauce. Thus, extra caution should be addressed when formulating fibre- and DHA-containing products.

3.6. Acknowledgements

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3.7. Bridge to Chapter 4

Chapter 3 supports that purified pectin has the potential to modify lipid digestion and even fatty acid bioaccessibility *in vitro*. Apples are a pectin-rich whole food that are widely consumed in North America. Whole (with skin) fresh apples are a good dietary source of both pectin and polyphenols (Henríquez et al., 2010), the latter of which is another bioactive exhibiting lipolysis-inhibiting effects *in vitro* (Sugiyama et al., 2007). Therefore, whole apples have the potential to alter lipid digestion and postprandial lipemia, when co-ingested with a fatty meal. This could be used as a dietary strategy to manage postprandial lipemia (PPL), which is a known risk factor for cardiovascular disease (Jackson, Poppitt, & Minihane, 2012). Therefore, the next study aimed to explore the acute effects of consuming whole apples on PPL in generally healthy but overweight and obese adults, who are at metabolic risk and tend to have impaired PPL (Mekki et al., 1999).
CHAPTER 4. The effects of whole apple consumption on postprandial lipemia, glycemia, insulinemia and gastric emptying in response to an oral fat tolerance test: A randomized crossover trial


This chapter is presented as the manuscript prepared for submission to the Journal of Nutrition.
4.1. Abstract

Background. Postprandial lipemia (PPL) is a risk factor for cardiovascular diseases and may be a target to manage disease risk, including in overweight and obese individuals who can have impaired PPL and higher metabolic risk. Whole apples are a source of pectin and polyphenols, both of which show potential to modulate PPL in in vitro and animal studies, although this has not been investigated in a human study.

Objective. This study aimed to use an oral fat tolerance test (OFTT) to explore the effects of whole apple consumption as a dietary strategy to alter postprandial lipemia, chylomicron metabolism, glycemia, insulinemia and gastric emptying, as a possible mediator of postprandial response, in generally healthy but overweight and obese adults.

Methods. A randomized, crossover acute meal study was conducted with 6 overweight and 20 obese participants (17 women and 9 men, mean ± SEM age of 45.5 ± 3.1 years, body mass index of 34.1 ± 0.2 kg/m², and fasting triacylglycerol (TAG) of 1.38 ± 0.08 mmol/L). Postprandial plasma TAG, apolipoprotein B-48 (ApoB48), chylomicron-rich fraction (CMRF) particle size and fatty acid composition, glucose, insulin and acetaminophen were analyzed for 6 hours after the consumption of the OFTT (1 g fat/kg body weight, containing 1500 mg acetaminophen per meal for estimating gastric emptying) ingested with and without three whole raw Gala apples (~200 g). Analysis of covariance was used to evaluate differences in postprandial response (i.e. mean concentration, peak concentration (C_max), time to peak (T_max) and incremental area under the curve) between treatments.

Results. Consuming whole apples with the OFTT did not alter postprandial TAG response (P > 0.05), nor did it change postprandial concentrations of ApoB48, chylomicron properties, glucose or acetaminophen (P > 0.05), except that ApoB48 peak concentration was higher with apple
consumption \((P < 0.01)\). Apple consumption was also associated with higher postprandial insulin between 20 – 180 min \((P < 0.05)\).

**Conclusions.** As a complex food matrix containing pectin and polyphenols, apples did not modulate PPL or gastric emptying of a high fat meal consumed by overweight and obese adults. This trial was registered at clinicaltrials.gov as NCT03523403.

Keywords: apple, cardiovascular risk, gastric emptying, overweight and obesity, pectin, polyphenol, postprandial lipemia
4.2. Introduction

Postprandial lipemia (PPL) is characterized by the dynamic changes in triacylglycerol (TAG) and TAG-rich lipoproteins (TRLs, i.e. chylomicrons, very-low-density lipoproteins (VLDL) and their remnants) that occur following lipid ingestion (Pirillo et al., 2014). Chylomicrons are intestinally-derived TRLs which transport TAG to the liver after they are reassembled in the enterocytes from dietary and endogenous lipids in the gastrointestinal tract (Tomkin & Owens, 2012). Chylomicron number (characterized by apolipoprotein B-48 (ApoB48) which is constitutive) and/or size increase when a fat-containing meal is consumed and return to baseline over several hours as TAG are cleared to tissues (Tomkin & Owens, 2012). Postprandial lipemic response is now recognized as an important risk factor for atherosclerosis-related diseases (Pirillo et al., 2014) and non-fasting TAG concentrations are a more significant risk factor for coronary heart disease events than fasting TAG (Nakajima et al., 2014). In light of this, oral fat tolerance tests (OFTTs) are increasingly performed to assess the impact of dietary strategies and to investigate the nature of PPL (Lairon et al., 2007). Such tests involve having an individual consume a standard high amount of fat, followed by serial blood sampling for determination of blood lipids. The response to an OFTT varies based on underlying health. For example, chylomicron overproduction is characteristic of individuals with insulin resistance, type 2 diabetes (Dias, Moughan, Wood, Singh, & Garg, 2017) and obesity (Wong, Chan, Pang, Watts, & Barrett, 2014); while smaller and more numerous chylomicrons have been reported in older versus younger populations (Milan et al., 2016) and in hypertriglyceridemic abdominal obese versus lean women (Mekki et al., 1999). The potential atherogenicity of chylomicrons and other TRLs is related to their ability to penetrate the arterial wall, reaching the subendothelial space, and inducing inflammation and oxidative stress (Proctor, Vine, & Mamo,
Smaller chylomicrons are also postulated to clear more slowly from circulating plasma (Nordestgaard & Freiberg, 2011). An OFTT can also be utilized to understand the role of other nutrients and foods on PPL. Such investigations are especially warranted in overweight and obese individuals as they tend to have impaired lipid metabolism, involving increased production and/or insufficient clearance of TRLs, resulting in elevated plasma TAG levels and prolonged PPL (Dias et al., 2017; Tiihonen et al., 2015).

Fruit and vegetable consumption is generally recommended to promote health, including for overweight and obese individuals to reduce their risk of atherosclerosis-related diseases, partly owing to the fact that fruits and vegetables are rich in dietary fiber (Fock & Khoo, 2013). For example, apple skin contains the viscosity-inducing soluble fiber pectin which shows potential to impact several parameters contributing to PPL. Specifically, apple pectin delayed gastric emptying (Di Lorenzo et al., 1988; Schwartz et al., 1982) and lowered fasting blood cholesterol (Brouns et al., 2012; Miettinen & Tarpila, 1977) in human studies. Purified pectin also reduced fasting plasma TAG in a hamster model (Trautwein et al., 1998) and pectin-rich dried beet pulp powder lowered the postprandial TAG response in pigs (Leclere et al., 1993). *In vitro*, pectin has also been shown to alter the rate and/or extent of lipid digestion (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, et al., 2014; Lin & Wright, 2018) and to bind bile salts (Cheewatanakornkool et al., 2012; Dongowski, 1995), which otherwise promote the solubilization of hydrolyzed lipids in the digestate. Apples are also rich in polyphenols, mainly flavanols (e.g. catechin and proanthocyanidins) and hydroxycinnamic acid derivatives (Vrhovsek, Rigo, Tonon, & Mattivi, 2004) that can play a role in PPL. For example, apple procyanidins inhibited pancreatic lipase activity *in vitro* and reduced postprandial TAG elevation in mice (Sugiyama et al., 2007). Apple procyanidins also reduced the secretion of TRLs in
human Caco-2/TC7 enterocytes in vitro, pointing to a possible hypolipidemic effect in vivo (Vidal et al., 2005). Another study with rats found that apple pectin more efficiently improved large intestinal fermentation and reduced fasting plasma TAG and cholesterol in the presence of apple polyphenols, suggesting synergy between apple fibers and polyphenols (Aprikian et al., 2003). That said, human studies related to apples and lipemia have largely focused on fasting blood lipids and yielded mixed results. In hyperlipidemic overweight men, 8-week consumption of 300 g/day golden delicious apple increased fasting serum TAG and VLDL (Vafa et al., 2011), while another study found that 4-week consumption of whole apples (unknown variety) (550 g/day) or apple pomace (22 g/day), compared to fiber-free apple juice (500 mL/day), decreased fasting low density lipoprotein (LDL)-cholesterol concentrations in healthy participants, highlighting the role of apple fibers in lowering LDL (Ravn-Haren et al., 2013).

In summary, apples as a complex food matrix show potential to alter fasting lipids as one of the cardiovascular health benefits (Bondonno et al., 2017). However, to date, their role in PPL, including in individuals at risk of metabolic disease, has not been investigated. Therefore, this study aimed to use an OFTT to explore the effects of consuming whole apples (Gala variety) on the postprandial lipemic response (plasma TAG, ApoB48, chylomicrons and remnants particle sizes and fatty acid (FA) composition), gastric emptying, glycemia and insulinemia in overweight and obese individuals. It was hypothesized that apple consumption would reduce the magnitude of PPL by slowing down the gastric emptying of the high fat meal (HFM).

4.3. Materials and Methods
4.3.1. Participants

Participants were recruited through flyer, internet, newspaper and radio advertisements in Guelph, Ontario, Canada and the surrounding communities. Inclusion criteria were: 18–75 years; body mass index (BMI) > 25.0 kg/m²; generally healthy; non-alcoholic; non-smoker; non-diabetic; free of digestive, cardiovascular, or inflammatory diseases/disorders; stable body weight (<5% fluctuation) for the previous 3 months; and no intention to gain or lose weight. Individuals were excluded if they were: hospitalized due to serious medical conditions within the last year; taking medications that could interfere with the study outcomes; allergic or intolerant to any ingredients in the test meals; pregnant, breastfeeding or post-menopausal. Of the 179 persons screened, 51 were invited for further screening, among which 28 met the inclusion and exclusion criteria and were enrolled in the study, all of which took place from January 2017 to September 2018.

4.3.2. Study Design and Protocols

This was a randomized, cross-over acute meal study nested within a larger parallel arm study comparing the effects of 6-week apple consumption on markers of lipid metabolism and inflammation. The study was conducted at the Human Nutraceutical Research Unit at the University of Guelph, approved by University of Guelph Human Research Ethics Board (REB# 16JA013), registered at clinicaltrials.gov (NCT03523403, The Apple Study: Investigating the Effects of Whole Apple Consumption on Risk Factors for Chronic Metabolic Diseases in Overweight and Obese Adults), and all participants provided written informed consent. Each participant attended two study visits separated by a one-week wash-out period and were randomized based on their order of study enrolment using a random number table (generated by Excel, Microsoft Ltd., organized by an external party) to consume either a dairy-based HFM
alone or the same HFM with apples at each visit. Due to the nature of the treatments, participants and researchers were not blinded at each study visit, although samples and data were coded based only on study visit day in order to conceal treatment allocation during their handling and analyses. Two weeks before the first study visit, participants were counselled to avoid foods rich in polyphenols and/or dietary fibers (e.g. berries, purple and red potatoes, and coffee), but to otherwise maintain their dietary and exercise habits which were recorded in a study diary. For 48 hours before each study visit, participants abstained from alcohol, exercise and over-the-counter medications (including acetaminophen-containing products). Participants consumed a standardized low fat (12.0 – 12.5 g fat per serving) dinner the night before each study visit. This consisted of one serving of vegetarian lasagna (President’s Choice Blue Menu), a cranberry almond granola bar (President’s Choice Chewy Trail Mix), a pudding (chocolate or vanilla flavored, Hunts), and a juice box (apple or fruit punch, President’s Choice). On the morning of each study visit, participants arrived at the research centre having fasted for 10-12 hours. Water consumption was encouraged for up to 1 hour before a phlebotomist inserted an intravenous catheter for fasting and periodic blood sampling over 6 hours. Participants consumed the test meals within 15 min and baseline (i.e. 0 min) was from when participants started to consume the meal. Throughout the day, participants remained seated in the research centre with only short breaks permitted to use the washroom.

4.3.3. Test Meal Treatments

On each study visit day, participants consumed the HFM which was a 500 mL mixture of 35% fat whipping cream and skim milk, standardized with additional skim milk powder such that each meal provided 1 g fat/kg body weight and 14.6 g protein. Extra Strength Tylenol (TYLENOL®, 00559407) containing 1500 mg acetaminophen was ground and added to each
HFM shortly before consumption by participants, for indirect assessment of gastric emptying (Willems et al., 2001). The HFM was well tolerated by most participants, although one participant vomited 20 min after its consumption and discontinued participation. Three Gala apples (provided by Martin’s Family Fruit Farm and Norfolk Fruit Growers Association in Ontario, Canada) were cored and sliced without removing the skin (~200 g total) and then consumed along with the HFM at one of the study visits. The pectin and total polyphenol contents of the Gala apples were measured using colorimetric assays. Briefly, whole apples were cored and sliced and then freeze dried (VirTis Genesis 35L pilot lyophilizer, Stone Ridge, NY, USA). For pectin analysis, alcohol insoluble solids were extracted from the dried apples and then hydrolyzed by sulfuric acid to galacturonic acid, which was reacted with m-hydroxydiphenyl and, finally, absorption was measured at 525 nm using a UV–vis spectrophotometer (Hewlett Packard 8451A Diode Array Spectrophotometer) and concentration was determined using a standard curve based on D-galacturonic acid (0 – 0.5 mg/mL) (Melton & Smith, 2001). For the total phenolic analysis, the dried apples were homogenized with acidified methanol to remove vitamin C and the extract was mixed with the Folin-Ciocalteu reagent and sodium carbonate solution, followed by absorbance measurements at 750 nm and using a standard curve based on gallic acid (0 – 0.156 mg/mL) (Almeida et al., 2011). The meals’ nutrient compositions are provided in Table 4.1.
Table 4.1. Nutrient composition of the test meals

<table>
<thead>
<tr>
<th>Nutrients (per meal)</th>
<th>HFM (500 mL)</th>
<th>HFM (500 mL) + Apples (200 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kCal)</td>
<td>792.7 – 1524.1</td>
<td>906.7 – 1638.1</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>14.6</td>
<td>15.8</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>20.0 ± 0.1</td>
<td>47.5</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
<td>11.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>5.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>3.3</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>71.6 - 154.0</td>
<td>71.6 – 154.0</td>
</tr>
<tr>
<td>FA Composition (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:0</td>
<td>2.1 ± 0.4</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>12:0</td>
<td>3.3 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>14:0</td>
<td>12.0 ± 0.1</td>
<td>12.0 ± 0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>36.7 ± 0.1</td>
<td>36.7 ± 0.1</td>
</tr>
<tr>
<td>16:1</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>18:0</td>
<td>11.6 ± 0.1</td>
<td>11.6 ± 0.1</td>
</tr>
<tr>
<td>18:1c9</td>
<td>22.1 ± 0.1</td>
<td>22.1 ± 0.1</td>
</tr>
<tr>
<td>18:2</td>
<td>2.4 ± 0.0</td>
<td>2.4 ± 0.0</td>
</tr>
<tr>
<td>Total Saturated FAs</td>
<td>67.8 ± 0.4</td>
<td>67.8 ± 0.4</td>
</tr>
<tr>
<td>Total Unsaturated FAs</td>
<td>32.2 ± 0.4</td>
<td>32.2 ± 0.4</td>
</tr>
<tr>
<td>Pectin (g)</td>
<td>-</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Total polyphenols (g)</td>
<td>-</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Acetaminophen (g)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

1 Values calculated based on nutrient reports in Health Canada Nutrients Database (Health Canada, 2015), unless otherwise stated. 2 Calculated based on product labels (for HFM) and nutrient reports in Health Canada Nutrients Database (for apples). 3 Depends on participants’ body weight, aiming to provide 1 g fat/kg body weight; 4 % total area count, measured by gas chromatography, as below; 5 Presented as galacturonic acid equivalents; 6 Presented as gallic acid equivalents. 10:0, decanoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1c9, oleic acid; 18:1c11, vaccenic acid; 18:2, linoleic acid. FA, fatty acid; HFM, high fat meal.

4.3.4. Blood Sampling and Analysis

Blood samples were taken at baseline, every 20 min within the first 3 hours, and at 4, 5, and 6 hours. All samples were drawn from the antecubital vein into EDTA vacutainer tubes via an intravenous line (Vacutainer®, K2EDTA, BD, NJ, United States). After centrifugation at 625 g (Allegra TM X-22R Centrifuge, Beckman Coulter Incorporated, CA, USA) at 4 °C for 10 min, plasma was collected, aliquoted into cryovials in small volumes, and immediately stored at
−80 °C until analysis by commercial assays. Plasma concentrations of the primary outcome, i.e. plasma TAG (Wako Diagnostics, USA) and secondary outcomes, i.e. ApoB48 (Cloud Clone, China), acetaminophen (Neogen, USA), glucose (Wako Diagnostics, USA) and insulin (Mercodia AB, Sweden) were determined according to the manufacturer’s instructions. The intra-assay variabilities were 10.0, 7.3, 5.3, 8.8 and 5.9 percent for TAG, ApoB48, acetaminophen, glucose and insulin, respectively. Fasting cholesterol concentrations were determined immediately from a drop of whole venous blood using the Cholestech LDX Lipid Analyzer (Cholestech, Hayward, CA, USA).

4.3.5. Chylomicron-rich Fraction (CMRF) Analysis

The CMRF was separated from plasma based on Vors et al. (2013) with slight modifications for determination of the secondary outcomes CMRF particle size and FA composition. Briefly, 3.0 mL plasma was overlaid with 2.5 mL saline solution (d = 1.006 g/mL) and centrifuged at 65,000 rpm at room temperature for 23 min. The top layer, which was white and cloudy, was aspirated for immediate particle size analysis by dynamic light scattering (NanoZetasizer S, Malvern Instruments, UK), using a refractive index of 1.450 for human plasma protein and absorption of 0.001 as input parameters for calculations (Milan, 2015). A portion was also stored in an Eppendorf tube at −80 °C until analysis of FA composition by gas chromatography (GC) within 1 month. For this, lipid extractions were performed based on the Folch method with slight modifications (Martin et al., 2010). 0.03 mL of CMRF was vortexed with 0.97 mL of 0.1 M KCl and 4 mL of chloroform:methanol (2:1) until well mixed. The mixture was flushed with N₂ and sat at 4 °C overnight before centrifugation at 1460 rpm for 10 min. The lower chloroform layer was collected and dried under nitrogen and then saponified by 2 mL of 0.5 M KOH in methanol at 100 °C for 1 h. After cooling to room temperature, methylation
was carried out with 2 mL hexane and 2 mL 14% BF$_3$-MeOH at 100 °C for 1 h. After the mixture was cooled, 2 mL water was added to stop the methylation and the mixture vortexed and centrifuged at 1460 rpm for 10 min. The top hexane layer containing the fatty acid methyl esters (FAMEs) was collected and dried down under N$_2$ and then reconstituted in 1 mL of hexane for analysis. FAMEs were separated by GC using an Agilent 6890 Network GC System (Agilent Technologies, CA, USA) with a Supelco SP 2560 fused-silica capillary column (100m×0.25mm i.d., 0.2 μm film thickness; Sigma-Aldrich, St Louis, MO, USA). The FA composition is presented as % total area count, calculated by dividing the peak area of the target FAs by the total area. Saturated and unsaturated FAs (SFA and UFA, respectively) from C10:0 to C24:0 were grouped separately for calculating their % total area counts and the ratio between them (SFA:UFA ratio). The same methods were used to determine FA profiles of the HFM.

4.3.6. Data and Statistical Analyses

All measurements were completed in triplicate, unless otherwise stated and analyzed using Statistical Package for the Social Sciences version 21 (SPSS; IBM Corporation) with a significance level of $P < 0.05$. Outlying values (in total two, based on Z-scores method) were excluded from the statistical analysis and data normality was assessed using Shapiro-Wilk testing. All normally distributed data are presented as the arithmetic mean ± SEM. When not normally distributed, data were log transformed and presented as the geometric mean ± average transformed SEM, as indicated. All fasting endpoints were compared between study visits 1 and 2 using paired-sample t-tests. Fasting glucose and insulin concentrations were used to calculate the homoeostatic model assessment of insulin resistance (HOMA-IR), using the HOMA2 Calculator (Version 2.2.3, Diabetes Trials Unit, University of Oxford). For the postprandial time-wise data, repeated measures ANCOVA were performed with centered baseline value (i.e.
individual baseline value minus mean baseline value) as a covariate, as suggested in Schneider et al. (Schneider, Avivi-Reich, & Mozuraitis, 2015), and Bonferroni post hoc testing. For each endpoint, the effects of treatment (HFM with or without apples), time (postprandial timepoints) and treatment x time interactions were accessed. Participant characteristics, including sex (male vs. female), age, body weight and BMI were added into the ANCOVA models as between-subject factors or covariates (for nominal and continuous values, respectively) to assess their effects on the endpoints, but only those with significant effects were retained in the models. Specifically, this was only sex, body weight and BMI in the acetaminophen data. Incremental area under the curve (iAUC) was calculated using the linear trapezoidal method (GraphPad Prism version 7.04 for Windows, San Diego California USA, www.graphpad.com) and compared using ANCOVA using centered baseline values as a covariate (Tiihonen et al., 2015). Maximum concentrations (C_max) of each endpoint were identified as the greatest value in an individual’s data set and maximum peak times (T_max) identified as the corresponding nominal sampling time. Mean C_max and median T_max are reported. Values of C_max were compared using paired-sample t-testing while T_max values were compared using median test for 2 independent samples test. Statistical analyses were also performed on change from baseline values, but no differences in results were observed from those based on absolute values (data not shown).

4.4. Results

4.4.1. Participant Characteristics

Of the 28 participants enrolled, 26 completed the study and 2 participants withdrew (Figure 4.1). Participant characteristics and fasting measurements are shown in Table 4.2. There were no differences in participant characteristics or fasting blood measurements between the two study visit days (P > 0.05 for all measurements, data not shown).
Figure 4.1. CONSORT flow diagram of the study. CONSORT, Consolidated standards of reporting trials
Table 4.2. Characteristics and fasting blood measurements for male (n=9) and female (n=17) participants$^1$

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean ± SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>45.5 ± 3.1</td>
<td>19 – 69</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>100.0 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>118.0 ± 7.5</td>
<td>91.6 – 154.0</td>
</tr>
<tr>
<td>Female</td>
<td>90.5 ± 4.1</td>
<td>71.6 – 131.6</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>34.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36.6 ± 2.3</td>
<td>27.2 – 48.2</td>
</tr>
<tr>
<td>Female</td>
<td>32.7 ± 1.3</td>
<td>25.4 – 41.5</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>112.8 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>122.1 ± 4.6</td>
<td>106.0 – 149.0</td>
</tr>
<tr>
<td>Female</td>
<td>107.9 ± 3.0</td>
<td>87.0 – 133.0</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.88 ± 0.12</td>
<td>2.59 – 6.70</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.14 ± 0.09</td>
<td>1.75 – 4.27</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.11 ± 0.05</td>
<td>0.57 – 2.06</td>
</tr>
<tr>
<td>Glucose (mmol/L)$^2$</td>
<td>4.98 ± 0.18</td>
<td>3.49 – 6.76</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>61.15 ± 9.54</td>
<td>14.50 – 183.50</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.14 ± 0.17</td>
<td>0.30 – 3.30</td>
</tr>
<tr>
<td>TAG (mmol/L)</td>
<td>1.38 ± 0.08$^3$</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>1.30 ± 0.11</td>
<td>0.75 – 1.89</td>
</tr>
<tr>
<td>Female</td>
<td>1.42 ± 0.10$^4$</td>
<td>0.66 – 2.31</td>
</tr>
<tr>
<td>ApoB48 (mg/L)</td>
<td>23.09 ± 1.90</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27.02 ± 3.23</td>
<td>15.62 – 40.74</td>
</tr>
<tr>
<td>Female</td>
<td>21.01 ± 2.25</td>
<td>1.29 – 38.53</td>
</tr>
<tr>
<td>CMRF particle size (nm)</td>
<td>55.25 ± 1.82</td>
<td>45.56 – 85.01</td>
</tr>
<tr>
<td>CMRF SFA:UFA ratio</td>
<td>0.50 ± 0.01</td>
<td>0.40 – 0.59</td>
</tr>
</tbody>
</table>

$^1$Values are the average of the fasting measurements on both study visit days. $^2$ n = 25 (one male participant outlying value eliminated from the datasets); $^3$ n = 25; $^4$ n = 16 (one female participant outlying value eliminated from the datasets). ApoB48, apolipoprotein 48; BMI, body mass index; CMRF: chylomicron-rich fraction; HDL, high density lipoprotein; HOMA-IR, homoeostatic model assessment of insulin resistance; SFA, saturated fatty acids; TAG, triacylglycerols; UFA, unsaturated fatty acids.

4.4.2. Postprandial TAG and ApoB48

Following consumption of both test meals, plasma TAG concentrations increased rapidly in the first 3 hours ($P < 0.05$) and started to decrease after 5 hours (Figure 4.2A). The consumption of apples did not have an overall main effect on postprandial TAG and there were no differences in TAG concentration between with and without apples at any time point throughout the 6-hour postprandial period ($P = 0.73$, Figure 4.2A). Similarly, peak TAG
concentration ($C_{\text{max}}$), TAG iAUC, and time to peak concentration ($T_{\text{max}}$) did not differ when the HFM was consumed alone versus with three apples ($P = 0.92, 0.39$ and $0.27$ respectively, Table 4.4). In terms of postprandial ApoB48 concentration, there were effects of time ($P < 0.01$) but not treatment ($P = 0.09$, Figure 4.2B) observed. ApoB48 iAUC and median $T_{\text{max}}$ values also did not differ between treatments ($P = 0.26$ and $0.38$ respectively, Table 4.4). However, ApoB48 $C_{\text{max}}$ was higher when the HFM was consumed with apples ($P < 0.01$, Table 4.4).
Figure 4.2. Postprandial plasma TAG (A) and ApoB48 (B) concentrations following consumption of HFM with and without apples. Data are presented as mean ± SEM, n = 26. $P_{time}$, $P_{treatment}$, and $P_{time*treatment}$ refer to results from repeated-measures ANCOVA, using centered fasting TAG (A) and ApoB48 (B) values as covariates, followed by Bonferroni post hoc testing. ApoB48, apolipoprotein B-48; TAG, triacylglycerol.
4.4.3. Postprandial Size and Fatty Acid Composition of the CMRF

CMRF particle size increased rapidly within 2 hours of HFM ingestion ($P < 0.05$), and reached a plateau, not changing between 4 and 6 hours ($P = 0.42$, Figure 4.3A). There were no differences in postprandial CMRF size between treatments over the 6 hours ($P = 0.94$, Figure 4.3A). The main FAs identified in the CMRF were myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1c9), vaccenic (18:1c11) and linoleic (18:2). The HFM also contained decanoic (10:0) and lauric (12:0) acids, but these species were not present in appreciable amounts in the CMRF (% total area count < 2% at peak value, data not shown). For both study visit days, the baseline CMRF SFA:UFA ratio was ~ 0.5 ($P = 0.11$). This ratio visibly increased following HFM ingestion, peaking at (at least) 6 hours (Figure 4.3B, i.e. $0.90 \pm 0.02$ and $0.84 \pm 0.02$, with and without apples respectively, $P < 0.01$), but there was no main effect of treatment ($P = 0.23$, Figure 4.3B). The proportion of individual saturated FAs, including 14:0, 16:0 and 18:0, did increase substantially with time (Table 4.3, $P_{\text{time}} < 0.01$). Correspondingly, the unsaturated FAs 16:1, 18:1c9 and c11, and 18:2 decreased over the same timeframe ($P_{\text{time}} < 0.01$, Table 4.3). However, apple consumption was not associated with any differences in CMRF FA composition.
Figure 4.3. Postprandial CMRF particle size (A) and SFA:UFA ratio (B) after consumption of HFM with or without apple. A: Data are presented as means ± SEM, n = 26 per treatment; $P_{time}$, $P_{treatment}$, and $P_{time\times treatment}$ refer to results from repeated-measures ANCOVA using centered fasting CMRF particle size (A) and SFA:UFA ratio (B) values as covariates, followed by Bonferroni post hoc testing. * $P < 0.01$ between with and without apples. CMRF, chylomicron-rich fraction; SFA:UFA ratio, the ratio between % total area count of saturated and unsaturated fatty acids; $Z$-average, intensity weighted harmonic mean size measured by dynamic light scattering.
Table 4.3. CMRF fatty acid composition at fasting and throughout the 6-hour postprandial period.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>0 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>ANCOVA Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$P_{time}$  $P_{treatment}$</td>
</tr>
<tr>
<td>14:0</td>
<td>1.7 ± 0.1</td>
<td>4.4 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>2.0 ± 0.1</td>
<td>4.3 ± 0.2</td>
<td>5.4 ± 0.2</td>
<td>5.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>24.1 ± 0.4</td>
<td>26.6 ± 0.4</td>
<td>29.2 ± 0.5</td>
<td>30.3 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>24.7 ± 0.4</td>
<td>26.9 ± 0.4</td>
<td>28.9 ± 0.4</td>
<td>30.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>16:1 c9</td>
<td>3.4 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>3.6 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>3.0 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>5.0 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td>7.4 ± 0.2</td>
<td>7.7 ± 0.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>5.4 ± 0.3</td>
<td>6.4 ± 0.2</td>
<td>7.4 ± 0.2</td>
<td>8.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>18:1 c9</td>
<td>32.6 ± 0.4</td>
<td>29.8 ± 0.4</td>
<td>27.7 ± 0.3</td>
<td>26.9 ± 0.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>31.9 ± 0.5</td>
<td>29.8 ± 0.4</td>
<td>27.8 ± 0.3</td>
<td>27.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>18:1 c11</td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>0.006</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>19.5 ± 0.5</td>
<td>15.9 ± 0.5</td>
<td>13.2 ± 0.4</td>
<td>12.5 ± 0.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>With apple</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without apple</td>
<td>18.3 ± 0.4</td>
<td>15.2 ± 0.4</td>
<td>13.1 ± 0.4</td>
<td>12.5 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are expressed as the percentage of total area counts (%) in gas chromatograph. 2 $P_{time}$ and $P_{treatment}$ refer to main effects of time and treatment in ANCOVA. C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1c9, oleic acid; C18:1c11, vaccenic acid; C18:2, linoleic acid; CMRF, chylomicron-rich fraction.

4.4.5. Postprandial Acetaminophen as An Indicator of Gastric Emptying

Acetaminophen concentration increased significantly after ingestion of the HFM ($P <$ 0.05) and slowly decreased towards baseline after 3 hours (Figure 4.4A). Treatment did not have an overall effect on postprandial acetaminophen concentration ($P = 0.08$, Figure 4.4A), nor was apple consumption associated with differences in acetaminophen iAUC, $C_{max}$ or $T_{max}$ ($P = 0.44$, 0.66 and 0.58 respectively, Table 4.4). Overall, females did have higher postprandial acetaminophen concentrations and higher iAUC values than males ($P_{male-female} = 0.007$, Figure 4.4B). Acetaminophen iAUC was also higher in females than males with apples ($P < 0.05$) and trended to be higher for females without apples ($P = 0.06$, Table 4.4). Sex did not have an effect.
on acetaminophen $C_{\text{max}}$ or $T_{\text{max}}$ within each treatment ($P > 0.05$ for all measurements). Like sex, body weight and BMI were inversely associated with plasma acetaminophen concentration ($P < 0.01$) and body weight contributed to iAUC and $C_{\text{max}}$ ($P < 0.01$).

![Graph A](image1)

**Figure 4.4.** Log-transformed postprandial plasma acetaminophen concentrations (A) and the influence of gender (B). A: Data are presented as geometric means ± average transformed SEM; $n = 26$ per treatment; $P_{\text{time}}$, $P_{\text{treatment}}$, and $P_{\text{time*treatment}}$ refer to results from repeated-measures ANCOVA with Bonferroni *post hoc* test. * $P < 0.05$ between with and without apples. B: Data are presented as geometric means ± average transformed SEM; $n = 9$ for the male group and $n = 17$ for the female group; $P_{\text{male-female}}$ refer to results from repeated-measures ANCOVA with Bonferroni *post hoc* test.
4.4.6. Postprandial Glucose and Insulin

Glucose concentration fluctuated significantly during the postprandial period \((P < 0.05)\) but did not differ between treatments \((P = 0.75, \text{Figure } 4.5\text{A})\). There was an expected, but statistically insignificant, early rise in plasma glucose with the apple treatment which was absent without apple. Overall, apple consumption also did not influence postprandial glucose \(i\text{AUC}, C_{\text{max}}\) and \(T_{\text{max}}\) values \((P = 0.45, 0.92\) and 1.00 respectively, Table 4.4\). Insulin concentration increased quickly after both HFM with and without apples consumption \((P < 0.05)\), and gradually returned to the fasting levels after 360 min (Figure 4.5B). With apple consumption, significantly higher insulin concentrations were observed from 20 to 180 min \((P < 0.05, \text{Figure } 4.5\text{B})\). \(i\text{AUC}\) and \(C_{\text{max}}\) values were also both higher with apple consumption \((P < 0.05, \text{Table } 4.4)\), although \(T_{\text{max}}\) was 60 min regardless of treatment \((P = 0.76, \text{Table } 4.4)\).
Figure 4.5. Postprandial plasma glucose (A), and log-transformed plasma insulin (B) concentrations. A: Data are presented as means ± SEM, n = 25 per treatment (one male participant outlying data removed from database); B: Data are presented as geometric means ± average transformed SEM, n = 26 per treatment. $P_{time}$, $P_{treatment}$, and $P_{time*treatment}$ refer to results from repeated-measures ANCOVA with Bonferroni post hoc test. * $P < 0.05$ between with and without apples.
Table 4.4. Postprandial TAG, ApoB48, acetaminophen, glucose and insulin iAUC, $C_{\text{max}}$ and $T_{\text{max}}$ values following ingestion of HFM with and without apples

<table>
<thead>
<tr>
<th></th>
<th>Without apples</th>
<th>With apples</th>
<th>$P$ value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAUC (mmol/L·h)</td>
<td>5.1 ± 0.7</td>
<td>5.4 ± 0.5</td>
<td>0.921</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (mmol/L)</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>0.385</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>300</td>
<td>240</td>
<td>0.266</td>
</tr>
<tr>
<td>ApoB48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAUC (mg/L·h)</td>
<td>30.9 ± 6.4</td>
<td>44.8 ± 10.0</td>
<td>0.257</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (mg/L)</td>
<td>32.7 ± 1.9</td>
<td>40.8 ± 3.5</td>
<td>0.007*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>180</td>
<td>240</td>
<td>0.375</td>
</tr>
<tr>
<td>Acetaminophen</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>iAUC (µmol/L·h)</td>
<td>448.2 ± 53.6</td>
<td>476.8 ± 47.5</td>
<td>0.440</td>
</tr>
<tr>
<td>Male</td>
<td>311.5 ± 64.0</td>
<td>345.1 ± 67.7</td>
<td>0.601</td>
</tr>
<tr>
<td>Female</td>
<td>520.5 ± 69.6</td>
<td>546.6 ± 57.4</td>
<td>0.577</td>
</tr>
<tr>
<td>$P_{\text{male-female}}$</td>
<td>0.062</td>
<td>0.041*</td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µmol/L)</td>
<td>146.8 ± 16.2</td>
<td>140.1 ± 13.0</td>
<td>0.661</td>
</tr>
<tr>
<td>Male</td>
<td>114.7 ± 25.7</td>
<td>118.7 ± 19.7</td>
<td>0.881</td>
</tr>
<tr>
<td>Female</td>
<td>163.7 ± 20.0</td>
<td>151.4 ± 16.7</td>
<td>0.521</td>
</tr>
<tr>
<td>$P_{\text{male-female}}$</td>
<td>0.154</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>120</td>
<td>120</td>
<td>0.577</td>
</tr>
<tr>
<td>Male</td>
<td>100</td>
<td>120</td>
<td>1.000</td>
</tr>
<tr>
<td>Female</td>
<td>140</td>
<td>120</td>
<td>0.732</td>
</tr>
<tr>
<td>$P_{\text{male-female}}$</td>
<td>1.000</td>
<td>0.399</td>
<td></td>
</tr>
<tr>
<td>Glucose$^1$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAUC (mmol/L·h)</td>
<td>2.3 ± 0.4</td>
<td>2.8 ± 0.5</td>
<td>0.446</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (mmol/L)</td>
<td>6.3 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td>0.923</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>160</td>
<td>120</td>
<td>1.000</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iAUC (pmol/L·h)</td>
<td>243.6 ± 38.0</td>
<td>434.6 ± 47.3</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (pmol/L)</td>
<td>250.8 ± 31.6</td>
<td>356.0 ± 30.2</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (min)</td>
<td>60</td>
<td>60</td>
<td>0.760</td>
</tr>
</tbody>
</table>

iAUC and $C_{\text{max}}$ of different treatment groups were compared using ANCOVA with fasting values as covariates, while $T_{\text{max}}$ were compared using Median test, iAUC and $C_{\text{max}}$ values are presented as means ± SEM and $T_{\text{max}}$ are medians; n = 26 per treatment. $^1$ n = 25, as one male participant outlying data removed from database. * $P < 0.05$.

4.5. Discussion

The present study aimed to evaluate the influence of consuming three whole raw Gala apples with a dairy-based HFM on postprandial TAG, CMRF properties, glycemia, insulinemia and gastric emptying to elucidate the role of apple consumption on PPL, as a risk factor for
atherosclerotic disease risk, in otherwise healthy overweight or obese participants. The effects of three whole Gala apples were explored because Gala apples are commonly available and consumed in Ontario, Canada, and ~ 200 g edible parts from three apples is a reasonable amount of fruits consumed in a single meal, given it is equivalent to the weight of one large apple (Health Canada, 2015). The main finding is that apple consumption had no major influence on PPL following an HFM. Gastric emptying, which may have been impacted by consuming whole apples, given that dietary fiber can slow down gastric emptying, in turn attenuating PPL (Lairon, Play, & Jourdheuil-Rahmani, 2007), was not implicated. Although a few human studies have examined their effects on fasting lipid profiles (Avci et al., 2007; Conceicao de Oliveira, Sichieri, & Moura, 2003; Dange & Deshpande, 2013; Ravn-Haren et al., 2013; Vafa et al., 2011), the influence of apple consumption on PPL, as an established independent risk factor for CVD, is understudied and remains equivocal. Therefore, this work contributes by uniquely evaluating the effects of whole Gala apples as a commonly consumed North American food in a postprandial setting to understanding impacts on PPL in overweight and obesity.

In the present study, participants’ BMI ranged from 25.4 to 48.2 kg/m², and 9 were classified as overweight (25 ≤ BMI < 30 kg/m²) and 17 as obese (BMI ≥ 30 kg/m²). The average fasting level of plasma TAG was 1.4 ± 0.1 mmol/L, but this ranged from 0.66 to 2.31 mmol/L. As such, both normolipidemic (< 1.69 mmol/L, n = 20) and hyperlipidemic (≥ 1.69 mmol/L, n = 6, 2 of whom were obese) individuals were included in the study. It is somewhat surprising that, of the 9 overweight participants, 4 were hyperlipidemic and, of the 17 obese participants, only 2 were hyperlipidemic. This may have contributed to the absence of expected correlations between postprandial TAG and BMI reported in other instances (Nogaroto et al., 2015). The average TAG level increased to $C_{\text{max}} 3.0 ± 0.3$ mmol/L around 4-5 hours after the HFMs were consumed.
and did not return to baseline by the end of the 6-hour postprandial period (2.3 ± 0.2 mmol/L, \( P < 0.05 \)). Thus, the extent and length of TAG elevations observed are comparable to previous OFTT studies which provided the same 1 g fat/kg body weight in healthy and overweight (Cohn, McNamara, Cohn, Ordovas, & Schaefer, 1988; Lopez-Miranda et al., 1997; Robinson et al., 2009), and obese (Robinson et al., 2009) participants.

The CMRF contains reassembled dietary TAG from intestinally-derived chylomicrons (Milan et al., 2016). The changes observed in the CMRF FA composition reflect the composition of the HFM, as expected (Mu & Høy, 2004), and did not differ with and without apple consumption. Specifically, the proportions of saturated FAs (16:0, in particular) increased in the CMRF as the unsaturated FAs (18:1c9 and 18:2) decreased over the postprandial period. The HFM had an SFA:UFA ratio of 2.1. In the CMRF, this ratio almost doubled, increasing from ~0.5 at fasting to 0.9 and 0.8 at 6 hours with and without apples, respectively, although the CMRF FAs did not exactly match that of the meals consumed. Chylomicrons can contain FAs from previous meals in the enterocyte storage pools (Mu & Høy, 2004). As such, the low-fat standardized pre-study evening meal was an important design parameter intended to minimize variability in participant CMRF FA composition (Lairon et al., 2007). We previously observed preferential aqueous phase solubilization of DHA from algal oil emulsions undergoing \textit{in vitro} digestion in the presence of a low level of purified apple pectin, suggesting the potential for alterations in FA absorption based on the presence of the fiber in the gastrointestinal tract, even when overall digestibility was unchanged (Lin & Wright, 2018). There is limited research investigating differences in postprandial chylomicron FA composition based on dietary fiber ingestion and food ingredient interactions, in relation to CVD risk. However, this is rationalized based on evidence that FA composition influences the conversion rate from chylomicrons to
remnants in rats (Rahman, Avella, & Botham, 1999), of interactions between chylomicron remnants with isolated rat hepatocytes (Lambert, Avella, Berhane, Shervill, & Botham, 2001), and of chylomicron remnant-like particles uptake by macrophages \textit{in vitro} (De Pascale et al., 2006), all of which have implications for CVD risk. Nevertheless, in the present study, no differences in the CMRF incorporation of specific FAs were evident between the meals, just as there were no differences observed in postprandial TAG response with and without apples. Of note, although age was previously associated with postprandial CMRF FA composition (i.e. elderly (60 – 75 y) versus young (20 – 25 y) adults had higher portions of oleic acid in their CMRF at 2 and 4 h after consuming an identical meal) (Milan et al., 2016), in the present study, age did not have any impact on CMRF FA composition when included as a covariate.

In terms of fasting ApoB48, there was a wide range of values observed (1.29 – 40.74 mg/L), possibly related to the variability in participants’ metabolic and health status, including fasting TAG levels. For example, with 335 normolipidemic and 253 hyperlipidemic participants, Sakai et al. (Sakai et al., 2003) observed that ApoB48 varied tremendously, although, generally, individuals with hypertriglyceridemia had significantly higher fasting ApoB48 levels, compared to the normolipidemic population (6.4 – 92.4 vs. 5.2 mg/L, respectively). Indeed, baseline TAG was found to be a significant covariate for postprandial ApoB48 (individually, but not when baseline ApoB48 was included as a covariate) in the present study \((P = 0.001)\). Although obesity has been associated with exaggerated postprandial ApoB48 elevations after a fat load (Wong et al., 2014), there was no difference in the postprandial increase of ApoB48 in obese participants comparing to overweight participants \(n = 9\) and \(17\) respectively, \(P = 0.65\), data not shown). The range of postprandial ApoB48 increase from baseline observed in the present study \((5 – 10\) mg/L) was similar to Sakai et al. (Sakai et al., 2003) in which participants consumed 30 g fat/m²
body surface of heavy cream (35% cream). CMRF particle size increased during the postprandial period, as expected when they are loaded with intestinally-derived reassembled TAG (Xiao & Lewis, 2012). The trends agree with previous observations that increases in particle size are much greater than particle number following ingestion of a HFM (Martins, Mortimer, Miller, & Redgrave, 1996; Xiao & Lewis, 2012). Smaller and higher numbers of chylomicrons and their remnants are proposed to increase atherosclerotic risk due to their ability to penetrate the arterial intima (Milan et al., 2016) and slower clearance from plasma (Martins et al., 1996). Consuming apples along with an HFM did not influence the number or size of circulating chylomicrons and remnants, in the current study. Hence apple consumption did not impact atherosclerotic risk related to postprandial chylomicron metabolism.

Overall, whole apple consumption did not alter postprandial lipemic response (TAG, ApoB48, CMRF properties) that occurred following ingestion of the HFM. Animal studies have shown attenuated postprandial TAG with ingestion of purified pectin and polyphenols (Leclere et al., 1993; Sugiyama et al., 2007), but such trends were not exhibited with whole apples, as a food matrix containing both pectin and polyphenols, in the present study. This could be because the amounts of these bioactives were lower than required to induce observable acute lipid lowering. For example, Leclere et al. (Leclere et al., 1993) fed 30 g dried sugar beet pulp fiber containing 4.11 to 7.32 g soluble fibers, mainly pectin (USDA National Organic Program, 2012), to pigs weighing ~30 kg. In the present study, participants weighed between 71.6 and 154.0 kg and consumed only 2.17 g pectin. Sugiyama et al. (Sugiyama et al., 2007) observed that the administration of an apple polyphenol extract at 1000 mg/kg body weight completely inhibited postprandial TAG elevation in mice. This is much higher than the dosage of apple polyphenols consumed from whole apples in the present study (i.e. 1.49 – 3.21 mg/kg body weight).
Therefore, even though ~200 g apples were ingested, the levels of pectin and polyphenols may have been insufficient to induce alterations in PPL. Moreover, compared with the HFM alone, the apple meal contained higher and different amounts of digestible carbohydrates, including fructose and sucrose (Table 4.1), which can promote postprandial hypertriglyceridemia when consumed with fat (Grant, Marais, & Dhansay, 1994; Lopez-Miranda et al., 2007; Saito, Kagaya, Suzuki, Yoshida, & Naito, 2013). With fructose, proposed mechanisms include a lower activation of adipose tissue lipoprotein lipase and reduced plasma TAG clearance (Chong, Fielding, & Frayn, 2007; Grant et al., 1994), improved de novo lipogenesis since fructose is a potent precursor (Chong et al., 2007; Parks, Skokan, Timlin, & Dingfelder, 2008), and a greater secretion of chylomicrons from enterocytes (Steenson, Umpleby, Lovegrove, Jackson, & Fielding, 2017). The latter would be indicated by a higher peak concentration of ApoB48 particles with apple consumption, although overall postprandial ApoB48 concentration was not altered. Therefore, fructose-induced postprandial hypertriglyceridemia with apple consumption may have counteracted any lipid-lowering potential of the pectin and polyphenols present. It should also be noted that the present study may have been under powered, given that a sample size calculation indicated 82 participants would be required, based on a statistical power of 80% and a significant reduction of 0.5 mmol/L in peak TAG concentration (the difference between normolipidemic and obese population observed in Tiihonen et al. (Tiihonen et al., 2015)). However, with 26 participants in this crossover study, the number of participants is within range of other postprandial lipemia studies where significant differences in plasma TAG have been observed with treatment (Hall et al., 2014; Kondo et al., 2004; Robinson et al., 2009).

Digestible and indigestible carbohydrates contribute to postprandial glycemia and insulinemia and can therefore influence lipid and lipoprotein metabolism through complex
processes (Lairon et al., 2007). As such, these endpoints are relevant in postprandial lipemia studies, especially when a carbohydrate-containing food such as apples is consumed along with the HFM. Although there was no oral glucose tolerance test performed, participants in this study generally showed fasting glucose and HOMA-IR values consistent with non-elevated risk for diabetes. All 26 participants had a fasting plasma glucose below 7.0 mmol/L, i.e. the cut-off value for diagnosis of diabetes (Diabetes Canada Clinical Practice Guidelines Expert Committee, 2018). Three did have HOMA-IR values above the cut-off (2.6) for diagnosis of insulin resistance in a population with normal glucose metabolism (Ascaso et al., 2003), although factors that could influence the HOMA-IR cut-off value, including ethnicity, gender and age (Gayoso-Diz et al., 2013), were not considered. The dairy-based HFM contained 20 g carbohydrate comprised of mainly lactose which is known for inducing a relatively low glycemic response (Ostman, Liljeberg Elmstahl, & Bjorck, 2001). As such, the minimal changes observed in postprandial glucose when the HFM was consumed alone were expected.

The 200 g apples provided additional digestible carbohydrates (i.e. 11.9 g fructose, 5.6 g sucrose and 3.3 g glucose, Table 4.1). However, no statistical differences were observed in postprandial glucose when apples were consumed with the HFM. There was a non-significant rise in glucose with apples at 30 minutes and a correspondingly significantly higher insulin response. Apples, in general, are known to have a relatively low glycemic index (~ 36) (Atkinson, Foster-Powell, & Brand-Miller, 2008). For example, when 50 g available sugar was consumed from apples, the glucose AUC was only 27% of that for 50 g pure glucose (Yusof, Talib, & Karim, 2005). This is partly attributable to the fact that much of apples’ available carbohydrate is fructose, which is known to induce a smaller postprandial plasma glucose rise than other common carbohydrates, including glucose, dextrose and sucrose (Crapo, Kolterman,
& Olefsky, 1980). When non-obese participants consumed 0.5 g/kg body weight of fructose with a mixed meal containing lipids and proteins (average body weight 63.7 kg, hence average fructose intake was 31.9 g per meal), the glycemic response did not differ (Theytaz et al., 2014). Therefore, with the present study’s much lower fructose intake (11.9 g per meal), large elevations in plasma glucose were not expected from the apples’ sugar content. Furthermore, the co-ingestion of fat with carbohydrates can reduce postprandial glycemia, possibly related to delayed gastric emptying in the presence of lipids (Collier, McLean, & O’Dea, 1984; Cunningham & Read, 1989). This mechanism cannot be confirmed in the present study, given an apple-only test meal was not included. Lastly, any potential rises in glucose from the apple carbohydrates may have been offset by attenuations in carbohydrate absorption with increased gut content viscosity contributed by the presence of soluble fiber (Flourie et al., 1984; Fuse et al., 1989).

Despite the non-significant rises in glucose, large postprandial increases in insulin were observed with and without apple consumption. Some of this would be in response to the glycemic load, but the dairy-based HFM also contained protein, including whey, which is known to promote postprandial insulin release (Nilsson, Stenberg, Frid, Holst, & Björck, 2004). There were variations in the amount of fat consumed by participants, but the HFM beverages were supplemented with skim milk powder to standardize the amount of total protein for all participants (14.58 g), minimizing variations in whey content. The elevation in insulin with the HFM alone in this study was similar, although slightly delayed, compared to that observed for whole milk containing similar amounts of protein and carbohydrates, but much less fat (Panahi et al., 2014). The differences were potentially related to delayed gastric emptying by the high fat content in the present study’s HFM. Significant rises in plasma insulin were observed with
apples between 20 and 180 min. This is similar to Theytaz et al. (Theytaz et al., 2014), where fructose consumption led to increased plasma insulin, despite no effects on plasma glucose. Insulin level is widely recognized as a modulator in TRL synthesis and secretion (Lairon et al., 2007). For example, in a study with healthy individuals without insulin resistance, postprandial insulin was positively correlated with ApoB48 and it was suggested that hyperinsulinemia exacerbates postprandial accumulation of chylomicrons in plasma (Harbis et al., 2001). However, according to a regression analysis, a similar correlation between insulin and ApoB48 concentrations was not found in the present study ($r^2 = 0.02$, $P = 0.17$, data not shown).

Nonetheless, the higher peak insulin concentration may, in part, explain the higher peak ApoB48 concentration when apples were consumed with the HFM. Therefore, in the present study, apples led to higher insulinemia and a higher ApoB48 $C_{\text{max}}$, although postprandial TAG did not differ. This highlights the complexity of food matrix effects and the interactions between lipid and carbohydrate metabolism. Further investigation is warranted, in particular to understand the contribution of mixed meals to PPL in individuals at risk of metabolic disease (Dias et al., 2017).

Gastric emptying is impacted by food composition and structure through various mechanisms (Ehrlein & Schemann, 2005). Postprandial glycemia, for example, has a bi-directional relationship with gastric emptying in that gastric emptying influences peak postprandial glucose concentrations, but the rate of emptying itself is modulated by acute hyperglycemia after meal consumption (Phillips, Deane, Jones, Rayner, & Horowitz, 2015). Pectin, as a soluble dietary fiber, has shown potential to delay gastric emptying in human studies in purified form and at relatively high doses ($10 – 20$ g per meal) (Di Lorenzo et al., 1988; Sandhu et al., 1987; Schwartz et al., 1982). In the current study, the apples contained only $2.17$ g pectin and had no impact on gastric emptying, as reflected in acetaminophen absorption after
HFM consumption. This is potentially because of the relatively low concentration of pectin present. Another factor that could have affected gastric emptying in the present study is the presence of milk proteins. For example, casein proteins coagulate at a low gastric pH, delaying gastric emptying and small intestine transit (Dalziel, Young, McKenzie, Haggarty, & Roy, 2017). In contrast, whey proteins are considered to be rapidly digested, remaining soluble in the stomach, and reaching the upper small intestine more quickly for nutrient-stimulated feedback, stimulating release of not only insulin, but also gut hormones that contribute to slower gastric emptying (Akhavan et al., 2014; Dalziel et al., 2017). It is conceivable that gastric emptying was already delayed by the HFM milk proteins, masking or counteracting any effects of whole apple consumption. Furthermore, consuming whole apples with the HFM introduced solids into the meal and this could also influence the rate of gastric emptying, differently, between the meals (Phillips et al., 2015). Also, the liquid HFM was 500 mL, i.e. a volume which is comparable to other OFTT studies (Bahceci et al., 2007; Marciani et al., 2009), although this substantially increased with the addition of the 200 g apples. Still, the total meal size (500 mL liquid + 200 g solids) is similar to other mixed meal studies (Coelho, Hermsdorff, Gomide, Alves, & Bressan, 2017; Tiihonen et al., 2015). An indirect consequence of this is that acetaminophen dilution could also have been affected with and without apples, although there were no main effects of treatment meal and postprandial acetaminophen. Another limitation of the gastric emptying method utilized is the fact that it more accurately represents liquid vs. solid content emptying (Willems et al., 2001). In addition to the physicochemical properties of the meal itself, gastric emptying is regulated by many factors, including gender. Animal and human studies have found that sex steroid hormones such as estrogen and progesterone inhibit gastric emptying such that females have slower gastric emptying comparing to males (Gandhi, Aweeka, Greenblatt, &
Blaschke, 2010). In the present study, females were found to have higher postprandial acetaminophen concentrations than males, but this is likely related to their lower body weight, as all participants, regardless of body weight, consumed 1500 mg acetaminophen. Body weight was indeed found to be a significant covariate for postprandial acetaminophen. The effect of body weight on gastric emptying has been extensively investigated, but results are conflicting. Some studies have indicated that obese versus lean participants have higher gastric emptying rates in the early postprandial period, but others found no differences or even delayed gastric emptying in obesity (Verdich et al., 2000), indicating a huge inter-individual variability. This may have contributed to challenges in observing differences in our study, and rationalizes using acetaminophen dosages standardized for body weight in studies comparing gastric emptying with different treatments, as is sometimes done (Medhus et al., 2001).

Overall, the consumption of apples did not influence postprandial TAG, chylomicron, glucose or gastric emptying that occurred with the consumption of the dairy-based HFM in overweight and obese participants. Conceivably the results could have been different for meals of different structures. In this case, the fat in the dairy-based liquid HFM was in the emulsified form, i.e. milk fat globules. In the stomach, bulk fat separates to the top of the gastric digestate, potentially leading to a delayed emptying of fat from the stomach. In contrast, emulsified lipids, which are stable and remain dispersed in the gastric environment, tend to empty from the stomach continuously as a uniform liquid (Marciani et al., 2009). These emulsion droplets enter the small intestine with a relatively large surface area compared to bulk fat, allowing more interactions with lipase and bile salts (Golding et al., 2011). As discussed in the introduction, such interactions could be influenced by the presence of whole apple components. Therefore, the effects of whole apples on gastric emptying and lipid digestion could be different for emulsified
lipids versus bulk fats. This highlights the importance of acknowledging the nature of fats, e.g. being in the bulk or emulsified form, in postprandial OFTT investigations.

4.6. Conclusions

In summary, this study examined the influence of consuming 200 g Gala apples, with skin, on postprandial lipemia, glycemia, and insulinemia, chylomicron size and number, and gastric emptying in generally healthy overweight or obese adults who ingested a high fat dairy-based meal. Apples, as a complex food matrix, did not affect most postprandial response, except for insulin, potentially owing to the relatively low levels of pectin and/or polyphenols and/or interactions between carbohydrate and lipid metabolism from the mixed meal. Differences in gastric emptying that might have mediated differences in nutrient absorption, were not observed using the indirect acetaminophen method. The practical relevance of this study includes the investigation of a mixed meal scenario and the observation that a commonly consumed fruit is not able to attenuate PPL as a CVD risk factor when an emulsion-based HFM is consumed by overweight and obese adults. Future research should explore the amount and type of apples or apple products that would realize any benefits of apple functional components on metabolism, paying close attention to interactions between food ingredients, such as lipids and carbohydrates in mixed meals and with larger numbers of participants. Overall, as postprandial lipemia is recognized as an important risk factor for CVD, the results in the present study have implications for dietary strategies that aim to reduce CVD risk in overweight and obese individuals.

4.7. Acknowledgement

We thank our study participants for their contributions; Emily Ward and Liam Cox for assistance with the study visits and glucose and insulin analyses; Premila Sathasivam, James
Turgeon, and Nina Andrejic for blood sampling; Dr. Amy Tucker for guidance and assistance with the research facility; and Dr. David W.L. Ma and Lyn Hillyer for support with the FA analysis. XL, DML, LER and AJW designed the research; XL, DML, HN, LER and AJW conducted the research; XL, DML, HN and AJW analyzed the data; XL and AJW performed the statistical analysis; XL and AJW wrote the paper; and XL, DML, LER and AJW had primary responsibility for the final content. All authors read and approved the final manuscript.
4.8. Bridge to Chapter 5

Apple ingredients such as pectin have shown potential to modify digestive processes that would impact postprandial lipemia. This was the main focus of Chapter 3. As such, subsequent work aimed to explore the potential for whole apples, as a source of lipid-lowering pectin and polyphenols, to influence emulsion digestion using both *in vivo* (Chapter 4) and *in vitro* methods. In Chapter 5 whole apple’s effects on lipid digestion were investigated using both static and dynamic *in vitro* digestion tools, with a secondary focus on comparing results obtained by the relatively crude static approach with that of the leading TIM-1 digestion simulator. Of note, timelines for carrying out the human trial (Chapter 4) meant that *in vitro* studies on whole apples were conducted mostly in advance of and somewhat concurrent with the human trial. The overall intention was for the *in vitro* methods to complement the human study results in order be able to offer mechanistic insights to any findings observed. In an attempt to best align the comparisons between the methods, a range of experimental conditions was applied in Chapter 5. For example, the test meal fat content in the human study varied with participants’ body weight to deliver 1 g fat/kg body weight, hence a range of test meal fat content was investigated and reported for the *in vitro* methods. Chapter 4 also highlighted the potential for complex interactions that might affect the results obtained from *in vitro* digestion experiments. With this in mind, different gastric pH were applied in some of the static *in vitro* digestion models, because gastric pH, varying with stomach content (Dressman et al., 1990; Minekus et al., 2014), was found to influence purified pectin’s effects on *in vitro* lipid digestion and fatty acid bioaccessibility in Chapter 3. Together, Chapters 3, 4 and 5 provide a comprehensive investigation into the effects of pectin and apples as a pectin-rich food matrix on lipid digestion and metabolism.
CHAPTER 5. In vitro investigations on the effects of whole apples on lipid microstructure, digestibility and bioaccessibility of a dairy-based high fat emulsion, using static and dynamic digestion models

Lin, X., Chen, P. X., Rogers, M. A. & Wright, A. J. In vitro investigations on the effects of whole apples on lipid microstructure, digestibility and bioaccessibility of a dairy-based high fat emulsion, using static and dynamic digestion models.

This chapter is in preparation for submission to Food & Function.
5.1. Abstract

Pectin and polyphenols can modulate lipid digestion and absorption in vitro. Therefore, pectin-containing apples may reduce postprandial lipemia, a risk factor for atherosclerosis-related diseases. To understand the postprandial lipemic response of healthy overweight and obese adults after consuming 500 mL dairy-based high fat emulsion (3 wt% protein; 14 – 31 wt% fat, depending on body weight) with or without 200 g apples (2.2 g pectin) observed in a randomized crossover human trial, similar emulsions (3 wt% protein; 10, 15 and 20 wt% fat) were digested with and without apples using static (i.e. adapted from INFOGEST standardized static model) and dynamic (TIM-1) in vitro methods to investigate lipid microstructure, digestibility and bioaccessibility in the presence of apples. For static digestion, two gastric pH levels, i.e. 3.0 as per the INFOGEST protocol and 6.5 to reflect anticipated buffering capacity of dairy products, were applied. Correlations between the in vitro and in vivo data were made to explore the suitability of these in vitro tools for lipid research. With in vitro digestion, the 10 wt% fat emulsion was influenced by gastric pH, but not apples. It was the opposite for the 15 wt% fat emulsion, potentially due to the lower protein-to-fat ratio. Similarly, the 20 wt% fat emulsion was destabilized by apples, but it did not lead to lower TIM-1 lipid bioaccessibility, agreeing with the no effect of apples seen in the human study, although limited bioaccessibility was observed. Overall, this work highlights the complexity of food matrix effects and the need to improve the physiological relevance of in vitro investigations.

5.2. Introduction

Apples are widely considered to be a healthy food, i.e. as the well-known adage goes, “an apple a day, keeps a doctor away”. There is substantial evidence to support this. For example, apple consumption by children in the United States was associated with higher intake of dietary
fibre and lower intakes of saturated fat and sodium, all of which have implications for lowering the risk of chronic diseases, including cardiovascular disease and type 2 diabetes (Nicklas, Neil, & Iii, 2015). Individual apple components have also been implicated in chronic disease risk reduction. For example, apples are rich in pectin, i.e. a soluble dietary fibre that has exhibited blood cholesterol-lowering effects in animals (Trautwein et al., 1998) and humans (Brouns et al., 2012), potentially by delaying gastric emptying and intestinal transit (Schwartz et al., 1982), binding bile acids (Dongowski, 1995), and modulating triacylglycerol (TAG) hydrolysis by inducing microstructural changes (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, et al., 2014; Lin & Wright, 2018; Verrijssen et al., 2015). That said, pectin functionality can be influenced by other molecules present in foods and in the gastrointestinal tract (GIT). For example, a study in rats found that apple pectin improved large intestinal fermentation and plasma lipid profiles more efficiently in the presence of a polyphenol-rich apple concentrate, suggesting synergistic interactions between fibres and polyphenols contained in apples (Aprikian et al., 2003). In addition, in Chapter 3 (Lin & Wright, 2018) we observed that the presence of apple pectin during simulated digestion of a docosahexaenoic acid (DHA)-rich emulsion either promoted or reduced emulsion stability under simulated gastric conditions, depending on gastric acidity, with subsequently different effects on in vitro lipid digestibility and DHA bioaccessibility. A range of gastric pH values (2.0, 3.0 and 4.8) was applied in that study, recognizing that gastric pH varies depending on physiological conditions (e.g. infants versus adults) and the type of foods (e.g. high pH-inducing dairy products) (Minekus et al., 2014). Gastric pH has also been shown to influence the bioaccessibility of food pollutants (Pelfrène, Waterlot, & Douay, 2011) and drugs (Gu, Rao, Gandhi, Hilden, & Raghavan, 2005) by changing the physicochemical properties of those compounds and/or the digestive environment, i.e.
something which was observed in Chapter 3, as well (Lin & Wright, 2018). As such, by modulating gastric pH, food matrices may directly and indirectly influence nutrient digestibility and bioaccessibility, which is a precursor for absorption. This complicates the ability to predict how ingredient interactions may alter postprandial metabolism and demands further investigation. It also highlights the importance of considering interactions between foods and/or digestive conditions, and the need to investigate how pectin-containing food matrices, including whole apples, affect lipid digestion and metabolism.

Milk ingestion is known to acutely increase the gastric pH in humans (Eyerly, 1940; Mason, 1962). Milk has a relatively high buffering capacity because of its protein, phosphates and calcium contents (Lutchman et al., 2006). Milk ingredients have also been shown to interact with soluble fibres, leading to interactions that could affect lipid digestion. Studies have shown that pectin can alter interfacial properties of lipid droplets stabilized with milk proteins in different ways, depending on factors such as the types of milk protein (Zhang et al., 2015) and pectin (Tokle et al., 2012), pectin concentration (Dickinson, Semenova, Antipova, & Pelan, 1998; Zhang et al., 2015), and solution ionic strength (Maroziene & De Kruif, 2000; Simo, Mao, Tokle, Decker, & McClements, 2012; Surh et al., 2006). Given that milk fat is dispersed as emulsified lipid droplets in many dairy products (Huppertz & Kelly, 2009), such changes could influence colloidal stability and hence droplet interfacial area available to digestive enzymes. For example, a low concentration (0.025%) of pectin improved the stability of lipid droplets emulsified with caseinate, and subsequently led to complete in vitro lipolysis (Zhang et al., 2015). Milk and dairy products are commonly consumed and have frequently been used as the fat source in postprandial lipemia studies (Guerci et al., 2000; Lovegrove, Brooks, Murphy, Gould, & Williams, 1997; Michalski, Vors, Lecomte, & Laugerette, 2016; Patsch et al., 1983;
Tholstrup, Sandström, Hermansen, & Hølmer, 1998; Weiss, Fields, Mittendorfer, Haverkort, & Klein, 2008), since they can be easily formulated to deliver large fat loads (Lairon et al., 2007) using high fat products (e.g. cream and butter). However, rarely is the possibility that the lipids present could be susceptible to interfacial and structural changes induced by factors such as gastric pH and interactions with dietary fibres, with subsequent influences on lipid metabolism, considered. For example, these and other factors may have combined to contribute to the observations in Chapter 4’s human study regarding whole apples’ effects on postprandial lipemia when ingested with a dairy-based high fat emulsion.

In this chapter, *in vitro* digestion models will be applied to better understand the digestive interactions occurring between apples and the same and similar high fat dairy beverages as consumed by study participants in Chapter 4. Although human studies offer the highest level of evidence of health effects of foods, *in vitro* digestion methods offer the advantages of lower costs and maintenance, no ethical restrictions, and ease of sampling so that digestive mechanisms can be investigated (Minekus et al., 2014). In the most simplest of *in vitro* digestion setups, static mono-compartmental models include different phases of digestion (most commonly gastric and duodenal) simulated in one container, where meal samples and simulated digestive fluids are mixed by agitation and the concentrations of enzymes and salts are not maintained during each phase (Minekus et al., 2014). Since static models do not account for evolutions of physicochemical conditions (pH, enzyme concentrations, ionic strength, etc.) over time (Dupont & Mackie, 2015), they are less physiologically relevant than dynamic models. However, static models are simple procedures, easy to operate, economical, can be applied to screen a relatively large number of samples, and can produce limited, but sometimes useful, insights. Use of these models has increased dramatically in recent years and they are widely used in practice. This
includes for studies of lipid bioaccessibility since solubilization of fatty acids is recognized as a rate-limiting step for absorption (Marze, 2015). They are also used to isolate specific factors such as ingredient interactions or the role of interfacial properties during GI digestion. Studies using static digestion models have been conducted using different digestive conditions, including concentrations of lipases and bile salts, residence times for each phase, pH, etc. At the same time, each of these important factors can, themselves, influence nutrient digestibility and, in the case of lipids and lipophilic nutrients, their solubilization into the aqueous phase (i.e. bioaccessibility) (Marze, 2015). As such, this can lead to discrepant results between studies and difficulties in comparing results between publications and laboratory groups (Minekus et al., 2014). In a move to address this, an international consensus methods paper reporting on a standardized static digestion method was published in 2014 (Minekus et al., 2014). Although this constituted an important step in improving and standardizing in vitro digestion experiments, it actually is not optimally adapted for all systems, including lipids. Specifically, with those methods, it is difficult to reach the desired lipase activity for high fat foods solely using pancreatin (Minekus et al., 2014), and without inducing excessive viscosity, so additional pancreatic lipases and co-lipases are required, the latter of which is very expensive. Also, lipolytic products (i.e. free fatty acids) are not removed during this and other static digestion models and this can lead to product inhibition of lipases unless calcium ions are continuously added to the system or the pH-stat is applied with ongoing titration (Minekus et al., 2014). In light of these and other challenges, the 2014 in vitro digestion consensus method is undergoing further improvements (Brodkorb, 2018).

The development and use of dynamic, multi-compartmental models, such as the TIM-1 from TNO Netherlands, has also increased in recent years. Such models are programmed to
reflect food transit in the GIT and the dynamic physiological conditions at different locations over time (Dupont & Mackie, 2015). For example, the TIM-1 consists of four compartments (stomach, duodenum, jejunum and ileum). The jejunum and ileum sections include dialysis membranes and filters with specific cut-offs to mimic intestinal absorption. Collected dialyzed samples can be analyzed to measure nutrient bioaccessibility (Minekus, 2015), including for lipids, since these molecules are absorbed in the small intestine. A major advantage of the TIM-1 over static methods is that this membrane arrangement removes lipolyzed fatty acids from the digestate milieu, minimizing product inhibition and enabling continued interfacial lipolysis. There is some, although very limited in the public domain, direct evidence that TIM-1 bioaccessibility closely approximates lipid absorption in humans following ingestion of a high fat meal. For example, one study observed a linear correlation between TIM-1 lipid bioaccessibility and postprandial serum TAG concentration from a human study of the same lipid meals (Thilakarathna et al., 2016). This supports the value of TIM-1 as a predictive tool in assessing postprandial lipemic response to a meal. Indeed, the TIM-1 model has become increasing popular with researchers seeking to understand digestive interactions and underlying mechanisms that determine absorption (Minekus, 2015). The greater physiological relevance of dynamic models like TIM-1 over static in vitro digestion methods is a major advantage, although maintenance and operational costs are relatively high.

Overall, this study aims to use in vitro methods of digestion to investigate the effects of whole apples on lipid digestibility and bioaccessibility. It aims to support a mechanistic understanding of the potential for apples to alter digestive events, and subsequently postprandial metabolism, as investigated in the previous chapters. The original intent was to utilize static digestion methods to study the same dairy emulsions, as consumed by study participants in
Chapter 4 and to build on Chapter 3, where the selection of simulated gastric pH (that might be buffered by dairy ingredients) was shown to influence lipolysis/digestibility and bioaccessibility and to integrate these observations with the human lipemic response data. However, the TIM-1 also became available during the course of study. This presented the unique opportunity to apply this more advanced model to explore the impact of apples on lipid bioaccessibility and also to compare between static and dynamic in vitro digestion experimental approaches. Therefore, a secondary aim became to compare between the results obtained from the static and dynamic models and to comment on the challenges and practical utility of both systems. It was hypothesized that both the presence of apples and gastric pH would influence the stability of the milk beverage under GIT conditions and hence alter the extent of lipolysis and bioaccessibility of release free fatty acids (FFA).

5.3. Materials and Methods

5.3.1. Materials

All chemicals and reagents were purchased from Sigma Chemical Co. (MO, USA), including pepsin (from porcine pancreas, P7012), porcine bile extract (B8631), pancreatin (from porcine pancreas, P1750), pancreatic lipase (from porcine pancreas, L3126), lipase from Rhizopus Oryzae (80612-25G), bovine pancreas (T9201), Nile Red (72485) and Fast Green FCF (F7252). Milk (Natrel skimmed milk, Canada), cream (Organic Meadow 35% whipping cream, Canada), apples (Gala, Martin’s family fruit farm and Norfolk Fruit Growers Association, product of Ontario, Canada) and Tylenol Extra Strength (500 mg acetaminophen) were purchased from a local supermarket. Low temp skim milk powder (376543, Parmalat Ingredients Canada) was purchased from Flanagan Foodservice in Kitchener, Ontario, Canada. Fresh porcine bile was collected from Conestoga meat packers (Kitchener, Ontario, Canada).
5.3.2. Overview and Rationale of the Experimental Variables

This study aimed to investigate the effects of the presence of apples in lipid digestion and bioaccessibility of high fat meals. Therefore, static and TIM-1 dynamic in vitro digestion experiments were carried out with and without apples. These were the same Gala apples consumed by study participants in Chapter 4 and contained 11 mg/g pectin and 1 mg gallic acid equivalent (GAE)/g total polyphenols (as reported in Chapter 4), i.e. levels which are similar to those reported for apples, previously (Colin-Henrion et al., 2009; Vrhovsek et al., 2004). In the static experiments, two levels of gastric pH were applied to reflect possible differences in food buffering capacity (Minekus et al., 2014; Simonian et al., 2005). This included pH 3.0 (high gastric acidity, HGA) which is used in the standardized protocol for in vitro digestion studies (Minekus et al., 2014) and pH 6.5 (low gastric acidity, LGA) which was the pH obtained when equal parts of simulated gastric fluid and the test meal were combined, without any pH adjustment. In this way, the LGA condition accounted for buffering from the dairy emulsion ingredients. This value of pH 6.5 is also similar to the gastric pH in human infants, which although was around 3 in the fasted state, buffered to near the initial pH of milk (i.e. pH 6.5 – 7) after milk consumption (Mason, 1962). Lastly, test meals with different fat contents (i.e. 10, 15 and 20 wt% fat, abbreviated as FM10, FM15 and FM20, respectively) were investigated. These were selected to represent the range of possible fat contents of high fat meals (HFM, providing 1 g fat/kg body weight) consumed by overweight and obese participants weighing 50, 75 and 100 kg, respectively. FM10 was included in both the static and TIM-1 experiments and represents the fat content our group typically uses in our static in vitro digestion investigations, including in Chapter 3. FM15 was investigated in the static model, expecting a mean participant body weight in the range of 75 kg. However, 75 kg ended up being an underestimation of participant body
weight and therefore, a higher fat content, i.e. 20% (FM20) was investigated in the TIM-1 model. With the static methods, samples of digesta were withdrawn periodically and analyzed for particle size distribution, microstructure and surface charge, and to determine lipid digestibility by quantifying FFAs in the digestate. At the end of the digestion, the aqueous phase containing the solubilized (i.e. bioaccessible) lipids was collected and the fatty acid (FA) composition of these lipids determined by gas chromatography (GC). Samples collected from the TIM-1 were dialysates separated from the jejunum and ileum compartments. FFAs were quantified for these samples to determine lipid bioaccessibility and their composition also determined by GC. A summary of the experimental variables investigated in this Chapter is provided in Table 5.1.

Table 5.1. Summary of test meal fat contents investigated with each digestion method, static gastric pH conditions utilized, and various sample analyses performed.

<table>
<thead>
<tr>
<th>Digestion model</th>
<th>Test meal</th>
<th>Gastric pH</th>
<th>Sample analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static digestion</td>
<td>FM10</td>
<td>HGA, LGA</td>
<td>Digestate physico-chemical properties(^1), Lipid digestibility, Lipid bioaccessibility, FA composition of the bioaccessible lipids</td>
</tr>
<tr>
<td>Static digestion</td>
<td>FM15</td>
<td>HGA, LGA</td>
<td>-</td>
</tr>
<tr>
<td>TIM-1</td>
<td>FM10</td>
<td>Dynamic(^2)</td>
<td>-</td>
</tr>
<tr>
<td>TIM-1</td>
<td>FM20</td>
<td>Dynamic(^2)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)Including lipid particle size, microstructure (assessed by confocal microscopy), and \(\zeta\)-potential for FM10, and only lipid particle size and \(\zeta\)-potential for FM15; \(^2\)See section 5.3.5. FM10, 10 wt% fat test meal; FM15, 15 wt% fat test meal; FM20, 20 wt% fat test meal; HGA, high gastric acidity (pH = 3.0); LGA, low gastric acidity (pH = 6.5)

5.3.3. Test Meal Composition and Preparation

The test meals were composed of a dairy mixture (skimmed milk and whipping cream) with or without cored whole apples (containing 1.1 ± 0.1 wt% pectin (galacturonic acid equivalent) and 0.1 ± 0.0 wt% total polyphenols (gallic acid equivalent), which were blended in a
Magic Bullet® blender (MBR-1701) to imitate the chewing process in human mouth. Each meal was prepared immediately prior to the start of the in vitro digestion experiment. The required meal volumes were different for the static digestion model and TIM-1, but all meals contained 2.9 wt% protein (standardized using low temperature skim milk powder) and 0.3 wt% acetaminophen (from crushed Tylenol tablets mixed in the dairy mixture, in order to replicate the meals applied in the human trial in Chapter 4).

**Static in vitro digestion:** The test meal contained 5.0 g of a milk-cream mixture containing 10 or 15 wt% fat (FM10 and FM15 respectively), with or without 2.0 g apples. Each milk-cream mixture was prepared by gently inverting a bottle containing the required amounts of 35% cream and skimmed milk to reach the desired fat content. Skim milk powder and crushed Tylenol were added to reach the standardized protein and acetaminophen contents before the bottle was inverted 6-8 times. When apples were included in the test meal, whole apples were cored and blended, with skin on, using the Magic Bullet® blender. Two grams of blended apples were weighed and added to the milk-cream mixture before digestion.

**Dynamic in vitro digestion with TIM-1:** The test meals consisted of 150 mL milk-cream mixture, with or without 60.00 g apples. The milk-cream mixtures were prepared, as above, but with two fat concentrations (i.e. 10 and 20 wt%, FM10 and FM20, respectively) for the TIM-1 experiments. When apples were included in the test meal, 60.00 g apples were blended with the skimmed milk, skim milk powder and the acetaminophen using the Magic Bullet® blender, followed by adding 35% whipping cream and inverting the container to achieve mixing. This mixture was not blended in order to avoid inducing structural changes.
5.3.4. Static Digestion Experiments

FM10 and FM15, with and without apples, were subjected to *in vitro* digestion experiments simulating gastric and duodenal conditions using a method slightly modified from Minekus *et al.* (2014). Simulated digestion fluids were prepared as described (Minekus *et al.*, 2014) with the exception that additional phospholipids (76 mg) were included in the 10 mL simulated duodenal fluid (SDF) (Lin & Wright, 2018; Mansbach II *et al.*, 2001). The digestions were performed in amber glass jars placed in a 37 °C 250 rpm shaking water bath (New Brunswick Scientific Co. Inc., NJ, USA). Five mL meal samples warmed at 37 °C for 15 min were then mixed with 5 mL simulated gastric fluids (SGF). The mixture contained 2,000 U/mL pepsin and 12.6 mg/mL pyrogallol (as an antioxidant) and was adjusted to pH 3.0 (high gastric acidity, HGA) or 6.5 (low gastric acidity, LGA) before being incubated for 2 h. The two different gastric pH levels (HGA and LGA) were applied to represent different gastric pH values depending on food buffering capacity (Minekus *et al.*, 2014; Simonian *et al.*, 2005). Ten mL of SDF were added to start the duodenal phase of digestion. The final digestion mixture contained 0.025 and 0.038 g/mL fat (for FM10 and FM15 respectively), 2,000 U/ml pancreatic lipase, 10 mg/mL bile extract (~10 mM bile salts), and 3.8 mg/mL (~8 mM) phospholipids. The duodenal digestate was adjusted to pH 6.0 by dropwise addition of 1 N sodium hydroxide and the digestion was carried out for 4 h. 0.1 mL aliquots of duodenal digestate were collected at 2, 5, 10, 15, 30, 60, 90,120, 180 and 240 min for later FFA analysis. A water control was digested and sampled at the same time points as the test meals to correct for any background contribution to the colorimetric determination of FFA. For the particle size measurements, sample and digestive fluid volumes were reduced to 1/5 of those stated above.
5.3.5. *TIM-1 Dynamic Digestion Experiments*

This study used the TIM-1 (TNO, Zeist, The Netherlands) simulated model of upper gastrointestinal tract with four compartments, including the stomach, duodenum, jejunum and ileum and experiments conducted according to the instrument manual. As reported in Ribnicky et al. (2014), the model was conditioned for the fed state after a high fat meal (Ribnicky et al., 2014), including peristaltic movements, nutrient and water absorption, gastric emptying, pH, enzyme secretion rates and transit times intended to approximate the human gastrointestinal environment. Specifically, pH of the stomach compartment was set up to evolve with time: 6.5 (0 min), 4.2 (30 min), 2.9 (60 min), 2.0 (120 min), 1.7 (210 min to 360 min). For the other compartments, pH was maintained throughout the digestion (5.9, 6.5, and 7.4 for the duodenum, jejunum and ileum, respectively). Simulated digestive fluids were prepared freshly on the day of the experiment, and enzyme solutions were stored between 0-5 ℃ before use, according to TIM-1 manual. Start residues (representing digestive fluids present in the gut during the fasted state) were prepared with electrolyte solutions and digestive enzymes and then added to each compartment before administering the meal. 150 g FM10 or FM20, with or without 60 g apples, was mixed with either 90 or 150 g gastric electrolyte solution (for with or without apples, respectively) to reach 300 g and the mixture added to the stomach compartment through a funnel. Six-hour simulated digestions were performed to mimic the suggested transit time for ingested materials to reach the cecum in adult humans. Small volumes of samples from the jejunal and ileal dialysates (containing the bioaccessible lipids (Minekus, 2015)) were collected at 40, 80, 120, 180, 240, 300, and 360 min (also 60 min for FM10 only) and immediately frozen at -80 ℃ until extraction, generally the next day. The dialysates were analyzed for total FFAs and the bioaccessibility of each major FA was determined, as described below. A water control was
subjected to the TIM-1 model and dialysates were sampled at the same time points to correct for any background contribution in the colorimetric determination of FFAs.

5.3.6. Determination of FFA Release during Static and TIM-1 Digestions

The jejunal and ileal dialysates collected from TIM-1 digestions and the digestate samples collected from static digestions were analyzed for FFA content using a non-esterified fatty acid (NEFA) kit (Wako Diagnostics, VA, USA), as per the manufacturer’s instructions (Nik et al., 2010). FFAs from 100 uL of sample were extracted into hexane (1000 μL) under acidic conditions (100 μL 1 M HCl) and the mixture vortexed for 10 s before centrifugation at 16,873 g for 30 min (5418 Laboratory Centrifuge, Eppendorf Hamburg, Germany). The hexane extract was then diluted to 3 mL and 5 μL was added to 225 μL of Reagent A in a 96-well plate (in duplicate) and the plate incubated at 37 °C for 10 min. 75 μL of Reagent B was then added with an additional 15 min incubation at 37 °C. FFAs were determined using a UV-VIS micro-plate spectrophotometer (Spectramax plus, Molecular Devices Corporation, CA, USA) by measuring the absorbance at λ\text{max} of 550 nm and a standard curve prepared using oleic acid ranging from 0.1 to 1.97 mM.

Static digestion results were expressed as lipolysis (%) which was calculated based on the moles of FFAs present in the digestate at each time point with respect to the total moles of FAs present initially (Lin & Wright, 2018), divided by 0.64 (including a contribution from glycerol) to obtain the percentage of TAG present initially. TIM-1 digestion results were expressed as cumulative lipid bioaccessibility (%), which was the accumulation of lipid bioaccessibility during each sample interval. For example, cumulative lipid bioaccessibility at 80 min is the sum of lipid bioaccessibility of dialysate samples collected between 0 and 40 min + lipid.
bioaccessibility between 40 and 80 min. The lipid bioaccessibility during each sample interval was calculated by the formula adapted from Fondaco et al. (2015):

\[
\text{TIM-1 Lipid bioaccessibility} \, (\%) = \frac{\text{FFA}_{\text{jej}} \, (g) + \text{FFA}_{\text{ileum}} \, (g)}{0.64 \times \text{total fat subjected to digestion} \, (g)} \times 100
\]

where \( \text{FFA}_{\text{jej}} \) is the weight of FFAs in jejunum dialysate collected during the sampling interval, \( \text{FFA}_{\text{ileum}} \) is that of ileum dialysate, 0.64 is the conversion rate from the weight of FFAs to weight of TAG, given that the average milk fat FA molecular weight is 780 g/mole (Walstra, 1999).

5.3.7. Isolation of the Aqueous Micellar Phase from Static Digestion

Digestate samples at the end of static digestions were immediately centrifuged at 144,000 g at 7 °C for 1 h using a Sorvall WX Ultra 80 Ultracentrifuge (Mandel Scientific, ON, Canada) to separate any undigested oil from the aqueous micellar phase and pellet. The aqueous fraction was collected with a fine tip disposable pipette, quantified using a graduated cylinder, and stored in glass vials under nitrogen at -80 °C until lipid extraction and analysis.

5.3.8. Analysis of FA Composition by Gas Chromatography (GC)

Lipids were extracted from the dialysate samples obtained from the TIM-1 and the aqueous fractions obtained from the static digestion experiments, based on the Folch method, as described (Martin et al., 2010). Afterwards, the samples were saponified, methylated and FAME quantified, as previously (Merino et al., 2011). FAMEs were separated by gas chromatography using an Agilent 7890A Network GC System (Agilent Technologies, CA, USA) with a Supelco SP 2560 fused-silica capillary column (100m × 0.25mm i.d., 0.2 μm film thickness; Sigma-Aldrich, St Louis, MO).
5.3.9. Particle Size, Microstructure and $\zeta$-potential of Static Digestate Samples

Mean droplet diameters ($D_{3,2}$: the surface area-weighted mean, $D_{4,3}$: the volume-weighted mean) before and after static gastric digestion were determined by laser diffraction (Mastersizer S, Malvern Southborough, MA, USA) using a refractive index of 1.46 for milk fat. Samples analyzed during the in vitro digestion experiments were drawn 0.5 cm from the bottom of the jars, under mixing at a speed of 250 rpm. A control dispersion of blended apples in water was also analyzed.

$\zeta$-potential of fat globules in the static digestate samples was measured using a particle electrophoresis instrument (NanoZS, Malvern Instruments Ltd, UK). Prior to analysis, in order to minimize multiple scattering effects, meal samples (pH = 6.6) were dispersed in water and gastric digestate fluids (pH = 3.4 and 5.0 after HGA and LGA, respectively) in citrate-phosphate buffer solutions at the pH of samples to achieve an oil concentration of 0.005 wt%.

Confocal laser scanning microscopy (CLSM) was used to investigate differences in milk fat globule microstructure before and after the static digestions. Undiluted emulsion and digestate samples were diluted to 1.6 % fat in phosphate buffer saline (pH 7.2), and then double-stained with Nile Red (a solution of 0.02 g/L for lipid staining) and fast green FCF (a solution of 0.1 g/L for protein staining) (Gallier et al., 2010). The staining was achieved by mixing 800 μL sample with 100 uL of both Nile Red and Fast Green FCF and let stand in an Eppendorf tube for 10 min. The dyed samples were then placed on concave glass slides, covered and sealed tight to prevent sample dryness. The Nile Red probes were excited with the 488nm line from the Ar+ laser and the filters set to collect the emitted light between 500 and 600nm; Fast Green FCF was excited with the 633nm line from the HeNe laser and the filter used was BA655-755 (Gallier et
al., 2010). The resulting images (1024×1024 pixels) were obtained using a 63 x oil immersion objective lens.

5.3.10. Statistical Analyses

All measurements were completed in triplicate, unless otherwise stated. Results are expressed as mean ± SEM. ANOVA (with Bonferroni’s post hoc testing) and multiple t tests (Bonferroni-Dunn method) was performed using GraphPad Prism version 7.04 for Windows 10 (GraphPad Software, San Diego California USA, www.graphpad.com) with a significance level of p < 0.05.

5.4. Results and Discussion

5.4.1. Emulsion Stability of FM10 and FM15 During Static Gastric Digestion at Different pH Levels (HGA and LGA)

The physicochemical properties of milk fat globules in the 10 and 20 wt% fat test meals (FM10 and FM15 respectively), without and with apples, were investigated after gastric digestion at an initial gastric pH of 3.0 (HGA) and 6.5 (LGA). The HGA and LGA conditions were included based on evidence that gastric pH, in the presence of pectin, alters lipid microstructure at the start of duodenal digestion (Lin & Wright, 2018) and this is known to play an important role in subsequent lipolysis by influencing fat globule surface area (Michalski et al., 2013). Of note, after 2 hrs of gastric digestion, the pH was found to increase from 3.0 to 3.4 in the case of HGA, but decreased from 6.5 to 5.0 with LGA, and these changes did not differ between with and without apples (p > 0.05). The changes were likely associated with proteolysis happening with HGA, but not LGA, because pepsin is active at pH 3 but limited above 6 (Piper & Fenton, 1965). The mechanisms behind the influence of released peptides and/or amino acids were not yet fully explored in this study. Characterizing the intact proteins and digestion
products in the test meals and gastric digestates would offer better insight into the differences. Overall, the particle size distribution, $\zeta$-potential and lipid microstructure of gastric digestates were measured at a pH of 3.4 for HGA and 5.0 for LGA. The particle size distributions (PSD) before and after static gastric digestion are presented in Figure 5.1, and corresponding values of fat globules $D_{3,2}$ and $D_{4,3}$ are listed in Table 5.2. $\zeta$-potential of FM10 and FM20 before and after gastric digestion was also investigated (Figure 5.2). In addition, CLSM was used to better understand fat globule microstructure, visually (Figure 5.3), but only with FM10.

5.4.1.1. Before gastric digestion

The particles in FM10 emulsion before digestion peaked between 1 – 10 µm, and the presence of apples resulted in a small peak in the range of 1000 µm (Figure 5.1A). However, the $D_{3,2}$ and $D_{4,3}$ values of FM10 emulsion before digestion did not change significantly with apples (Table 5.2). This indicates that, instead of destabilized lipid droplets, the large particles were mainly introduced by the blended apples, which peaked around 1000 µm when measured by itself (data not shown). The CLSM images also showed that the addition of apples did not change the morphology or size of lipid droplets in FM10 before digestion (Figure 5.3). Some proteins were observed around the fat globules (Figure 5.3). They were probably embedded in the milk fat globule membrane (MFGM), which stabilizes the milk fat globules in the aqueous phase of milk (Goff, 2017). Nevertheless, apples did lower the $\zeta$-potential of FM10 emulsion by half ($p < 0.05$, Figure 5.2), indicating a reduced electrostatic repulsion between lipid droplets. This loss of surface charge may be associated with depletion flocculation induced by excessive non-adsorbed pectin, because both apple pectin (dissociation constant (pKa) $\approx$ 3.5) and MFGM proteins (isoelectric point (pI) = 4 – 5) possessed negative charges at the pH of FM10 (6.6) (Beysseriat et al., 2006; Simo et al., 2012). However, it is unclear if the concentration of pectin
(0.04 wt%) in FM10 reached the critical concentration for depletion flocculation. In either case, the reduction in electrostatic repulsion between droplets was not extensive enough to cause emulsion destabilization in FM10, as evidenced by PSD and CLSM images. In addition, glycoproteins embedded in MFGM may also have helped maintaining FM10 emulsions when the electrostatic repulsion was decreased. They have been reported to be more resistant to proteolysis by pepsin comparing to proteins in the serum phase, which provides a steric barrier that prevents milk fat globules from coalescing and aggregating (Le et al., 2012).

The PSD of FM15 without apple was similar to that of FM10, and their mean droplet diameters (i.e. D₃,₂ and D₄,₃) did not differ. Similarly, the ζ-potential of FM15 emulsion was reduced by the presence of apples as well, to a similar extent of FM10 (Figure 5.2). However, extensive destabilization of emulsion droplets was observed in FM15 with apples, as reflected in PSD (Figure 5.1B) and mean droplet diameters (Table 5.1). This may be attributed to the high fat content in FM15. As both FM10 and FM15 contained 2.9 wt% protein, the fat to protein ratio in FM15 was much higher, which may result in insufficient protein coverage on the lipid droplet surface (Tomas, Paquet, Courthaudon, & Lorient, 1994). In addition, the protein composition in FM10 and FM15 might be different too. FM15 was composed of more 35 wt% fat cream and less skim milk than FM10, hence it contained less protein (2.7 wt%) before adding skim milk powder to reach the protein content of FM10 (2.9 wt%). Although the emulsifying property of MFGM components fragmented from skim milk powder have been reported (Phan et al., 2013), without homogenization in the current study, the skim milk powder in FM15 was not expected to behave in the same way as the MFGM proteins in FM10. Instead, the skim milk powder proteins likely remained in the serum phase of the FM15 emulsions. Hence, a smaller portion of the proteins in FM15 was MFGM proteins, including the glycoproteins providing steric repulsion.
Therefore, FM15 emulsions may be more prone to oil-water interface changes induced by pectin-containing apples, which explains the significant increase in lipid droplet size with apples in FM15 compared to no influence in FM10.

5.4.1.2. After gastric digestion: the effect of apples

In terms of PSD of the FM10 emulsions, the presence of apples led to the small peak near 1000 µm in gastric digestate samples, similar to the one observed in the PSD before digestion (Figure 5.1C and E). Despite this, apples had limited influence on the location of the main peak, regardless of gastric pH (Figure 5.1C and E). Similarly, apples had limited influence on digestate mean droplet diameters, except that for digestion with HGA, the value of D_{4,3} was increased (p < 0.01, Table 5.2). CLSM images also showed that the size of fat globules and the presence of protein did not seem to differ drastically between with and without apples (Figure 5.3). The larger particles in FM10 emulsion with apples were not shown in CLSM images, probably because the large particles contributed by apples contained limited lipid and protein and hence were not visible in the CLSM images. Therefore, the higher D_{4,3} but unchanged D_{3,2} observed with apples can be mainly attributed to the large particles from the blended apples, since D_{4,3}, the volume weighted mean, is more sensitive to the presence of large particles comparing to D_{3,2}, the surface weighted mean (Allen, 1981). Overall, the presence of apples did not appear to have a major impact on the particle size or surface charge of FM10 emulsions after gastric digestion. In contrast, the stability of FM15 emulsions were much more sensitive to the presence of apples, based on the right-shifted main peak in the PSD (Figure 5.1D and F) and significantly increased mean droplet diameters (p < 0.05, Table 5.2). Unfortunately, CLSM was not applied for FM15. In summary, lipid droplet stability in the FM15 emulsions, but not FM10, was influenced by the presence of apples during gastric digestion. This could be attributed to the fact that, before
digestion, FM15 emulsions were already destabilized by the presence of apples, while FM10 droplets remained dispersed. This indicates that the emulsion destabilization induced by apples before digestion was not reversed or modified after gastric digestion. In Chapter 3, 0.06 wt% purified apple pectin improved the gastric stability of a soy lecithin-stabilized emulsion at a pH of 3.0, but applesauce containing the same amount of pectin did not exhibit the same effect (Lin & Wright, 2018). Similarly, in the current study where whole apples were studied, the stabilizing effect of apple pectin was not observed at a similar pH (HGA). This highlights the importance of food matrix effects and suggests that other apple components may have contributed to the destabilization of dairy emulsions. For example, apples are rich in polyphenols (e.g. catechins and proanthocyanidins) (Vrhovsek et al., 2004), which have been extensively studied for their ability to form complexes with milk proteins, naturally through hydrophobic interactions and hydrogen bonds (Yildirim-Elikoglu & Erdem, 2018). A few studies have investigated the effect of protein-polyphenol complex on emulsion stability. Sabouri et al. found no difference between particle sizes of sodium caseinate-stabilized emulsion droplets with or without epigallocatechin-gallate extracted from tea (Sabouri, Geng, & Corredig, 2015). Furthermore, von Staszewski et al. indicated that green tea polyphenols decreased the initial droplet size of a fish oil emulsion stabilized with β-lactoglobulin, and at the same time improved its physical and oxidative stability (von Staszewski, Pizones Ruiz-Henestrosa, & Pilosof, 2014). In contrast, Lamothe et al. discovered that polyphenols from green tea extract reduced the in vitro proteolysis of dairy products in the gastric phase, potentially by interacting with milk proteins to limit the access of pepsin (Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014). Many factors can influence the interaction between polyphenols and proteins, including polyphenol type and structure, protein structure, polyphenol/protein ratio, and pH. The first three
factors were not thoroughly studied in the current study and hence are out of the scope in this paper. However, the effect of pH will be discussed in the following section.

5.4.1.3. After gastric digestion: the effect of an initial gastric pH of 3.0 (HGA) or 6.5 (LGA)

Many dairy proteins, including sodium caseinate (Dickinson et al., 1998; Surh et al., 2006), casein micelles (Maroziene & De Kruiif, 2000), and β-lactoglobulin (Simo et al., 2012), can interact with pectin, but the impact of pectin-protein interactions on the stability of milk fat globules depends on the aqueous solution conditions, including pH. Therefore, the effect of gastric pH was specifically investigated. Of note, after 2 hrs gastric digestion, the pH was found to increase from 3.0 to 3.4 in the case of HGA, but decreased from 6.5 to 5.0 with LGA, and these changes did not differ between with and without apples (p > 0.05). As above, the changes were likely associated with proteolysis occurring with HGA, but not LGA, given that pepsin is active at pH 3, but has limited activity above pH 6 (Piper & Fenton, 1965).

For the PSD of FM10 emulsions, gastric digestion with an initial pH of 3.0 (HGA) led to the main peak shifted towards a larger size range (10 – 100 µm, Figure 5.1C). This distribution was comparable to that observed in another study where bovine milk was digested at a gastric pH of 1.5 (Gallier, Ye, & Singh, 2012). In contrast, digestion with an initial pH of 6.5 (LGA) did not have a significant effect on PSD (Figure 5.1E). These results indicate that more large particles were present after digestion with HGA versus LGA. This effect of gastric pH was similarly reflected in the values of D_{3,2} and D_{4,3} of milk fat globules. Both D_{3,2} and D_{4,3} values of FM10 emulsion increased after gastric digestion with HGA (p < 0.01, Table 5.2). In contrast, D_{3,2} increased (p < 0.001) while D_{4,3} remained unchanged (p > 0.05) after LGA (Table 5.2). Comparing between HGA and LGA digestions, LGA resulted in smaller increase of D_{3,2} (p <
0.001), but the D_{4,3} values after HGA and LGA digestions were not significantly different (p > 0.05, Table 5.2).

A similar trend was also observed in the CLSM images of FM10. With HGA, the proteins formed aggregates instead of surrounding the fat globules (Figure 5.3). The formation of such protein aggregates was also reported by Gallier et al. after 120-min gastric digestion of bovine milk at pH 1.5 (Gallier et al., 2012). This is expected when the simulated gastric pH decreased the pH of the FM10 emulsion to below the pI of the MFGM proteins (i.e. pH 4 - 5) (Corredig & Dalgleish, 1998). In fact, the ζ-potential of FM10 fat globules diminished to around zero after HGA digestion (p < 0.05, Figure 5.2), indicating a loss of electrostatic repulsion between fat globules. Correspondingly, CLSM images show larger fat globules in the HGA digestates of FM10 emulsion (Figure 5.3), suggesting coalescence of the milk fat globules. The loss of surface charge of FM10 emulsion droplets could also be attributed to proteolysis of the charged MFGM proteins, which probably occurred during the gastric digestion with HGA, as pepsin is quite active between pH 1.5 – 4 (Piper & Fenton, 1965). Furthermore, casein micelles in the serum phase of milk (pI = 4.6) can aggregate during gastric digestion due to the low pH and the proteolysis of pepsin, and the aggregated casein micelles may lead to bridging flocculation of the fat globules (Ye, Cui, & Singh, 2011). Lastly, some fat globules may be trapped inside the protein aggregates formed at the acidic gastric pH, leading to the presence of large particles (Gallier et al., 2012). Although the interactions between fat globules and protein aggregates could not be demonstrated in the CLSM images in the present study, the abovementioned mechanisms may have contributed to the greater droplet diameters of FM10 emulsion observed after digestion with HGA.
Comparing to HGA, much smaller protein aggregates (Figure 5.3) and higher absolute values of FM10 emulsion ζ-potential (Figure 5.2) were observed at the higher gastric pH (LGA). This can be attributed to the relatively small changes in pH of the FM10 emulsion with LGA digestion, so limited aggregation of milk proteins had occurred. Even though some MFGM proteins have a pI of 5.0 (Corredig & Dalgleish, 1998), the gastric pH with LGA did not decrease to 5.3 until the last 30 min of the digestion (data not shown), leaving insufficient time to induce destabilization of FM10 emulsion droplets. Also, a gastric pH of 5.0 was probably not low enough to induce aggregation of all MFGM proteins or casein micelles in the serum phase. In addition, proteolysis was probably inhibited because pepsin has limited activity above pH 5 (Piper & Fenton, 1965). Therefore, extensive changes in protein structure were not expected with LGA, and the fat globules appeared to remain intact and dispersed. Considering all the results above, gastric acidity had a significant influence on fat globule microstructure of FM10 emulsion; HGA (i.e. a low initial gastric pH of 3.0) caused destabilization of proteins, resulting in larger particles in gastric digestates.

FM15 emulsions with 15 wt% fat were investigated for particle size and ζ-potential, as well. Opposing to the FM10 emulsions, FM15 samples were not as sensitive to gastric digestion or the different gastric pH conditions investigated. In the absence of apples, neither HGA nor LGA induced any changes in PSD (Figure 5.1B, D and F) or mean droplet diameters (p > 0.05, Table 5.2), although ζ-potential of the FM15 emulsions was lowered (Figure 5.3). With HGA, ζ-potential was reduced from \(-45.03 \pm 0.18\) to \(-9.65 \pm 1.41\) mV, which again could be attributed to the pH being below the pI of milk proteins. With LGA, ζ-potential was lower than before digestion, but still mainly preserved \((-37.52 \pm 2.77\) mV). The unchanged PSD and mean droplet diameters suggest that the lipid droplets in FM15 were stable, regardless of changes in ζ-
potential. This could be attributed to the glycoproteins in MFGM, which are relatively more resistant to proteolysis and provide steric repulsion between fat globules (Le et al., 2012). Given the same amount of proteins in FM10 and FM15 emulsions, a larger portion of proteins would be expected in the serum phase of FM15, i.e. likely casein and whey proteins provided by the additional skim milk powder. With the same pepsin activity for FM10 and FM15, the portion of casein micelles affected by proteolysis may have been less in FM15, resulting in less bridging flocculation of fat globules induced by casein micelle aggregates. Nevertheless, this needs to be confirmed with CLSM imaging of different protein species and should be investigated in future studies. In the presence of apples, digestion with LGA still did not influence PSD (Figure 5.1F) or mean droplet diameter ($p > 0.05$, Table 5.2), but greater mean droplet diameters were observed with HGA (Table 5.2), in agreement with the observation for the FM10 emulsions. Therefore, the structural changes of emulsion droplets caused by gastric pH were exaggerated in the presence of apples and this may be explained by the susceptibility of FM15 emulsions to apple-induced destabilization, related to the low protein to fat ratio (as discussed in section 5.4.1.1). Overall, gastric digestion at acidic versus near-neutral pH did not impact the lipid droplet stability in the FM15 emulsions, except in the presence of apples.

It should be noted that, the effects of apples and gastric pH were not independent of each other. Firstly, apple pectin is highly methylated (i.e. high methoxyl pectin, HMP) (Voragen, Schols, & Pilnik, 1986) and one of the two requirements for HMP gelation is an acidic environment (pH < 3.6), i.e. a condition satisfied in the HGA digestions (Thakur et al., 2009). However, another requirement is a high concentration of co-solutes (e.g. > 55 wt% sucrose) that can stabilize junction zones in the HMP gel network (Thakur et al., 2009). This condition was unlikely achieved in the HGA digestate samples. Secondly, the physicochemical properties of
apple pectin and dairy proteins heavily depend on the pH of the emulsion system, given that the pKa of pectin is around 3.5 (Surh et al., 2006) and the pI of dairy proteins ranges from 4 to 5 (Corredig & Dalgleish, 1998). During HGA digestions (pH 3.0 – 3.4), pectin may have lost its negative charge because the pH was close to pectin’s pKa, leading to a decreased ability to interact with dairy proteins. For example, the adsorption of pectin onto cationic sodium caseinate-coated emulsion droplets was dramatically decreased at a pH of 3 (ζ-potential close to zero) (Surh et al., 2006), similar to the ζ-potentials observed with HGA digestions (Figure 5.2).

On the other hand, at a higher pH of 5 (similar to LGA digestions), anionic pectin was found adsorbed onto anionic sodium caseinate-coated emulsion droplets, regardless that both compounds possess a negative charge (Surh et al., 2006). It was suggested that the anionic carboxylate groups on pectin molecules can form linkages with the cationic ammoniumyl groups in proteins (Surh et al., 2006). Conceivably such interactions may have contributed to the higher ζ-potentials observed for FM10 and FM15 during the LGA versus HGA digestions. Another study demonstrated that pectin adsorbed onto casein micelles, leading to bridging flocculation when pectin concentration (0.05 – 0.1 wt%) was high enough to allow the sharing of pectin molecules between micelles, but insufficient to fully cover the surface of micelles, whose concentration in the system was 2.5 wt% (Maroziene & De Kruif, 2000). In the LGA digestate, the concentration of pectin was 0.02 wt% and casein micelles approximately 1.10 wt% (assuming a ratio of 80:20 for casein to whey in milk protein, and 95% of casein is in the form of micelles (Huppertz & Kelly, 2009)), leading to a pectin to casein micelles ratio (1.8 wt%) slightly lower than the reported minimum concentration to induce bridging flocculation (2.0 wt%). Therefore, bridging flocculation of casein micelles may have not occurred in LGA digestions, leading to the formation of fewer protein aggregates and emulsion destabilization.
compared to with the HGA digestions. Lastly, pH could have influenced emulsion stability by affecting polyphenol-milk protein complexes that potentially formed in FM10 and FM15 in the presence of apples. When the pH of the aqueous system is close to a protein’s pI, the affinity between proteins and polyphenols seems to be higher, which could be attributed to conformational changes in protein molecules (Yildirim-Elikoglu & Erdem, 2018). Polyphenol-protein interactions can lower protein solubility, but this depends on many factors such as amino acid composition and sequence and polyphenol type (Yildirim-Elikoglu & Erdem, 2018). Due to the limited details regarding the type and amount of polyphenols present in the current study, it is impossible to confirm the effects of pH on the polyphenol-protein complex and subsequent emulsion stability, but this should be considered in future studies.

Table 5.2. Values of D₃₂ and D₄₃ for FM10 and FM15 fat globules before and after static HGA (pH 6.5) or LGA (pH 3.0) digestions, with and without apples.

<table>
<thead>
<tr>
<th>Digestate samples</th>
<th>Without apples</th>
<th>With apples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D₃₂ (µm)</td>
<td>D₄₃ (µm)</td>
</tr>
<tr>
<td>FM10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before digestion</td>
<td>2.96 ± 0.01ₐᴬ</td>
<td>9.54 ± 0.78ₐᴬ</td>
</tr>
<tr>
<td>After gastric digestion</td>
<td>13.10 ± 0.90ₐᴮ</td>
<td>39.98 ± 8.07ₐᴮ</td>
</tr>
<tr>
<td>HGA</td>
<td>5.94 ± 0.18ₐᶜ</td>
<td>9.72 ± 0.37ₐᴬ</td>
</tr>
<tr>
<td>LGA</td>
<td>4.06 ± 0.08ₐᴬ</td>
<td>15.04 ± 1.87ₐᴬ</td>
</tr>
<tr>
<td>FM15</td>
<td>4.42 ± 0.29ₐᴬ#</td>
<td>12.17 ± 1.7₀ₐᴬ</td>
</tr>
<tr>
<td>Before digestion</td>
<td>4.98 ± 0.87ₐᴬ</td>
<td>17.50 ± 4.97ₐᴬ</td>
</tr>
<tr>
<td>After gastric digestion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM, n = 3, analyzed by two-way ANOVA with Bonferroni post hoc testing. For each test meal, within each of D₃₂ and D₄₃, different uppercase letters (A, B) indicate significant differences between with and without apples and different lowercase letters (a, b, c) indicate significant differences among before digestion, after 120 min HGA and LGA digestions (p < 0.05). # indicates FM15 was significantly different from FM10 (p < 0.05).
Figure 5.1. Particle size distributions of FM10 and FM15 before digestion (A and B respectively), after 120 min gastric digestion with HGA (C and D respectively) and LGA (E and F respectively), with and without apples (n = 3). FM10, 10 wt% fat test meal; FM15, 15 wt% fat test meal; HGA, high gastric acidity (pH = 3.0); LGA, low gastric acidity (pH = 6.5).
Figure 5.2. $\zeta$-potential values for FM10 and FM15 before digestion and after 120 min gastric digestion with HGA and LGA, with and without apples ($n = 3$). FM10, 10 wt% fat test meal; FM15, 15 wt% fat test meal; HGA, high gastric acidity (pH = 3.0); LGA, low gastric acidity (pH = 6.5). Different uppercase letters (A, B) indicate significant differences within each stage (before digestion, after HGA and LGA digestions), and different lowercase letters (a, b, c) indicate significant differences among before digestion, after 120 min HGA and LGA digestions ($p < 0.05$).
Figure 5.3. Microstructure of FM10 before and after 2 h of gastric digestion with HGA (pH 3.0) and LGA (pH 6.5), with and without apples. Scale bars represent 5 μm. Lipids were labelled using Nile Red and proteins with Fast Green FCF.

5.4.2. Lipid Digestibility of FM10 and FM15 in the Static Model – Effects of Apple Treatments and Gastric pH Conditions (i.e. HGA and LGA)

After HGA digestion, the duodenal lipolysis for FM10 reached ~ 30% by 2 min, increased rapidly until 60 min, and complete lipolysis was achieved by the end of the 240 min (Figure 5.4A). A similar pattern of lipolysis was observed following the higher gastric pH (i.e. LGA, Figure 5.4B). The presence of apple did not have an overall effect in the extent of lipolysis over the 240 mins (p > 0.05). However, gastric acidity did have an influence on lipolysis (p < 0.01). This may be associated the differences observed in fat globule microstructures, as
described above. Specifically, when apples were present, gastric acidity had an overall effect on lipolysis ($p = 0.038$), and there was specifically higher lipolysis at 180 min with LGA compared to HGA ($99.32 \pm 6.45\%$ versus $83.95 \pm 4.58\%$, $p = 0.036$). Although differences were not observed at the beginning of the duodenal phase, this overall effect of gastric acidity could be attributed to the smaller fat globules observed with at the higher gastric pH (i.e. LGA) which is expected to provide a larger surface area for pancreatic lipases activity (Golding, 2014).

Comparing to FM10, FM15 was digested to a lesser extent. Depending on the presence/absence of apple and gastric pH, the lipolysis after 2-min duodenal digestion was between $7 - 15\%$ and reached $53 - 70\%$ by the end (Figure 5.4C and D). The lower extent of lipolysis with FM15 is probably owing to the presence of 50% more fat in the digestate. Given the duodenal lipase activity was 2,000 U/mL in all static experiments, there conceivably was insufficient lipase to completely hydrolyze FM15. Furthermore, a higher fat content lowers the ratio of calcium to fat present, leading to less effective precipitation and micellization of FFAs that accumulate on the surface of lipid droplets (Li, Hu, & McClements, 2011). Most in vitro static lipid digestion studies, including our previous work (Lin & Wright, 2018), have used test meals containing in the range of or less than 10% fat (Golding et al., 2011; Verrijssen et al., 2016; Zhang et al., 2015; Zhu et al., 2013). FM10 and FM15 provided 25 and 38 mg/mL lipids, respectively, in the duodenal digestate, which were higher than 8 mg/mL lipids suggested for duodenal digestions with a pH-stat titration method (Li et al., 2011). That said, the lipase activity suggested for pH-stat digestions is also much lower (i.e. 240 – 960 U/mL versus 2,000 U/mL) than in the current study (Minekus et al., 2014). In terms of the so-called consensus static digestion protocol, a suitable test meal fat content was not addressed beyond a statement that high fat foods are not suitable, with no definition of “high fat” (Minekus et al., 2014).
Recommendations for the range of fat contents suitable for static models would help to further standardize the protocol.

Relative to the effect of gastric acidity (p < 0.05), apples had a more pronounced overall effect on FM15 lipolysis (p < 0.001). For digestion with HGA, apples led to significant lower lipolysis at most time points throughout the 240 min (Figure 5.4C), while with LGA, the difference was only observed at the last two hours (Figure 5.4D). Overall, the effect of apples was observed with FM15 but not with FM10. This observation correlates well with the stability of milk fat globules in the presence of apples (as discussed in section 5.4.1). Comparing to FM10, FM15 emulsions were significantly destabilized in the presence of apples, before and after gastric digestions, regardless of gastric pH. Therefore, the lipid droplets in FM15 with apples had less surface area for lipase adsorption when they entered the duodenal phase. Nevertheless, the lower extent of initial lipolysis with apples was only observed for the HGA digestions, but not LGA, suggesting that milk fat globule destabilization in the LGA digestions was reversed when mixed with the simulated duodenal fluids. Indeed, limited proteolysis is expected in the LGA digestions due to the near-neutral gastric pH, and the destabilization induced by the presence of apples was depletion and/or bridging flocculation. Such destabilization was more quickly reversible, comparing to the destabilization caused by losing surface charges from proteolysis, which likely occurred at the highly acidic pH, i.e. HGA digestions. However, the presence of apples suppressed the last two hours of lipolysis following LGA digestion (Figure 5.4D), indicating that apples impeded the lipolysis of milk fat globules in ways other than by influencing their gastric stability. In fact, both pectin (Tsujita et al., 2003) and apple polyphenols (Sugiyama et al., 2007) have been reported to reduce pancreatic lipase activity.
in vitro in a dose-dependent manner, and pectin exhibited the effect by reducing pancreatic lipase adsorption on the surface of emulsion droplets (Tsujita et al., 2003).

![Graphs showing lipolysis (%)](image)

Figure 5.4. Lipolysis (%) of FM10 (A and B) and FM15 (C and D) during 240-min static in vitro duodenal digestion, after gastric digestion with HGA (left) and LGA (right) (n = 3). * p < 0.01 between with and without apples at specified time point. Data reported as mean ± SEM. Overall ANOVA p < 0.05 (A and D), p < 0.01 (B), and p < 0.001 (C) for time × apple treatment interaction. HGA, high gastric acidity (pH = 3.0); LGA, low gastric acidity (pH = 6.5).

5.4.3. TIM-1 lipid bioaccessibility of FM10 and FM20 – effects of apple treatments and gastric pH conditions (i.e. HGA and LGA)

The static methods discussed above quantified lipid digestibility, i.e. FFA release. Lipid bioaccessibility is commonly investigated as well. This is defined as the portion of digested lipids which become solubilized in the aqueous phase structures and are therefore available to
cross the unstirred water layer for absorption through the gut wall (Etcheverry, Grusak, & Fleige, 2012; Minekus, 2015). As such, lipid bioaccessibility includes lipids which have specifically solubilized within the aqueous phase of the digestate and are not simply those that have been hydrolyzed (Marze, 2015). The efficiency of FFA solubilization (i.e. bioaccessibility) depends on many factors, including fatty acid chain length and unsaturation, bile salt concentration (Marze, 2015), and soap formation with calcium (Michalski et al., 2013). When FM10 was digested, the TIM-1 cumulative lipid bioaccessibility significantly increased with time (p < 0.001) and seemed to slow down after 300 min, both without and with apples. Overall, the presence of apples led to a lower cumulative bioaccessibility starting from 180 min of the TIM-1 digestion (p < 0.001, Figure 5.5A). After 360 min, the cumulative bioaccessibility was 65.44 ± 8.14% versus 98.92 ± 7.41 % for with and without apples, respectively (p < 0.001, Figure 5.5A). This indicates that, without apples, the fat in FM10 was fully digested and bioaccessible. As such, in these experiments, apples were observed to impede lipid bioaccessibility. This could be attributed to limited lipid hydrolysis and/or limited uptake in the jejunum and ileum.

Based on results from the static model, apples had no effect on the amount of FFAs released from FM10. The lack of differences in static FFA release, with differences observed in TIM-1 bioaccessibility (i.e. FFA solubilization and uptake) suggests involvement of the apple components in the formation of mixed micelles and other aqueous phase structures that form and need to pass through the TIM-1 membrane filters (with a pore size of approximately 50 nm). Pectin has been shown to interfere with micelle formation by binding bile salts (Dongowski, 1995) and lipids (Falk & Nagyvary, 1982), although those studies were conducted using relatively simple model solutions and purified pectin versus the complex digestive environment and whole apples in the present work. Another way in which apples could influence lipid uptake
in the TIM-1 is by increasing viscosity of the intestinal fluids, leading to restricted transport of micelles through the filters. Indeed, pectin has been found to increase the thickness of the unstirred water layer in animal (Gerencser et al., 1984) and human (Flourie et al., 1984; Fuse et al., 1989) intestine, and this was associated with delayed lipid (Fuse et al., 1989) and glucose (Flourie et al., 1984; Fuse et al., 1989) absorption. In this study with whole apples, apple polyphenols could have contributed to the reductions in FM10 lipid bioaccessibility. For example, apple polyphenols previously exhibited a dose-dependent inhibitory effect on pancreatic lipase \textit{in vitro}, and suppressed postprandial TAG elevation in humans after HFM consumption, although this was with a higher dose of polyphenols than used in the current study (15 mg per g fat, versus 4 mg per g fat for FM10) (Sugiyama et al., 2007). Furthermore, as discussed in section 5.4.1.2, Lamothe et al. reported that \textit{in vitro} gastric proteolysis of dairy products was reduced by green tea polyphenols (Lamothe et al., 2014) and this may influence lipolysis by changing the integrity of the MFGM when it reaches the duodenal phase. Indeed, a moderate inhibition of lipolysis (6 - 9\%) was observed in the study by Lamothe et al., in which a polyphenol to protein ratio of 10.5 mg GAE/g protein was applied, i.e. fairly close to the 13.7 mg GAE/g protein in the current study. Although apples were not found to induce lipolytic differences in the static digestions of FM10, this may have occurred in the TIM-1 model because it is a closer approximation of actual GI conditions, with continuous secretion of digestive fluids and removal of digestion products.

An emulsion based on 20\% fat (i.e. FM20) was also analyzed using the TIM-1 simulator, in order to represent the average fat content in the test meals consumed by overweight and obese participants in the human trial (Chapter 4). In contrast to the findings with the 10\% fat emulsion (i.e. FM10), lipid bioaccessibility was relatively limited, regardless of apple treatment (p > 0.05).
Specifically, the cumulative lipid bioaccessibility was 49.83 ± 7.71% and 48.82 ± 0.87% at the end of the duodenal digestion with and without apples, respectively (p > 0.05, Figure 5.5B). Significant increases were observed over time (p < 0.001), but these rises were slower than for FM10 and increased in a linear fashion over the 360 min. This limited bioaccessibility may be attributed to the high fat content of FM20. Firstly, similar to the higher fat meal tested in the static digestion model (i.e. FM15), FM20 had an even lower protein to fat ratio, leading to greater susceptibility to emulsion destabilization. As such, we can postulate that the FM20 lipid microstructure was less likely to be intact going into the jejunum and ileum compartments, leading to decreased interfacial area and with more limited initial lipolysis. Secondly, the apparent pKa of FFAs in the presence of lipid droplets was likely higher than the “true” pKa measured in aqueous solution, because FFAs tends to interact with the lipid phase, making it more difficult for them to partition into the aqueous phase (McClements & Li, 2010). For example, the apparent pKa of lauric acid was increased from 4.9 in an aqueous solution to 11.2 in a 50% fat system (Domínguez de María, Fernández-Álvaro, ten Kate, & Bargeman, 2009). The higher fat content in FM20 may have contributed to higher apparent pKa values for FM20, leading to more non-ionized FFAs accumulating in the lipid phase (McClements & Li, 2010) instead of being available for solubilization by bile salt micelles. Therefore, compared to FM10, FM20 may have had less potential for the hydrolyzed FFAs to be solubilized in the aqueous micellar phase. Nevertheless, lipid droplets were present in the initial duodenal digestates of both FM10 and FM20, and it is unknown if this association between increased pKa and fat content would be dose-dependent, so further investigation is warranted.

Furthermore, experimental conditions may have limited the potential to achieve complete hydrolysis with the high fat content in FM20. Even though TIM-1, being a dynamic model,
includes the gradual secretion of digestive fluids, as well as the absorption of water and liberated nutrients that minimize product inhibition of lipolysis (Minekus, 2015) it does not allow for feedback based on meal characteristics (Minekus, 2015), including fat content. In healthy adult humans, lipid digestion is generally very efficient, as pancreatic lipase levels tend to adapt to the amount of lipid ingested (Armand, 2007). Therefore, the results observed with the TIM-1 cannot fully account for the human conditions and the bioaccessibility observed with TIM-1 may not fully correspond to the digestion in vivo. To the authors’ knowledge, a test meal containing 20 wt% fat has not been tested in any published TIM-1 studies. The official manual of TIM-1 recommends ~9 wt% fat for a standardized high fat meal, i.e. similar to that advised for drug delivery experiments by U.S. Food and Drug Administration and Center for Drug Evaluation and Research (Ribnicky et al., 2014). From a meal context, it is indeed rare that a liquid or emulsion-based food item would contain 20% lipid and be consumed in a large amount in one setting. For example, a McDonald’s vanilla triple thick milkshake contains only ~4 wt% fat and ice creams generally contains 8 – 15 wt% fat (up to 18 wt% for super-premium ice cream) (Goff & Hartel, 2013). Therefore, it is conceivable that the higher fat content investigated has not been explored with the TIM-1 digestion model, because it does not constitute a realistic food-digestion scenario. That said, high fat meals used for oral fat tolerance tests commonly involve having participants ingest a high amount of fat (e.g. 1 g fat/kg body weight) that is standardized for high body weight. As in Chapter 4 with overweight and obese individuals, this results in fat contents that are very similar to or even higher than the 20 wt% applied in this instance. As such, the TIM-1 may not be suitable for mimicking these scenarios. Oral fat tolerance tests are commonly applied in clinical trials to challenge the capacity of lipid metabolism in order to detect underlying deficits in individuals at metabolic risk, including individuals with obesity (Lairon et
al., 2007). Until there is a clearer understanding about the physiological parameters for lipid digestion and absorption in these specific populations, application of the TIM-1 to study those scenarios may be limited. For example, compared to a healthy body weight, obesity has been associated with faster initial gastric emptying in humans (Mushref & Srinivasan, 2013) and lower secretion of pancreatic pro-colipase in rats (Erlanson-Albertsson & Larsson, 1988), i.e. both factors that can contribute to variations in lipid metabolism. Thilakarathna et al. compared the results from a TIM-1 in vitro lipid digestion study with those of a human trial with healthy and obese participants, using a stearic-rich HFM providing 1 g fat/kg body weight. In that study, TIM-1 lipid bioaccessibility was significantly correlated with the postprandial serum TAG concentrations in healthy participants, but not in obese participants (Thilakarathna et al., 2016). The authors understand that TNO may be seeking validation of TIM-1 for scenarios involving obesity, but, to the best of our knowledge, there are no publications addressing this. Thus far, TIM-1 digestive conditions have been adapted and the results validated against in vivo data to mimic protein digestion in infant (Havenaar et al., 2013; Ménard et al., 2014) and elderly (Denis et al., 2016) populations.
Figure 5.5. Cumulative lipid bioaccessibility (%) of FM10 (A) and FM20 (B) during 360-min in vitro TIM-1 digestion (n = 3). Data reported as mean ± SEM. * Significant treatment differences (p < 0.001) observed between with and without apples. Overall ANOVA p < 0.001 (A) and p > 0.05 (B) for time × apple treatment interaction.
5.4.4. FA Composition of Bioaccessible Lipids in the Static and TIM-1 Digestion Models

Based on the results of Chapter 3, it was postulated that pectin has the potential to influence the bioaccessibility of specific FAs (such as docosahexaenoic acid). Bioaccessibility is a pre-requisite for lipid absorption, and the FA composition of absorbed lipids is reflected in chylomicrons carrying reassembled TAG (Milan et al., 2016). The FA composition of chylomicrons can affect their clearance and uptake (De Pascale et al., 2006; Lambert et al., 2001; Rahman et al., 1999), which has implications in terms of cardiovascular health. Nevertheless, work is very limited in the area of dietary fibres’ effect on FA composition of bioaccessible lipids and/or chylomicrons. Therefore, in this study, the effect of apples on the FA composition of bioaccessible lipids was investigated for both TIM-1 (throughout digestion) and the static (at the end of digestion) methods. Of note, although milk fat contains FAs ranging from as low as four carbons in length (Månsson, 2008), due to methodological limitations, only medium to long chain FAs (≥10 carbons) were quantified. These longer chain length fatty acids also have more relevance to chylomicron metabolism investigated in the human study, given that short, as well as medium chain length FAs, tend to be absorbed through the portal vein (Mu & Høy, 2004).

As expected, the main FAs present in the FM10 and FM20 beverages included myristic (14:0), palmitic (16:0), stearic (18:0) and oleic (18:1n9) acids (Table 5.3). A small proportion of linoleic acid (18:2n6) was present, as well (Table 5.3). In total, these FAs accounted for more than 85% of the total area count of the lipids analyzed (Table 5.3). This FA composition is very similar to that reported previously for bovine milk fat (Månsson, 2008). Lastly, as expected, there was no difference between the FA compositions of FM10 and FM20 (p > 0.05).

For the TIM-1 digestions, the FA composition of the jejunal and ileal bioaccessible lipids was determined throughout the 360 min. Overall, it differed between FM10 and FM20 (p = 0.01
for oleic acid and p < 0.001 for other FAs), indicating an effect of fat content on specific FA bioaccessibility. For FM10, the proportions of the main FAs changed over time (p < 0.05, Figure 5.6A, C, E, G and I). More specifically, rapid increases in myristic and oleic acids were observed, while the other FAs decreased substantially within the first 80 min. In addition, palmitic, stearic and oleic acid showed a trend of returning to their original proportions in the last 2 hours (Figure 5.6, left panel). There were no differences observed in terms of FA composition of the bioaccessible lipids with the inclusion of apples in FM10 (p > 0.05). Compared with FM10, the changes in FA composition over time with FM20 were less substantial. Palmitic and stearic acids did not change throughout the 360 minutes (p > 0.05, Figure 5.6D and F) and the change in oleic acid was marginal (p = 0.05, Figure 5.6H). Also, the absence/presence of apples appeared to have a greater influence on FA composition with FM20 versus at the lower fat content. Despite no influence on myristic and linoleic acids, apples blunted the fluctuations in palmitic, stearic and oleic acids, over time. The presence of apples also led to a lower proportion of the saturated FAs (palmitic and stearic acids) and relatively more unsaturated FAs (oleic acid) becoming bioaccessible, both at the beginning and the end of the digestion period (p < 0.05, Figure 5.6D, F and H). Overall, with FM20, the presence of apples was associated with reductions in the proportion of saturated FAs and increases in the proportion of unsaturated FAs in the bioaccessible lipids. This was despite the fact that apples did not induce any significant differences in the magnitude of lipid bioaccessibility of FM20 (as evidenced in section 5.4.2).

A few factors may have contributed to the observed higher unsaturated FA solubilization with apples in FM20. First of all, as discussed before, the higher fat content of FM20 could have influenced the actual pKa and partitioning of FFAs, reducing their solubilization in the aqueous micellar phase, and this effect may be more dominant in palmitic and stearic acids than in oleic
acid because they are inherently less soluble (Smith & Lough, 1976). Also, apple contain polyphenols, which are antioxidants which conceivably may have helped protect any unsaturated fatty acids from oxidation (von Staszewski et al., 2014) during digestion, hence improving the proportion of unsaturated FFAs in the bioaccessible lipids. In addition, in the presence of apples, and at the pH of the jejunum and ileum compartments, anionic pectin may bind cationic long chain FFAs, especially saturated FFAs which are more charged comparing to unsaturated FFAs given the same chain length (Kanicky & Shah, 2002). This could have assisted in the removal of liberated saturated FAs from the lipid droplet surface. Furthermore, FFAs (mainly saturated) were also shown to be removed from the oil-water interface by forming soaps with calcium ions (Ye, Cui, Zhu, & Singh, 2013). Palmitic and stearic acids were the only FAs observed to form soaps during the early stage (10 – 30 min) of an in vitro digestion protocol (Ye et al., 2013). Therefore, if apples indeed facilitated the removal of FFAs in the initial duodenal stage (first 40 min), the impact could mainly be on palmitic and stearic acids. Less solubilization of palmitic and stearic acids would translate into higher proportions of the other FAs in the bioaccessible lipids. Of note, within the first 40 min of TIM-1 FM20 digestion, very limited lipid bioaccessibility was observed (~1%, Figure 5.5B). This bioaccessibility may be from lipids contributed by the digestive fluids, including phospholipids in the bile extract, although this contribution should be standardized across the TIM-1 digestions, as well as a small amount of bioaccessible lipids from FM20.

This unsaturated FA-promoting effect of apples was not observed with FM10, i.e. where lipid bioaccessibility was attenuated with apples. This suggests that, with FM10, even though apples increased the proportion of unsaturated FAs in the bioaccessible lipids, the effect may have been counteracted by the overall lower bioaccessibility with apples. With the TIM-1
digestion model, it is not possible to determine if observed lower bioaccessibility is owing to reduced lipolysis or reduced solubilization of FFAs, or both. In Chapter 3, a low-level of purified pectin (1.1 mg/g pectin in a 10 wt% fat lecithin-stabilized emulsion) promoted lipolysis (moderately) and DHA bioaccessibility (drastically) after a gastric treatment at pH 3.0. This was because the pectin protected the emulsion droplets from destabilizing at the low pH. In the current study, the pectin content was higher (5.9 mg/g pectin in FM10), and the gastric pH changed (6.5 to 1.7) over the course of 6 hours. MFGM proteins are negatively charged above their pI (pH 4.5-5) and become aggregated when treated with pH 3.0 (evidenced in section 5.4.1). Therefore, the change in gastric pH might have destabilized the milk fat globules in the gastric compartment, especially with the rapid decrease in pH within the first hour (i.e. 6.5 to 2.9).

Pectin, present in the form of whole apples, likely did not serve to maintain the intact milk fat globule structure in FM10 during the TIM-1 digestion (nor did it protect FM10 emulsion microstructure in the static digestion), which agrees with the observed lower bioaccessibility.

In terms of the static digestion model, the FA composition of bioaccessible lipids in the aqueous phase was only determined at the end of the 240 min duodenal digestion and only with the 10% fat test meal (i.e. FM10). With the most acidic gastric pH (i.e. HGA) and without apples, there was generally no difference between the FA composition of the FM10 meal and composition of the bioaccessible lipids (except for linoleic acid). However, the presence of apples significantly lowered palmitic acid bioaccessibility and increased that of oleic and linoleic acids (p > 0.05, Figure 5.7A). With LGA, the bioaccessibility differences between with and without apples disappeared. That is, at pH 6.5, regardless of whether or not apples were present or not, lower bioaccessibility of saturated FAs (myristic, palmitic and stearic acids) and higher bioaccessibility of unsaturated FAs (oleic and palmitic acids) compared to the meal, were
observed (Figure 5.7B). Regardless of gastric pH, the proportion of linoleic acid was much higher in the bioaccessible lipids compared to the emulsions (p < 0.05, Figure 5.7A and B). This elevation in linoleic acid was probably contributed by the phospholipids (soy lecithin, contain ~63% linoleic acid) (Thornton, Johnson, & Ewan, 1944) added to the duodenal digestive fluid. Within the apple treatments, gastric pH also had a significant influence on the composition of bioaccessible FAs (p < 0.01) and this varied between FAs. When only the test meal was present, digestion with LGA lowered the proportion of palmitic and stearic acids and increased oleic acid (p < 0.05), while digestion with HGA did not lead to any changes in those three FAs comparing to before digestion (p > 0.05). Overall, when FM10 was digested with LGA, there was no difference between apple treatments, but with the lower gastric pH (i.e. HGA), apples reduced palmitic acid and improved oleic and linoleic acid bioaccessibility comparing to without apples. This observation agrees with the TIM-1 results and may be associated with the anionic pectin at the duodenal pH (6 – 7), which may assist in the removal of cationic saturated FFAs (as discussed in the previous paragraph). Nevertheless, this theory needs to be confirmed with more mechanistic studies, and more research is needed to assess the effects of dietary fibres on specific FA uptake during lipid digestion.
Table 5.3. Fatty acid compositions of the FM10 and FM20 emulsions.

<table>
<thead>
<tr>
<th>FA species (% total area count)</th>
<th>FM10</th>
<th>FM20</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>12.0 ± 0.0</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td>C16:0</td>
<td>36.8 ± 0.2</td>
<td>36.6 ± 0.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>11.7 ± 0.6</td>
<td>11.6 ± 0.0</td>
</tr>
<tr>
<td>C18:1n9</td>
<td>22.2 ± 0.2</td>
<td>22.1 ± 0.0</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>2.9 ± 0.0</td>
<td>2.9 ± 0.0</td>
</tr>
<tr>
<td>Sum of the above FAs</td>
<td>85.6 ± 1.0</td>
<td>85.2 ± 0.1</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM, n = 3, p > 0.05.
Figure 5.6. FA composition of bioaccessible lipids in the jejunal and ileal dialysates from the TIM-1 digestions of FM10 (A, C, E, G, I) and FM20 (B, D, F, H, J), with and without apples. * p < 0.05 indicates significant difference between with and without apples at the specified time point. For time × apple treatment interaction in ANOVA, p > 0.05 (A, B, C, G), p = 0.05 (I), p < 0.05 (D), and p < 0.01 (E, F, H, J). FM10, 10 wt% fat test meal; FM20, 20 wt% fat test meal.
Figure 5.7. FA composition of FM10 before digestion and of the aqueous micellar phase after static duodenal digestion with or without apples following gastric simulation at pH 3.0 (i.e. HGA, A) and pH 6.5 (i.e. LGA, B) apples (n = 3). Different letters (a, b, c) indicate significant differences between test meal FA composition and the bioaccessible FA compositions with and without apples. FM10, 10 wt% fat test meal; HGA, high gastric acidity (pH = 3.0); LGA, low gastric acidity (pH = 6.5).
5.4.5. *Comparisons between the Static Model and TIM-1 Dynamic Model*

With the static digestion model lipid digestibility was quantified. This captures the total amount of fatty acids released during TAG hydrolysis, whether or not they were solubilized in the aqueous phase. This is a direct measure of the hydrolytic capacity by pancreatic lipase and is a pre-requisite for bioaccessibility. In contrast, lipid digestibility cannot be directly measured in the TIM-1 model. Instead, the TIM-1 assesses the amount of hydrolyzed fatty acids (FFAs) solubilized in the aqueous phase and transported through a simulated intestinal wall and is a measure of lipid bioaccessibility. For each digestion model, a low fat (FM10) and a high fat (FM15 for the static model and FM20 for TIM-1) meal were investigated. FM10 without apples was fully hydrolyzed in the static digestion model and fully bioaccessible in the dynamic TIM-1 model. In contrast, the higher fat test meals did not reach complete digestibility or bioaccessibility.

Generally, apples were found to have a suppressive effect on lipid digestibility in the static model and bioaccessibility in the dynamic model. However, the effect of apples depended both on digestion model as well as test meal fat contents. For the FM10 emulsions, apples demonstrated a suppressive effect on lipid bioaccessibility in the TIM-1 experiments, but showed no effect on digestibility in the static experiments. This could be attributed to differences in FM10 digestion between the models, i.e. mixing conditions and transit as well as methodological differences. For example, in the static digestions, apples were blended by themselves and then added to the FM10, because of the small volume of the test meals used. In contrast, for the TIM-1 experiments, apples were blended with the skim milk, skim milk powder and acetaminophen and then mixed with whipping cream. This was necessitated because blending apples by themselves generated large chunks that tended to get stuck in the TIM-1. Therefore, for the TIM-
experiments, apples ended up being more finely blended, potentially influencing the interfacial properties of milk fat globules to a greater degree, owing to different microstructures and more extensive release of intracellular compounds, including pectin and polyphenols. Such a fine blend of apples, as required for the method, may not realistically reflect chewing processes in the mouth, hence leading to differences between in vitro and in vivo GI behaviors of the test meals.

Also, test meal transit patterns were very different between the two digestion models. In the TIM-1 platform, the digestate could have had a phase separation or fat globule destabilization occurring in the gastric phase, induced by the presence of apples, resulting in fat being retained in the stomach compartment for a longer period of time. In contrast, in the static experiments, gastric and duodenal digestions were simulated in the same container. Even though the presence of apples led to partial fat globule destabilization by the end of gastric digestion (evidenced by the additional peak of larger sizes observed in section 5.4.1), the fat may have been rapidly re-emulsified by surfactants (e.g. bile salts and phospholipids) present in the duodenal digestive fluids. This may have obscured the ability to detect possible differences between with and without apples in the static model. That said, a significant effect of the presence of apples was observed with FM15 in the static model. This may be because FM15 was more sensitive to emulsion destabilization induced by apples, potentially due to the lower protein to fat ratio compared to FM10 (as discussed in section 5.4.1.1). Overall, the in vitro lipid digestibility in the static model was in line with changes observed in lipid microstructure during gastric digestion (as discussed in section 5.4.1). With the TIM-1, differences in lipid bioaccessibility were not observed at the higher fat content (FM20), probably because of the high fat content and limited hydrolysis. Even if the apples led to retention of some fat in the stomach compartment, the amount of fat reaching the jejunum and ileum compartments was likely sufficient or even
excessive for the lipase activity programmed in TIM-1. Therefore, it is conceivable that all fat reaching the jejunum and ileum was fully digested, and the effect of apples delaying fat transit was hindered.

Aside from the study’s main outcomes (lipid digestibility versus bioaccessibility), there are other differences between the static and dynamic models that should be considered when interpreting the results. Regarding enzyme and salt concentrations in the digestive fluids, TIM-1 has a pre-set program allowing enzymes and digestive fluids to be continuously secreted (Minekus, 2015). In contrast, the static model only included the enzymes added at the beginning of each stage. TIM-1 might therefore be able to support the digestion of a larger amount of fat, given that lipases and bile salts are supplied over the course of the digestion. Still, the total lipase output does not change with meal fat content (nor did it change with the static model, either), which was the opposite in healthy humans (Armand, 2007). Also, the gradual secretion of lipases and bile salts may be a rate-limiting step when a high fat meal is present, because, when the fat supplied exceeds the capacity of lipid hydrolysis pre-set in the TIM-1, only a limited portion of fat can be digested between each time points, potentially leading to lipolysis in a linear manner. Since all the lipases and bile salts are provided at the beginning of static digestion, it is common to observe a rapid initial increase in lipolytic products, followed by a slowing down and plateau being reached after some time, both with pH-stat titration and other methods, including the current study. In static models where there is no removal of digestion products substrate inhibition can occur at the oil-water interface, limiting enzyme activity (Reis et al., 2009). In contrast, lipolytic products were continuously removed through solubilization and filtration in the TIM-1, promoting lipid digestion and allowing the collection of dialysates that can be used for estimating lipid bioaccessibility, i.e. a closer approximation of absorption than simple lipid
digestibility. This is an advantage of the TIM-1, although an advantage of the static models is the relative ease with which samples can be obtained from different stages during the static model, allowing for investigation of microstructure. Nevertheless, the static digestion model only permitted investigation of the composition of bioaccessible lipids accumulated by the end of the duodenal digestion. With the TIM-1 model the composition of bioaccessible lipids could be monitored throughout digestion. Therefore, TIM-1 allows for the study of FFA solubilization kinetics and transit.

5.4.6. **Integration of in vitro Digestion Observations and Postprandial Lipemia in Human**

In Chapter 4, it was found that consumption of apples with a high fat meal (HFM, a dairy-based beverage providing 1 g fat/kg body weight, prepared with same ingredients as the ones in the current study) did not modulate postprandial lipemia in overweight and obese participants. That finding should be considered in the context of the in vitro digestion observations. While in vitro digestion methods are now routinely applied, rarely is there opportunity to assess the results against human data. From the in vitro experiments, we can conclude that apples have the potential to alter lipid digestibility and bioaccessibility, interactively with emulsion fat content, gastric pH and based on the in vitro digestion model utilized. For the 10 wt% fat emulsion (i.e. FM10), opposite trends were observed from the static versus TIM-1 models, i.e. TIM-1 bioaccessibility was reduced in the presence of apples, while no difference was observed with the static digestibility. Trends were relatively more consistent between the static (with HGA) and dynamic models comparing the higher fat content emulsions, i.e. FM15 (static) or FM20 (TIM-1) where digestibility and bioaccessibility, respectively, were more limited, and apples showed no effects. The presence of apples was found to significantly destabilize the FM15 emulsions, but not the FM10, i.e. the lower fat system. This might be
attributable to a lower protein to fat ratio in FM15, leading to insufficient emulsifier being present to stabilize the lipid droplets, leaving them more prone to interfacial changes induced by the pectin-containing apples (as discussed in Section 5.4.1.1). Emulsion stability has been related to \textit{in vivo} gastric emptying. For example, Marciani et al. demonstrated consistent gastric emptying for an acid stable emulsion, whereas an emulsion that was destabilized by an acidic gastric pH showed rapid emptying of the aqueous contents and slower emptying of the bulk fat layer which floated on the top of the gastric contents (Marciani et al., 2009). In this study, however, the emulsion destabilization induced by apples \textit{in vitro} did not translate into observed differences in human gastric emptying. This could be related to various factors.

Firstly, FM15 contained 15 wt% fat to correspond to the HFM consumed by participants weighing 75 kg. This was actually on the lower end of the range of participant body weights in the human trial (71.6 – 154.0 kg). Therefore, for most participants, the meal they ingested had a protein to fat ratio lower than that of the FM15 emulsion. Also, the \textit{in vitro} methods did not focus on mimicking digestion in the oral phase. Protease present in human saliva (especially with a higher activity in obese versus normal weight people (Vors et al., 2015)) is expected to contribute to destabilization of milk fat globules even before the meal arrived in the stomach, with or without the presence of apples, potentially leading to no difference observed in gastric emptying. This highlights the importance of including an oral digestion in simulated \textit{in vitro} digestions. It could be an important step influencing lipid microstructure, depending on the food type (e.g. solid versus liquid) (Minekus et al., 2014). Secondly, in the \textit{in vitro} digestion models, the apples and the emulsion were exposed to the digestive fluids together. In contrast, in the human trial, participants were not able to eat the apples and drink the HFM at the same moment, and the consumption period was up to 15 min. Therefore, the solid apples and HFM (as a thick
liquid) may not have arrived in the stomach at the same time, contributing to differences in the effects of apples on emulsion gastric stability. Lastly, gastric emptying in the human trial was measured indirectly using plasma acetaminophen. Since acetaminophen is water-soluble (Willems et al., 2001), even if apples promoted phase separation of the HFM during gastric digestion, the plasma acetaminophen response was likely more reflective of the emptying of the aqueous phase contents, versus the entire meal and, in particular, any retained, separated fat.

In terms of lipid digestibility, the destabilization of FM15 emulsions by apples led to differences in the \textit{in vitro} lipolysis, specifically the extent. The effect of apples was more pronounced at an initial gastric pH of 3.0 (HGA) than 6.5 (LGA); with HGA, differences in lipolysis were observed throughout the duodenal digestion, but for LGA they were only seen at the end of digestion (Figure 5.4C and D). It was suggested that the milk fat globules were more stable in the LGA digestions because of limited proteolysis, leading to higher surface area of the lipid droplets entering the duodenal digestion. In the human trial, gastric pH could not be determined, but is expected to have been buffered more similarly to LGA (versus HGA) with ingestion of the HFM, due to the strong buffering capacity of dairy components (Lutchman et al., 2006). Although food consumption triggers gastric acid secretion and the pH decreases over time to fasting levels (Minekus et al., 2014), a large portion of the lipids in the gastric digestate might could have left the stomach before then, leaving more intact milk fat globules for lipid hydrolysis in the small intestine. Meanwhile, the destabilization induced by apples at a higher gastric pH was postulated to be mainly reversible (i.e. bridging or depletion flocculation), leading to less impact on lipolysis in the presence of other surfactants in the small intestine (as discussed in section 5.4.2). The observed limited difference caused by apples in the initial \textit{in vitro} lipolysis,
after gastric digestion with LGA, is a dynamic that may have existed and contributed to the no
effect of apples observed in terms of postprandial TAG elevation.

In addition to digestibility, lipid bioaccessibility is a pre-requisite for postprandial
lipemia. The effects of apples on lipid bioaccessibility were investigated with the TIM-1
dynamic model using 10 and 20 wt% emulsions (FM10 and FM20 respectively), the latter of
which represented the lipid content of the HFM for the average participant body weight (100 kg)
in Chapter 4. The presence of apples did not affect lipid bioaccessibility of FM20, in agreement
with the lack of difference in postprandial lipemic response with and without apples in the
human trial.

There was a significant correlation found between the amount of FFAs collected from the
jejunum and ileum compartments of the TIM-1 model throughout the 6 hour in vitro digestion
and the human postprandial plasma TAG, (R² = 0.6383, p < 0.001, Figure 5.8). However, the
ultimate lipid bioaccessibility reached was very low for FM20 without apple (≈ 50%, Figure
5.5B), but not FM10 without apple (≈ 100%, Figure 5.5A). This suggests the possibility of
incomplete lipolysis and/or FFA solubilization with the higher fat content. In contrast, lipid
digestion is generally very efficient in healthy adult humans, because the secretion of pancreatic
lipase adjusts to the amount of lipid ingested (Armand, 2007). It could be argued that any effects
of apples were therefore obscured by the limited lipolysis/FFA solubilization in those TIM-1
digestions. Nonetheless, limited lipolysis was observed with FM15 in the static model as well,
although apples were able to further suppress the lipolysis, after gastric digestion at the less
physiologically relevant pH (HGA, Figure 5.4C). Therefore, the lack of effects of apples on
TIM-1 lipid bioaccessibility may not be due to limited lipolysis. The above discussion, overall,
supports the alignment of this TIM-1 lipid bioaccessibility result and the postprandial TAG
response in Chapter 4, despite the discrepancy in the lipid digestion capacity \textit{in vitro} and \textit{in vivo}, especially in terms of the inability to respond to meal fat content and to acknowledge the potential differences in lipid metabolism in obese participants (as discussed in section 5.4.4). Of course, lipid bioaccessibility does not fully dictate postprandial lipemia, which is a dynamic process; absorbed lipids will be packed into chylomicrons, released into blood, and cleared by lipoprotein lipases at tissue-levels (Lambert & Parks, 2012), all of which occur throughout the postprandial period and impact the plasma TAG level.

The clearance and uptake of chylomicrons which is associated with cardiovascular risks, are influenced by their FA composition (De Pascale et al., 2006; Lambert et al., 2001; Rahman et al., 1999). Thus, composition of the bioaccessible FAs were determined for both the static and TIM-1 digestion models. Only the results with FM20 emulsions in the TIM-1 digestion model are discussed here, because its fat content was within the range of HFM consumed in the human trial in Chapter 4. The trend of FA proportional changes over time was quite different between the TIM-1 and \textit{in vivo} data. Specifically, the proportion of palmitic and stearic acids generally increased while oleic acid decreased over time in the human trial, but the trends for these FAs were the opposite \textit{in vitro}. The enzymes present in the digestion may have contributed to this discrepancy between the TIM-1 and \textit{in vivo} data. Lipase from \textit{Rhizopus Oryzae} (used as the gastric lipase) and pancreatic lipase from porcine pancreatin, which are both sn-1,3 regioselective (Houde, Kademi, & Leblanc, 2004), were used in TIM-1 digestion. In contrast, in human, milk TAG was also hydrolyzed by lingual and gastric lipases (Margit Hamosh, Bitman, Larry, & Hamosh, 1985), which preferentially act at sn-3 (Hennessy, Ross, Fitzgerald, Caplice, & Stanton, 2014). This stereoselectivity of pre-pancreatic enzymes may promote the early release of FAs located at sn-3, mainly oleic acid (and short chain FAs which are not discussed
here as they are absorbed through portal vein (Månsson, 2008)). This may have contributed to the higher proportion of oleic acid in chylomicrons at the beginning of the postprandial period observed in the human trial. In addition, samples collected from the TIM-1 model and human inherently represent different stages of absorption/metabolism. The TIM-1 samples consisted of FFAs considered available for absorption in the jejunum and ileum. For the human trial, the absorbed FFAs were reassembled into TAG, which were transported into blood by chylomicron-rich fraction (CMRF). Therefore, more steps beyond simple absorption (i.e. TIM-1 bioaccessibility) contributed to the human study results. For example, oleic acid may have been reassembled and incorporated in chylomicrons faster than other FAs. In a Caco-2 cell model, oleic acid induced higher secretion of apolipoprotein-48 (ApoB48), and oleic acid-rich TAG was more efficiently incorporated in chylomicrons released from a Caco-2 cell model (Van Greevenbroek, Van Meer, Erkelens, & De Bruin, 1996). This suggests a substrate specificity of microsomal triglyceride transfer protein, which translocate TAG into the nascent particle of chylomicrons (Van Greevenbroek et al., 1996). Therefore, in the human trial, given that a certain amount of oleic acid was present in the HFM, a faster transfer of oleic acid into chylomicrons could lead to a higher proportion in their FA composition at the beginning. The proportion of oleic acid could have decreased over time because less oleic acid became available, and the proportion of other FAs changed correspondingly because it was calculated based on the percentage of the total area count, not an actual quantification of the FAs. Because the solubilized FFAs collected in TIM-1 digestion samples did not go through the reassembly or translocation of TAG, this preferential incorporation of oleic acid into chylomicrons cannot be reflected. Similarly, the inability to mimic TAG reassembly and chylomicron formation in the TIM-1 model may have led to the disagreement about apples’ effect. The increase in unsaturated
FAs and decrease in saturated FAs observed with apples in TIM-1 digestion was attributed to a few physicochemical mechanisms during digestion and absorption (see section 5.4.5), but they might have been counteracted by post-absorption events, such as the interactions between carbohydrate and lipid metabolism. The readily digestible carbohydrates present in apples, especially fructose, can induce hyperinsulinemia (as observed in Chapter 4), which has been shown to be positively associated with the synthesis and secretion TAG-rich lipoprotein (Harbis et al., 2001) and was a relationship observed in Chapter 4. The exact mechanism by which fructose-induced hyperinsulinemia could influence FA incorporation in chylomicrons is unclear and unfortunately cannot be studied using the TIM-1 digestion model. The differences in FA bioaccessibility trends between the in vitro and in vivo analyses, while perhaps not unexpected, highlight limitations in the in vitro methodologies.

Overall, to some extent, the results obtained from both in vitro digestion models (static and TIM-1) were consistent with the observations in the human trial. For the static digestion model, agreement with in vivo trend of no effect of apples was only observed with a gastric pH that reflected the anticipated buffering capacity of the food consumed. This supports the need to consider what is the physiological range of gastric pH for in vitro static experiments. For the TIM-1 dynamic model, the no-effect of apples on the lipid bioaccessibility was also in line with the observed with the insignificant in vivo postprandial TAG response differences. In fact, the FFA release from TIM-1 digestion correlates well with plasma TAG concentrations, despite the likely discrepancy in lipolysis capacity between TIM-1 and humans, especially regarding the studied population (normal weight population represented by TIM-1 and overweight and obese participants). In terms of FA composition of the absorbed lipids, the results from TIM-1 digestion and human did not correlate well, potentially because the samples were, ultimately,
collected at different stages of the lipid metabolism (i.e. absorption versus circulation for TIM-1 and human data, respectively). Therefore, it is critical to acknowledge the difference between the experimental outcomes of the in vitro and in vivo methods, in order to accurately correlate and interpret the results.

![Graph](image)

Figure 5.8. Linear regression analysis between 6 hours TIM-1 FFA release data for FM20 (mg, error bars indicate SEM, n = 3) and human study plasma TAG concentrations (mmol/L, error bars indicate the SEM, n = 26).

5.5. Conclusions

The objective of this study was to use in vitro digestion methods to investigate the effect of whole apples and gastric pH on emulsion stability, lipid digestibility and bioaccessibility of a dairy emulsion-based test containing levels of fat contents as applied in a postprandial human trial. Overall, interactions between the presence of apples, gastric acidity, and test meal fat content were observed. In general, the destabilizing effects of low gastric pH and presence of apples were more substantial with the test meals possessing a low protein to fat ratio in the meal.
Emulsion microstructural changes were observed related to acidity of the gastric environment and digestibility and specific FA bioaccessibility was shown to be modifiable. The effects of meal (with or without apple, and fat content) also depended on the simulated digestion model utilized (static or TIM-1), related to difference in their experimental parameters, e.g. meal preparation, chyme transit, enzyme concentration. This highlights the importance of considering and prioritizing various possible factors when comparing the results of in vitro lipid digestion studies and drawing conclusions. It also emphasizes the need to develop experimental conditions better accommodating to specific meals conditions and the gastrointestinal physiology of different populations, specifically obesity. The in vitro results were also integrated with those of the human study, and correlations were found to exist, to some extent, with some conditions. However, even acknowledging that the in vitro methods were not optimized for GI conditions in the human study overweight and obese participants, caution is urged when using in vitro digestion methods to predict postprandial lipemic response.

5.6. Acknowledgements

We thank Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and Ontario Apple Growers for the financial support and providing the apples (Martin’s Family Fruit Farm and Norfolk Fruit Growers Association). We also thank Hannah Neizer and Erika Marshall for the assistance in sample collection and analysis. In addition, we thank Natalie Ng for TIM-1 experiments, Dr. David W.L. Ma and Lyn Hillyer for fatty acid analysis, Dr. Michaela Strüder-Kypke for assistance with the confocal laser scanning microscopy, and the department of Food Science at the University of Guelph related to use of equipment.
CHAPTER 6. Integrated Discussion & Conclusions

6.1. Overall Objective

This thesis aimed to understand how the presence of pectin, as a dietary fibre, and pectin-containing food matrices, specifically apples, modulate lipid metabolism, given the implications for nutritional strategies to target cardiovascular disease risks. In particular, the work focused on the effects of pectin and/or apple pectin-containing matrices on the lipid microstructure, digestibility, bioaccessibility and bioavailability of oil-in-water emulsions. Both in vitro and in vivo methodologies were applied, allowing for exploration of in vitro-in vivo correlations.

6.2. Summary and Discussion of Major Findings

In study 1 (Chapter 3), the digestive stability, digestibility and bioaccessibility of a soy lecithin-stabilized algal oil emulsion rich in docosahexaenoic acid (DHA) was studied using a standardized static in vitro digestion model. Four meal samples containing different amounts of apple pectin were digested using three different gastric pH conditions, i.e. representing the variable pH potential induced by different food compositions. It was hypothesized that pectin would destabilize the emulsion and hence reduce lipid digestibility and DHA bioaccessibility. Differences in emulsion microstructural changes were observed based on the combinations of meal composition and gastric pH. Also, these microstructural changes were shown to modulate in vitro duodenal lipolysis and DHA bioaccessibility. Specifically, a low pectin concentration (1.1 mg/g emulsion) was found to improve lipid stability, digestibility and DHA solubilization when a gastric pH of 3.0 was applied, while a high pectin level (20 mg/g emulsion) reduced emulsion stability, digestibility and FA bioaccessibility at any gastric pH. These findings partially agree with the original hypothesis and indicate possible impacts of interactions between
food components and gastrointestinal factors on lipid digestion. There are implications for dietary recommendations and design strategies for lipid-based foods and natural health products, including those containing fish oil and DHA which have increased in popularity.

Building on the main finding in study 1 (Chapter 3, i.e. that pectin can impact emulsion stability and digestibility in vitro), whole apples, as a food matrix rich in pectin, were investigated for their potential to modulate postprandial lipemia, using a randomized crossover human trial in study 2 (Chapter 4). It was hypothesized that consumption of apples would slow down gastric emptying and delay/limit the extent of postprandial lipemia after ingestion of the OFTT. 26 overweight or obese participants consumed a high fat dairy beverage (standardized as per typical oral fat tolerance test (OFTT) methods to provide 1 g fat/kg body weight) with or without ~200 g whole apples providing 4.3 mg pectin/g emulsion. Gastric emptying and postprandial lipemic response were characterized for 6 hours and compared between with and without apples. Counter to what was hypothesized, apples did not affect gastric emptying, measures of postprandial lipemia (i.e. triacylglycerols (TAG), plasma FA composition or glycemia. However, it was found that apple consumption induced hyperinsulinemia, which may be contributed by the digestible sugars in apples, leading to a higher secretion of TAG-rich lipoproteins. This was supported by a higher peak ApoB48 peak concentration observed with apples. These findings showed that, as a whole food matrix, apples, despite their pectin and polyphenol contents, did not induce lipid-lowering benefits in a group of participants susceptible to elevated postprandial lipemia. The associated postprandial response was summative of the effects of all food matrix components. This highlights the complexity of food matrix effects, which is often neglected in in vitro studies.
To gain mechanistic insights into the potential impacts of apples on digestive processes, the same high fat dairy beverages, with or without apples, as consumed by the participants in study 2 were investigated using two in vitro simulated digestion models (i.e. dynamic TIM-1 and static model) in study 3 (Chapter 5). It was hypothesized that whole apples would decrease lipid droplet stability, digestibility and bioaccessibility in vitro, and that the dynamic digestion model would be the most appropriate model for predicting postprandial lipemic response. This was partially confirmed because apples’ effects on emulsion stability, digestibility and bioaccessibility were shown to depend on various factors, including the fat content of the emulsion and the simulated gastric pH utilized. In the static digestion, a low initial gastric pH, but not apples, led to emulsion destabilization with a high protein-to-fat ratio, but the opposite was seen when a lower protein-to-fat ratio was present in the emulsion. Destabilization of the low protein-to-fat ratio emulsion did not translate into lower lipid bioaccessibility in the TIM-1 digestion however, in agreement with the observation that apples did not alter postprandial TAG in humans. That said, incomplete digestibility and low lipid bioaccessibility were achieved in vitro, even with fat contents similar to those applied in the human trial, pointing to insufficient capacity for lipid digestion/absorption in vitro comparing to that in humans, and, in particular, for the study population investigated, e.g. overweight and obese participants. This discrepancy rationalizes further improvements in in vitro methodologies to better simulate the gastrointestinal dynamics in vivo, including for metabolically at risk individuals who may be best served by diet-based strategies aimed at altering lipemic response.

6.3. Future Directions, Strengths and Limitations

Pectin, as a soluble dietary fibre, has been shown and postulated to have various health benefits related to its behaviour in the GI tract and impact on lipid digestion, but this relies on
many factors. As evidenced by us (Lin & Wright, 2018) and in other *in vitro* (Beysseriat et al., 2006; Dickinson et al., 1998; Roudsari et al., 2006; Verrijssen et al., 2014) and *in vivo* (Flourie et al., 1985; Fuse et al., 1989) publications, pectin concentration is critical to its physicochemical and physiological functions related to lipid digestion and metabolism. In addition, this work explored the lipid-lowering potential of different pectin-containing food matrices (i.e. purified pectin, applesauce, and whole apples), demonstrating the importance of complex food matrix effects. For example, despite reported potential lipid-lowering effects for purified pectin (Beysseriat et al., 2006; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, & Narváez-Cuenca, 2014; Leclere et al., 1993; Verrijssen et al., 2015; Zhang et al., 2015), consuming three apples with a high fat meal actually had no influence on postprandial lipemia (Chapter 4). Furthermore, other food components co-ingested with pectin could influence its functionality directly (e.g. emulsifiers interacting with pectin (Verrijssen et al., 2015; Zhang et al., 2015)), and indirectly (e.g. dairy products buffering the pH of gastric digestate (discussed in Chapter 5)). Lastly, although not discussed in depth in this thesis, other physicochemical properties of pectin, including degree of methylation (Brouns et al., 2012; Dongowski, 1995; Trautwein et al., 1998) and amidation (Hagesaether & Sande, 2007), can influence its gastrointestinal behaviour. For instance, native apple pectin is mainly highly methylated (degree of methylation > 71%) (Voragen et al., 1986), so it will form gel in the presence of an acidic environment and co-solute, instead of crosslinking with calcium ions the way low methylated pectin does (Thakur et al., 2009). In this thesis, the apple pectin in the whole apples and applesauce is expected to be highly methylated and not amidated, i.e. should be similar to native apple pectin used in study 1. Nevertheless, confirming the degree of methylation and amidation of the pectin applied in this thesis can better help with explaining the observations. All these factors could be investigated in
studies of food synergy (i.e. determining how food matrix enhances functionality and bioavailability) and should be considered when designing food products containing lipids and pectin, in order to realize potential health benefits.

It was also shown in this thesis that gastric pH plays an important role in emulsion stability and subsequent lipid digestion and solubilization during simulated digestion (discussed in Chapters 3 and 5). However, the current standardized protocol for in vitro static digestion applies one pH (i.e. 3.0) throughout the 2 hr gastric phase (Minekus et al., 2014). This approach overlooks the dynamic pH changes which can occur and be significantly influenced by food consumption (Dressman et al., 1990; Mojaverian, 1996). As such, it could obscure any effects of ionic strength on emulsion droplet interfacial properties and subsequent lipid digestion and absorption. In contrast, the TIM-1 digestion model accounts for the gastrointestinal dynamics and the gastric pH, as monitored, decreased from 6.5 to 1.7 within 3 hours of digestion, i.e. similar to the pH profile reported for healthy individuals (Dressman et al., 1990). However, TIM-1 instrumentation is not yet widely available as it is expensive and requires considerable skill and effort to maintain and operate. A semi-dynamic gastric digestion model using the pH-stat titration approach became available recently (Ferreira-Lazarte et al., 2017; Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2017). It involves continuous secretion of simulated gastric fluids, rabbit gastric extract and pepsin to achieve the required 2,000 U/mL protease activity in the final gastric digestate, and the gastric pH after administration of dairy products was similar to the gastric pH profile of TIM-1 (Mulet-Cabero et al., 2017). This and similar developments are important to offer researchers affordable dynamic digestion options that approach physiological relevance, at least for certain scenarios. Further in vitro research should be undertaken with the aim to reflect more accurately the pH buffering effects associated with food consumption.
Although TIM-1 is a dynamic model, complete lipid bioaccessibility was not seen after 6 hours when the 20 wt% fat emulsion was digested, indicating insufficient capacity for TAG hydrolysis and/or fatty acid absorption at this high of a fat content (discussed in Chapter 5). This inability of \textit{in vitro} digestion models to respond to meal fat content is in contrast with the lipolysis capacity in human. It limits the utility of the TIM-1 and other platforms to predict postprandial lipemic response to higher fat foods and to compare the results with standard oral fat tolerance test (OFTT) results in overweight and obese participants who since these tests commonly utilize 1 g fat/kg body weight. Therefore, a limitation of this work is the fact that the human study involved participants who were overweight or obese, but generally healthy, and the \textit{in vitro} digestion experimental parameters utilized may not approximate the GI conditions of these individuals. For example, compared to individuals with normal body weight, obesity has been associated with faster initial gastric emptying (Mushref & Srinivasan, 2013) and lower secretion of pro-colipase (Erlanson-Albertsson & Larsson, 1988), which can contribute to differences in the rate or efficiency of lipid digestion. It would be extremely valuable to characterize the lipid digestive conditions reflective in obese population and apply it in TIM-1 digestion to achieve a more accurate prediction of postprandial lipemic response. This would tailor the methods to a metabolically at risk population who could benefit from strategies that alter postprandial lipemia and also enable greater comparisons to results from OFTT methods where meal fat content is generally standardized based on body fat and hence far above those typically used in \textit{in vitro} studies. Lastly, unfortunately any post-absorptive metabolic events are not mimicked by or reflected in the results of the TIM-1 or any other stand-alone \textit{in vitro} digestion method, at present. As such, various factors that can influence lipid metabolism are not accounting for, including the presence of insulinemia-promoting fructose in apples (discussed in
Chapter 4). Cell culture models (e.g. Caco-2) (Van Greevenbroek et al., 1996) may be applied in future studies to better assess the effects of apples on TAG reassembly and lipoprotein secretion, although those methods also are not without limitations. For example, bile salts can be toxic to the cells, requiring dilution of digestate samples (Alminger et al., 2012) to levels that may alter their structure, compromising physiological relevance.

This work has limitations, as discussed throughout the thesis, and summarized here. Firstly, in vitro evidence, especially in study 1, was generated using a specific set of digestive conditions that are standardized. It has been shown in this work that digestive experimental parameters can have a major influence on the observations, so the conclusions drawn from such evidence may be specific to those parameters (e.g. fat content and gastric pH). In addition, the in vitro digestion models applied did not necessarily reflect the physiological conditions in the overweight and obese participants in the human study, as discussed above. Also, the sample size of human participants in Chapter 4 was relatively small, especially given the variability in magnitude of the postprandial lipemia (i.e. TAG and ApoB48) observed, which could have obscured the ability to tease out effects of apples. To minimize this, a greater sample size and considering genetic and other physiological factors that could influence the functionality of pectin and/or apples at the time of participant recruitment would be interesting.

Nevertheless, this work is unique in terms of taking food matrix effects into consideration, so that in vitro effects demonstrated by pectin should be closer to those observed in realistic meal scenarios in vivo. The results have implications for designing nutrient delivery systems that maximize bioavailability. Another strength of this work is the multi-level investigation of pectin’s effects on lipid digestion, which allowed for the comparisons and the assessment of correlations between evidence from a human trial, as well as different in vitro
methodologies. This contributes to the understanding of the physiological relevance of \textit{in vitro} digestion models and provides some insights for future improvements.

6.4. Conclusions

This work examined 1) the interactive effects between pectin and gastric pH on \textit{in vitro} lipid microstructure, digestibility and DHA bioaccessibility from a soy-lecithin stabilized emulsion; 2) the influence of whole apples on gastric emptying and postprandial response of TAG, ApoB48, glucose and insulin after consumption of a dairy-based high fat meal by overweight and obese yet healthy participants; and 3) the mechanisms by which apples affect lipid metabolism and the physiological relevance of \textit{in vitro} digestion models (standardized static model versus dynamic TIM-1 model). Overall, the presence of pectin and apples was shown to influence \textit{in vitro} emulsion stability, digestibility and bioaccessibility, but only under specific combinations of experimental conditions, including pectin concentration, gastric pH buffered by food and fat content. TIM-1 lipid bioaccessibility trends agreed with our observations in the human trial, which was no influence of apples on postprandial TAG response. Despite the alignment of this result, the discrepancies in terms of the digestive process and capacity between \textit{in vitro} models and human should not be ignored. This should be taken into consideration when using \textit{in vitro} methodologies to predict \textit{in vivo} lipemic responses.
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responses to a breakfast meal. The University of Auckland.


States of America, 80(5), 1449–1453.


Metabolic fate of fructose ingested with and without glucose in a mixed meal. *Nutrients, 6*, 2632–2649.


Vors, Cécile, Pineau, G., Gabert, L., Drai, J., Louche-pélissier, C., Defoort, C., ... Michalski, M.


APPENDICES

Appendix A. University of Guelph Research Ethics Board Certificate of Approval for Study 2

RESEARCH ETHICS BOARDS
Certification of Ethical Acceptability of Research
Involving Human Participants

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<th>APPROVAL PERIOD:</th>
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<td>PRINCIPAL INVESTIGATOR:</td>
<td>Robinson, Lindsay (<a href="mailto:lrobinso@uoguelph.ca">lrobinso@uoguelph.ca</a>)</td>
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<td>OMAF-University of Guelph Partnership</td>
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<tr>
<td>TITLE OF PROJECT:</td>
<td>The Apple Study: The effect of 6 week whole apple consumption on blood lipids and inflammatory markers in obese adults</td>
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The members of the University of Guelph Research Ethics Board have examined the protocol which describes the participation of the human participants in the above-named research project and considers the procedures, as described by the applicant, to conform to the University’s ethical standards and the Tri-Council Policy Statement, 2nd Edition.

The REB requires that researchers:
- Adhere to the protocol as last reviewed and approved by the REB.
- Receive approval from the REB for any modifications before they can be implemented.
- Report any change in the source of funding.
- Report unexpected events or incidental findings to the REB as soon as possible with an indication of how these events affect, in the view of the Principal Investigator, the safety of the participants, and the continuation of the protocol.
- Are responsible for ascertaining and complying with all applicable legal and regulatory requirements with respect to consent and the protection of privacy of participants in the jurisdiction of the research project.

The Principal Investigator must:
- Ensure that the ethical guidelines and approvals of facilities or institutions involved in the research are obtained and filed with the REB prior to the initiation of any research protocols.
- Submit an Annual Renewal to the REB upon completion of the project. If the research is a multi-year project, a status report must be submitted annually prior to the expiry date. Failure to submit an annual status report will lead to your study being suspended and potentially terminated.

The approval for this protocol terminates on the EXPIRY DATE, or the term of your appointment or employment at the University of Guelph whichever comes first.

Signature: [Signature]
Date: March 16, 2018

L. Valls
Chair, Research Ethics Board-NPES
Appendix B. Participant Recruitment Poster for Study 2

Apple Study

Participates are needed to study the effects of eating whole apples on risk factors for obesity-related chronic diseases

Participants must be:
- 18 - 75 years old
- Overweight or obese (BMI ≥ 25.0 kg/m²),
- or waist circumference of ≥ 40” for males or ≥ 30” for females
- In generally good health
- Able to tolerate acetaminophen-containing products (e.g. Tylenol)

Study participation involves:
- Eat 3 apples per day for 6 weeks, or no apples per day for 6 weeks if randomized to the “control” group
- Attend 2, 7-hour study visits: drink a high-fat dairy product and provide intravenous blood samples
- Attend 2, 15-minute study visits: answer questions about your health and lifestyle
- Collect 6 fecal samples: 3 before, and 3 after the 6-week study
- Record a 3-day food diary on 3 separate occasions: twice before and once during the 6-week study

*Participants will be asked to limit consumption of polyphenol-rich foods (e.g. berries, coffee, chocolate, etc.)

Total time commitment ≈ 18 hours over 6 weeks

*Financial Compensation Provided!

Up to $300

To find out more about the study and your eligibility as a participant, please contact:

apples@uoguelph.ca

or

519-824-4120 x56967

This project has been reviewed by the Research Ethics Board for compliance with federal guidelines for research involving human participants (REB# 16IA013)
# Apple Study

## PHONE SCREENING QUESTIONNAIRE

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| Date: | Time: |

| Participant’s preferred method of contact: | Email | Phone |

| If by phone, preferred days/times: |

## Apple Study interest questions:

1. How did you hear about The Apple Study?

   - _____ Friend
   - _____ Poster:
   - _____ Newspaper:

   - _____ Social media:
   - _____ Website:
   - _____ Other:

2. If you participate in this study, you will be asked to eat 3 apples per day for 6 weeks if you are randomized to the apple

   | YES | NO |

197
intervention group, or no apples per day for 6 weeks if you are randomized to the control group. Would you be comfortable with this?

<p>| 3. If you are found to be eligible for the study based on this phone questionnaire, you will be asked to visit the HNRU at your earliest convenience for a 1-hour in-person screening visit to confirm your eligibility. Would you be able to accommodate this? | YES | NO |
| 4. If you are found to be eligible for the study based on that in-person screening visit, you will be asked to visit the HNRU at your earliest convenience for a 1-hour orientation session. Would you be able to accommodate this? | YES | NO |
| 5. If you participate in this study, you will be asked to visit the HNRU for 2, 7-hour study visits scheduled 6 weeks apart, and starting between 7:30 - 8:30 am. Would you be able to accommodate this? | YES | NO |
| 6. If you participate in this study, you will be asked to fast for 10-12 hours overnight before the in-person screening visit and before the 2, 7-hour study visits. Water is permitted up until 1 hour before each visit. Would you be comfortable with this? | YES | NO |
| 7. If you participate in this study, you will be asked to drink a high-fat dairy product composed of cream, milk, and water at the 2, 7-hour study visits. This is a research standard to measure your post-prandial response to a high-fat meal. Would you be comfortable with this? | YES | NO |
| 8. If you participate in this study, you will be asked to visit the HNRU for 2, 15-minute visits scheduled 2 weeks apart to check-in with a study coordinator and to collect your apples if you are randomized to the apple intervention group. Would you be able to accommodate this? | YES | NO |
| 9. If you participate in this study, you will be asked to transport approximately 50 apples at a time from the HNRU to your home, and then store them in a cool, dry, and dark place, such as in your fridge. Would you be able to accommodate this? | YES | NO |</p>
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<td>10. Are you comfortable providing blood samples by finger-prick?</td>
<td>YES</td>
<td>NO</td>
</tr>
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<td>11. Are you comfortable providing blood samples intravenously, through a line (catheter) in your arm?</td>
<td>YES</td>
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<td>12. If you participate in this study, you will be asked to collect 6 fecal samples: 3 before the 6-week study, and 3 at the end of the 6-week study. You will be provided with the necessary materials and detailed instructions. Would you be comfortable with this?</td>
<td>YES</td>
<td>NO</td>
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<td>13. If you participate in this study, you will be asked to limit (not eliminate, but limit) polyphenol-containing foods, such as berries, coffee, and chocolate for the entire study period according to the dietary guidelines that we will provide. Polyphenols are nutrients found in plants and plant products that pose health benefits, so limiting your consumption of polyphenol-containing foods during the study will be very important because we want to determine the health benefits of only apple polyphenols. Would you be able to accommodate this?</td>
<td>YES</td>
<td>NO</td>
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<td>14. If you participate in this study, you will be asked to keep a 3-day food diary on 3 occasions to ensure that your diet does not significantly change during the study period. Would you be comfortable with this?</td>
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<td>15. If you participate in this study, you will be asked to maintain your weight during the study period. Would you be comfortable with this?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>16. If you participate in this study, you will be asked to maintain your activity/exercise habits during the study period. Would you be comfortable with this?</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

**Health and lifestyle questions:**

17. How old are you?
<table>
<thead>
<tr>
<th>Question</th>
<th>Answer Options</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Must be 18 – 75 years old</strong></td>
<td></td>
</tr>
<tr>
<td><strong>18. When is your birthday?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>19. What sex do you identify with?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>20. What is your height, approximately?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>21. What is your weight, approximately?</strong></td>
<td></td>
</tr>
<tr>
<td><strong>BMI calculation: weight (kg) / height² (m²)</strong></td>
<td></td>
</tr>
<tr>
<td>Must be ≥ 25.0</td>
<td></td>
</tr>
<tr>
<td><strong>22. Are you currently trying to lose or gain weight?</strong></td>
<td>YES</td>
</tr>
<tr>
<td><strong>23. (Female callers only) Are you pregnant or breast feeding?</strong></td>
<td>YES</td>
</tr>
<tr>
<td><strong>24. Do you smoke?</strong></td>
<td>YES</td>
</tr>
<tr>
<td><strong>25. Do you use recreational drugs?</strong></td>
<td>YES</td>
</tr>
<tr>
<td><strong>26. Do you consume alcohol?</strong></td>
<td>YES</td>
</tr>
<tr>
<td>If YES, if one drink = 1 bottle of beer, or 1 glass of wine, or 1 oz liquor:</td>
<td>YES</td>
</tr>
<tr>
<td>Question</td>
<td>Yes</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>How many drinks per week?</td>
<td></td>
</tr>
<tr>
<td>Must be ≤ 14</td>
<td></td>
</tr>
<tr>
<td>How many drinks per sitting?</td>
<td></td>
</tr>
<tr>
<td>Must be ≤ 4</td>
<td></td>
</tr>
<tr>
<td>27. This study requires participants to avoid alcohol for 48 hours before and after the 2, 7-hour study visits. Would you be comfortable with this?</td>
<td>YES</td>
</tr>
<tr>
<td>28. Have you ever been diagnosed with any major physical or mental disorders or diseases? For example, liver or kidney disease, arthritis, diabetes, cardiovascular disease, anxiety, depression, etc.</td>
<td>YES</td>
</tr>
<tr>
<td>If YES, please describe.</td>
<td></td>
</tr>
<tr>
<td>29. Do you have any gastrointestinal disorders or diseases? For example, Celiac disease, irritable bowel syndrome, gluten/lactose intolerance, etc.</td>
<td>YES</td>
</tr>
<tr>
<td>If YES, please describe.</td>
<td></td>
</tr>
<tr>
<td>30. Are you currently taking any medications? That includes both prescribed and over-the-counter/unregulated medications.</td>
<td>YES</td>
</tr>
</tbody>
</table>
If YES:

<table>
<thead>
<tr>
<th>Medication:</th>
<th>Used to treat:</th>
<th>Taken for (weeks/months/years):</th>
</tr>
</thead>
</table>

**PI will use discretion if taking medication for >3 months, depending on what it is and what it is for**

31. Would it be possible for your medications to remain stable for the entire study period?    
*Participants are encouraged to consult their physician.*  
| YES | NO |

32. Do you routinely use acetaminophen-containing products, such as Tylenol, without complications, or did you in the past without complications?  
| YES | NO |

33. Do you have any known allergies or sensitivities to acetaminophen-containing products?  
| YES | NO |

34. Are you comfortable avoiding any over-the-counter/unregulated medications (including acetaminophen-containing products like Tylenol) for 48 hours **before and after** each study visit?  
*Participants are encouraged to consult with their physician.*  
| YES | NO |

35. Are you currently taking any natural health products or supplements? This includes all vitamins and minerals, herbal remedies, protein powder, etc.  
<p>| YES | NO |</p>
<table>
<thead>
<tr>
<th>36. Are you comfortable avoiding any natural health products or supplements for the entire study period if they were NOT recommended to you by a healthcare professional?</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>37. Do you have any food allergies or intolerances?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>If YES, please describe.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YES is only exclusionary for allergies related to dairy products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>38. Do you have any anaphylactic or life-threatening allergies?</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>If YES, please describe.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If YES:

<table>
<thead>
<tr>
<th>NHP: Recommended by healthcare professional?</th>
<th>Taken for (weeks/months/years):</th>
</tr>
</thead>
</table>

YES is only exclusionary if the NHPs were recommended by a health care professional
PI will use discretion if taking NHP for >3 months
<table>
<thead>
<tr>
<th><strong>39. Are you vegan or vegetarian, or do you have any other dietary restrictions?</strong></th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>If <strong>YES</strong>, please describe.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PI will use discretion depending on dietary restrictions</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>40. Have you taken oral antibiotics in the past 3 months, or do you have any reason to take oral antibiotics in the next 3 months?</strong></th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>If <strong>YES</strong>, please describe.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>41. Did you finish participating in any other research study within the past 3 months, or do you plan to start participating in any other research study within the next 3 months?</strong></th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>If <strong>YES</strong>, please describe.</td>
<td></td>
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</tbody>
</table>
Appendix D. In-person Screening Consent Form and Questionnaire for Study 2

The Apple Study

The effects of whole apple consumption on risk factors for chronic metabolic diseases in overweight and obese adults

CONSENT TO IN-PERSON SCREENING

INTRODUCTION
You are being asked to participate in a screening visit for the Apple Study, a research study conducted by PhD student Danyelle Liddle, and directed by Professors Lindsay Robinson and Amanda Wright of the department of Human Health and Nutritional Sciences (HHNS) at the University of Guelph. The results of this research will contribute to the theses of PhD students Danyelle Liddle and Xinjie (Lois) Lin, and to the research activities of MSc and BSc students at the University of Guelph. This research is funded by the Ontario Ministry of Agriculture, Food and Rural Affairs and the Ontario Apple Growers.

APPLE STUDY CONTACT INFORMATION
If you have any questions or concerns at any time, please feel free to contact the Apple Study research team at apples@uoguelph.ca or 519-824-4120 x56967.

RESEARCHER CONTACT INFORMATION
Or, contact the researchers directly:

Danyelle Liddle, MSc  Lindsay Robinson, PhD  Amanda Wright, PhD
Lead study coordinator  Principal Investigator  Faculty Co-investigator
PhD student, HHNS  Associate Professor, HHNS  Associate Professor, HHNS
Email: dliddle@uoguelph.ca  Email: Irobinso@uoguelph.ca  Email: ajwright@uoguelph.ca
Phone: 519-824-4120 x56967  Phone: 519-824-4120 x52297  Phone: 519-824-4120 x54697
PURPOSE OF THE SCREENING VISIT

The purpose of the screening visit is to determine your eligibility to participate in the Apple Study. This study will determine the effects of one-time (i.e. acute) and long-term (i.e. chronic) consumption of Ontario-grown Gala apples on gastric emptying rate, lipid digestion and absorption, inflammatory mediator production, biomarkers of metabolism, and the gut microbiota profile in obese participants with elevated blood triglycerides, who are therefore at risk of developing chronic metabolic diseases, such as type 2 diabetes or cardiovascular disease.

The screening visit will take place at the Human Nutraceutical Research Unit (HNRU):

Room 144 of the Food Science-Guelph Food Technology Centre (building #88)

88 McGilvray St., University of Guelph, N1G 2W1

Phone: 519-824-4120 x53925

Website: http://www.uoguelph.ca/hnru/index.html

You will receive a $5 Tim Horton’s gift card as a token of our appreciation for your time and commitment.

PROCEDURES

Prior to the screening visit

You will be asked to avoid alcohol, exercise and over-the-counter medications for 48 hours prior to your screening visit. You are encouraged to discuss with your physician the avoidance of any recommended over-the-counter medications. Also, you will be asked to eat a standard evening meal and then fast for 10-12 hours overnight before your morning appointment at the HNRU. You are advised to drink water during the fast up to 1 hour before your screening visit.

During the screening visit

If you volunteer to participate in the screening visit, you will be asked to:

- Have one fasting finger-prick blood sample taken by a trained study coordinator to immediately measure blood glucose, insulin, triglyceride and cholesterol levels. After your fasting finger-prick blood sample, you will be provided with a juice box and a granola bar.
- Complete a questionnaire detailing information about your diet, lifestyle and overall health.
• Have your height, weight, waist circumference, and blood pressure taken by a trained study coordinator.

Total time for the screening visit will be approximately 60 minutes.

Importantly, results from this screening visit will not be obtained from a licensed medical laboratory and thus should not be used for diagnostic purposes; if you have concerns about your results you are advised to see a physician.

After the screening visit
The study coordinator will discuss the results of your (blood and anthropometric) measurements and questionnaire with the research team to determine your eligibility for the study. This process may take up to 5 days. Therefore, a study coordinator will contact you within one week of your screening visit, whether or not you are eligible for the study. If you meet the eligibility requirements, you will be invited to participate in the Apple study. A study coordinator will answer any questions that you might have and arrange for a study orientation visit, during which you will learn all about the participant responsibilities and benefits, and review the study consent form. If you do not meet the eligibility requirements, your data will be immediately destroyed.

POTENTIAL RISKS AND DISCOMFORTS
There are minimal risks associated with participation in the screening visit, but may include:

• Discomfort or a slight pinch from the fasting finger-prick blood sample.
• Dizziness, headache and/or mild nausea due to fasting for 10-12 hours overnight. However, following your fasting blood sample, you will be immediately provided with a granola bar and juice box.
• Discomfort and/or embarrassment about answers to the questionnaire and/or body measurements. However, questions will be asked discreetly using a written questionnaire, and body measurements will be taken in a private area with a trained study coordinator.

The research team will make every effort to ensure your safety and comfort during the screening visit. In the unlikely event of a study-related injury, we will engage appropriate emergency response services to assist in your care.

POTENTIAL BENEFITS TO PARTICIPANTS AND/OR TO SOCIETY
There are no direct benefits to participants for participating in this screening visit. However, your potential participation in this study will generate results that contribute to an understanding of how bioactive-rich foods, such as apples, can impact obesity-related chronic disease risk, and may lead to an increased consumer awareness about the health benefits of apples. More generally, the knowledge gained from this study may contribute to dietary recommendations for individuals to manage their risk of developing obesity-related chronic
diseases. This research may also benefit the Ontario apple industry by providing evidence to support apple health benefits.

**COSTS FOR PARTICIPATION**

There is no direct cost for participating in the screening visit. However, you will be responsible for any costs related to attending your scheduled screening visit at the HNRU, such as gas money, public transportation fees, parking fees, etc.

**CONFIDENTIALITY**

Every effort will be made to ensure confidentiality of any identifying information that is obtained in connection with this study. You have been assigned a coded study number to use in place of your name on all study documents, and all coded study documents will be stored in a locked filing cabinet in a locked office, and compiled in a password-protected computer file. Results from the study may be published or presented, but will be so as grouped data. The master list linking your name with your study code will locked in the Principal Investigator’s office, and will be shredded after publication of results. At that time, all coded data will become anonymized, and all anonymized study documents and data (hard and electronic copies) will be kept indefinitely for potential future use in studies conducted by the research team, investigating apple health benefits in obese adults. By following these procedures, your confidentiality as a participant will be maintained to the best of our ability.

If requested, direct access to your research records for this study will be granted to study monitors, auditors, the University of Guelph Research Ethics Board, and regulatory authorities for the verification of study procedures and/or data. Your confidentiality as a study participant will not be violated during this process, to the extent permitted by applicable laws and regulations. By signing this written informed consent form you are agreeing to authorize such access.

**PARTICIPATION AND WITHDRAWAL**

You can choose whether or not to participate in the screening visit. You may withdraw your consent at any time and discontinue participation without penalty. Even if you meet the eligibility requirements for this study, your participation is not obligatory and you may choose not to participate. You may refuse to answer any questions that you do not want to answer and still continue with the screening visit. If information becomes available that may be relevant to your willingness to continue participating with the screening visit, you will be informed in a timely manner. The Principal Investigator may withdraw you from this research if circumstances arise that warrant doing so.
RIGHTS OF RESEARCH PARTICIPANTS

You do not waive any legal rights by agreeing to take part in this study. This project has been reviewed by the Research Ethics Board for compliance with federal guidelines for research involving human participants. If you have any questions regarding your rights and welfare as a research participant in this study (REB# 16JA013), please contact: Director, Research Ethics; University of Guelph; reb@uoguelph.ca; 519-824-4120 x56606.

SIGNATURE OF RESEARCH PARTICIPANT

I have read the information provided for the study “The Apple Study: The effects of whole apple consumption on risk factors for chronic metabolic diseases in obese adults” as described herein. My questions have been answered to my satisfaction, and I agree to participate in this study. I have been given a copy of this form.

_______________________________________________
Participant Name (please print)  Signature  Date

SIGNATURE OF WITNESS

______________________________________________________________________________
Witness Name (please print)  Signature  Date
The Apple Study

The effects of whole apple consumption on risk factors for chronic metabolic diseases in overweight and obese adults

IN-PERSON SCREENING QUESTIONNAIRE

Study coordinator:

Date: 

Time: 

Thank you for your interest in The Apple Study. The purpose of this questionnaire is to gather more information about you and to ensure your safety as a participant in this study. You may refuse to answer any questions that you are uncomfortable answering. Please feel welcome to ask a study coordinator any questions at any time. All information provided in this questionnaire will be kept confidential.

*You are encouraged to consult with your physician about your responsibilities as a participant in the Apple Study.

1. In the past 10-12 hours, did you refrain from all food and drink, except water?

   If NO, please describe.

   YES / NO
2. In the past 48 hours, did you consume any alcohol?
   If **YES**, please describe.
   **YES** / **NO**

3. In the past 48 hours, did you refrain from any intense exercise?
   If **NO**, please describe.
   **YES** / **NO**

4. In the past 48 hours, did you refrain from any over-the-counter/unregulated medications?
   If **NO**, please describe.
   **YES** / **NO**

**Health questions**

1. How are you feeling today (e.g. well, tired, stressed, sick, etc.)?
2. Has your body weight changed in the past:

   3 months? **YES / NO** If **YES**, how much did you **LOSE / GAIN**?

   \[ \geq 5\% \text{ of initial body weight} \text{ LOSE/GAIN} \]

   1 year? **YES / NO** If **YES**, how much did you **LOSE / GAIN**?

3. This study requires that participants maintain their weight for the entire study period. Would you be comfortable with this? **YES / NO**

4. Do you currently have, or have a history of any major diseases or medical conditions? For example, liver or kidney disease, arthritis, diabetes, cardiovascular disease, etc.

   If **YES**, please describe in this space.

   **YES / NO**

   However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.

5. Do you have any gastrointestinal disorders? For example, Celiac disease, irritable bowel syndrome, gluten or lactose intolerance, etc.

   If **YES**, please describe in this space.

   **YES / NO**

   However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.
6. Have you had any surgeries or any health events requiring hospitalization in the past 6 months?

    If YES, please describe in this space.

**YES / NO**

However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.

7. Are you currently taking any medications? This includes hormonal contraceptives, hormonal replacement therapy, and any prescription and over-the-counter/unregulated (e.g. Tylenol) medications.

**YES / NO**

However, the PI and lead study coordinator will decide on a case-by-case basis if the infrequent use of over-the-counter medications is significant enough to compromise the study results.

    If YES, please list.

<table>
<thead>
<tr>
<th>Product name</th>
<th>Reason for use</th>
<th>Dose</th>
<th>How often</th>
<th>How long</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>
8. Would it be possible for your medication(s) to remain stable during the entire study period?  
*Participants are encouraged to consult with their physician

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

9. Do you routinely use acetaminophen-containing products (e.g. Tylenol) without complications, or did you in the past without complications?

The research team cannot be confident that participants can tolerate the acetaminophen added to the high-fat meal if they do not have current or past experience with acetaminophen-containing products without complications.

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

10. Do you have any known allergies or sensitivities to acetaminophen-containing products?

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

11. This study requires that participants avoid taking over-the-counter/unregulated medications (including acetaminophen-containing products, e.g. Tylenol) that were NOT recommended by a physician for 48 hours before each study visit. Would you be comfortable with this?

*Participants are encouraged to consult with their physician

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
</table>

12. This study requires that participants avoid acetaminophen-containing products (e.g. Tylenol) for 48 hours after each study visit. Would you be comfortable with this?

*Participants are encouraged to consult with their physician

| YES | NO |
13. Are you currently taking any natural health products or supplements? This includes all vitamins and minerals, herbal remedies, protein powder etc.

**YES** / **NO**

However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.

If **YES**, please list.

<table>
<thead>
<tr>
<th>Product name, active ingredients</th>
<th>Reason for use</th>
<th>Dose</th>
<th>How often</th>
<th>How long</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

14. Are you comfortable avoiding any natural health products or supplements for the entire study period if they were NOT recommended to you by a healthcare professional?  

*Participants are encouraged to consult with their physician*  

**YES** / **NO**

15. Have you taken oral antibiotics in the past 3 months, or do you have any reason to take oral antibiotics within the next 3 months?  

**YES** / **NO**
16. Do you have ANY allergies or intolerances? For example, to foods, medications, ragweed or pollen, etc.

**YES / NO**

If **YES**, please list:

<table>
<thead>
<tr>
<th>Allergy/intolerance</th>
<th>Type of allergic reaction (e.g. anaphylaxis, difficulty breathing, swelling, etc.)</th>
</tr>
</thead>
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</tr>
</tbody>
</table>

17. (FEMALES ONLY) Are you pregnant or breastfeeding? **YES / NO**

18. (FEMALES ONLY) Have you gone through menopause? **YES / NO**

19. (FEMALES ONLY) If **NO** to question 18, do you have regular monthly menstrual cycles?

   If **NO**, please describe the irregularities.

**YES / NO**

---

**Lifestyle questions**

1. Do you smoke? **YES / NO**

   If **NO**, have you ever smoked? **YES / NO**

   If **YES**, how long since you quit? < 6 months
2. Do you use recreational drugs?  

YES / NO

3. If 1 alcoholic drink = 12 oz beer, 5 oz wine, or 1.5 oz hard liquor, approximately how many alcoholic drinks do you consume in:

1 typical sitting? ___________________________ ≥ 4 drinks/sitting

1 week? ___________________________ ≥ 14 drinks/week

4. This study requires that participants avoid alcohol for 48 hours before and after each study visit, as well as limiting red wine during the entire study period. Would you be comfortable with this?  

YES / NO

5. Do you consume caffeinated beverages? For example, coffee, tea, pop, energy drinks, etc.

If YES, please list what types of beverages, and how many you consume per day in this space.

YES / NO

6. This study requires that participants limit (but not eliminate) coffee consumption (decaffeinated and caffeinated) during the entire study period. Would you be comfortable with this?  

YES / NO

7. How would you describe your activity level? Please circle one.

Very low    Low    Moderate    High    Very high
8. Do you participate in any of the following:

   i) Weight training?  **YES / NO**  If **YES**, how often? ____________________________

   ii) Running/jogging?  **YES / NO**  If **YES**, how often? ____________________________

   iii) Aerobics?  **YES / NO**  If **YES**, how often? ____________________________

   iv) Team sports?  **YES / NO**  If **YES**, how often? ____________________________

   v) Other?  **YES / NO**  If **YES**, please describe in this space.

9. This study requires that participants **DO NOT** participate in intense exercise for 48 hours before each study visit. Would you be comfortable with this?  **YES / NO**

10. Do you have any unusual sleep patterns? For example, insomnia, shift work, etc.

    If **YES**, please describe in this space.

    **YES / NO**
11. How many hours of sleep do you usually get per night? Please circle one.

   0 – 3          4 – 6          7 – 9          10+

---

**Dietary questions**

1. Are you following a vegan or vegetarian diet, or do you have any other dietary restrictions?

   If **YES**, please describe in this space.

   **YES** / **NO**

However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.

2. Do you consume breakfast on a regular basis? **YES** / **NO**

3. How many meals do you eat per day?

4. How many snacks do you eat per day?

5. How many apples do you eat:

   Per day? ________________________

   Per week? ________________________
6. This study may require participants to eat 3 apples/day for 6 weeks, if randomized to the intervention group. Would you be comfortable with this?  

**YES / NO**

7. Please review the Dietary Guidelines: the foods to avoid and the foods allowed during the 2-week run-in period for the study. Would you be able to accommodate this?  

**YES / NO**

8. Please review the Dietary Guidelines: the foods to avoid, the foods to limit, and the foods allowed during the 6-week apple intervention. Would you be able to accommodate this?  

**YES / NO**

9. Please list what you ate and drank for dinner last night, as well as the amount of each food and beverage:

<table>
<thead>
<tr>
<th>Food/beverage</th>
<th>Amount</th>
</tr>
</thead>
</table>

**Study logistics**

1. **If you are eligible and interested in participating in this study, you will be asked to:**

   a) Visit the HNRU at the University of Guelph for a 1-hour orientation session, starting between 4:00 - 7:00 pm, depending on your and your fellow participants’ availability. Would you be able to accommodate this?  

   **YES / NO**
If **YES**, is there a particular weekday that you absolutely **CAN NOT** schedule the 1-hour orientation session on? Please circle all that apply.

Monday  Tuesday  Wednesday  Thursday  Friday

If **NO**, please explain why so that the research team can determine an alternative procedure.

b) Visit the HNRU at the U of G for two, 7-hour study visits starting between 7:30 - 8:30 am, 6 weeks apart. Would you be able to accommodate this?  **YES / NO**

If **YES**, is there a particular weekday that you absolutely **CAN NOT** schedule a 7-hour study visit on? Please circle all that apply.

Monday  Tuesday  Wednesday  Thursday  Friday

If **YES**, is there a particular weekday that you prefer to schedule a 7-hour study visit on? Please Circle all that apply.

Any day  Monday  Tuesday  Wednesday  Thursday  Friday

c) Fast for 10-12 hours overnight before the two, 7-hour study visits. Water is permitted until 1 hour before each study visit. Would you be comfortable with this?  **YES / NO**
d) Consume a high-fat dairy product (cream + water) at the two, 7-hour study visits. Would you be comfortable with this? | YES / NO

e) Visit the HNRU at the U of G for two, 15-minute visits between 9:00 am - 5:00 pm, 2 weeks apart to check-in with a study coordinator and to pick-up your apples. Would you be able to accommodate this? | YES / NO

f) Pick-up 42 apples every 2 weeks (at study visit #1, and check-in visits #1 and #2) and then store them in a cool, dry, dark place (e.g. refrigerator). Would you be able to accommodate this?

If NO, please explain why so that the research team can determine an alternative procedure.

YES / NO

g) Providing finger-prick blood samples. Would you be comfortable with this? | YES / NO

h) Providing blood samples through an intravenous (IV) catheter in your arm. Would you be comfortable with this? | YES / NO

i) Collect 6 fecal samples: 3 before and 3 at the end of the 6-week study. Would you be comfortable with this? | YES / NO

j) Record a 3-day food diary on 3 separate occasions: before and at the end of the 2-week run-in period, and again at the end of the 6-week study. Would you be comfortable with this? | YES / NO
2. The Apple Study has two phases: acute (one time) and chronic (long-term) apple consumption. The acute phase will involve eating 3 apples in one sitting, whereas the chronic phase will involve eating 3 apples or no apples every day for 6 weeks. All 60 eligible participants will be included in the chronic phase, and will be invited to also participate in the acute phase. There will be no consequences for refusing to also participate in the acute phase. 30 of the 60 participants will be included in the acute phase on a “first come, first served” basis and, upon completion of the acute phase, they will immediately start the chronic phase of the study. The weekly timelines for the acute + chronic, and chronic only phases of the study are depicted in Figure 1 and Table 1, appended. Please review these and feel welcome to ask a study coordinator any questions.

Are you interested in participating in the acute phase of the Apple Study, in addition to the chronic phase?

| YES / NO |
|-----------------|-----------------|
| YES / NO |

If YES, the acute phase of the study requires participants to visit the HNRU at the U of G for another 7-hour study visit starting between 7:30 - 8:30 am. Would you be able to accommodate this?

| YES / NO |
|-----------------|-----------------|
| YES / NO |

3. Are you currently involved in a research study? If YES, please describe in this space.

| YES / NO |
|-----------------|-----------------|
| YES / NO |

However, the PI and lead study coordinator will consider all participants’ eligibility on a case-by-case basis.
4. Have you ever been involved in a research study before? If **YES**, please describe in this space.

**YES / NO**

Thank you for completing this questionnaire. A study coordinator will now review the questionnaire with you and answer any questions that you may have.

Thank you again for your time today and your interest in the Apple Study!

______________________________

*Study coordinator signature*

---

**Body measurements**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td><strong>Must be ≥ 25.0 kg/m$^2$</strong></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td><strong>Must be ≥ 40” for males or ≥ 30” for females to remain eligible.</strong></td>
</tr>
</tbody>
</table>
*Either of the BMI or waist circumference minimum (or both) must be satisfied to remain eligible.

<table>
<thead>
<tr>
<th>Blood pressure (mmHg)</th>
<th>1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2)</td>
</tr>
</tbody>
</table>

**Blood measurements**
Appendix E. Study Consent Form for Study 2

The Apple Study

The effects of whole apple consumption on risk factors for chronic metabolic diseases in overweight and obese adults

CONSENT TO PARTICIPATE IN RESEARCH

INTRODUCTION

You are being asked to participate in a research study conducted by PhD students Danyelle Liddle and Xinjie (Lois) Lin, and directed by Professors Lindsay Robinson and Amanda Wright of the Department of Human Health and Nutritional Sciences (HHNS) at the University of Guelph. The results of this research will contribute to the theses of PhD students, and to the research activities of MSc and BSc students at the University of Guelph. This research is funded by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and the Ontario Apple Growers.

APPLE STUDY CONTACT INFORMATION

If you have any questions or concerns at any time, please feel free to contact the Apple Study research team at apples@uoguelph.ca or 519-824-4120 x56967.

RESEARCHER CONTACT INFORMATION

Or, contact the researchers directly:

<table>
<thead>
<tr>
<th>Danyelle Liddle, MSc</th>
<th>Lindsay Robinson, PhD</th>
<th>Amanda Wright, PhD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead study coordinator</td>
<td>Principal Investigator</td>
<td>Faculty Co-investigator</td>
</tr>
<tr>
<td>PhD student, HHNS</td>
<td>Associate Professor, HHNS</td>
<td>Associate Professor, HHNS</td>
</tr>
<tr>
<td>Email: <a href="mailto:dliddle@uoguelph.ca">dliddle@uoguelph.ca</a></td>
<td>Email: <a href="mailto:lrobinso@uoguelph.ca">lrobinso@uoguelph.ca</a></td>
<td>Email: <a href="mailto:ajwright@uoguelph.ca">ajwright@uoguelph.ca</a></td>
</tr>
<tr>
<td>Phone: 519-824-4120 x56967</td>
<td>Phone: 519-824-4120 x52297</td>
<td>Phone: 519-824-4120 x54697</td>
</tr>
</tbody>
</table>
PURPOSE AND DESCRIPTION OF THE STUDY

Whole apples (with the peel) are a very good source of dietary polyphenols and the soluble fibre, pectin; bioactives that likely account for their health benefits. The purpose of this study is to assess the effects of acute and chronic consumption of Ontario-grown Gala apples on gastric emptying, lipid digestion and absorption, inflammatory mediator production, biomarkers of metabolism, and the gut microbiota profile in overweight and obese participants, who are therefore at risk of developing chronic diseases, such as type 2 diabetes or cardiovascular disease.

The Apple Study has two phases: acute (one time) and chronic (long-term) apple consumption. The acute phase will involve eating 3 apples in one sitting, whereas the chronic phase will involve eating 3 apples or no apples every day for 6 weeks. All 60 eligible participants will be included in the chronic phase, and will be invited to also participate in the acute phase. There will be no consequences for refusing to also participate in the acute phase. 30 of the 60 participants will be included in the acute phase on a “first come, first served” basis and, upon completion of the acute phase, they will immediately start the chronic phase of the study. The weekly timelines for the acute + chronic, and chronic only phases of the study are depicted in Figure 1 and Table 1.

If you volunteer to participate in this study, each study visit will take place at the Human Nutraceutical Research Unit (HNRU), located in room 144 of the Food Science-Guelph Food Technology Centre Building, 88 McGilvray St. at the University of Guelph (phone: 519-824-4120 x53925).

STUDY PROCEDURES

Dietary Guidelines

You will be asked to avoid polyphenol-containing foods as listed in The Apple Study Handbook, Table 2 for the 2-week (14 days) run-in period prior to your study visit #1, and for a third week prior to your study visit #2 should you volunteer to participate in the acute + chronic phases of the study. During the 6-week chronic phase of the study, you will be asked to limit polyphenol-containing foods as listed in The Apple Study Handbook.

Please note that eating 3 apples in one day and/or every day for 6 weeks, and the study visit high-fat meal (whipping cream + water) may increase your daily calorie intake. However, apart from the dietary guidelines listed in The Apple Study Handbook, we ask that you maintain your normal dietary, exercise and lifestyle habits for the entire study duration.

3-Day Food Record
You will be asked to keep a 3-day food record on 3 separate occasions according to the instructions in The Apple Study Handbook. Food records will be submitted to a study coordinator to determine any drastic dietary changes during the study. It is important to complete food records honestly and accurately to avoid compromising the results of the study. Total time to complete 3 days of a 3-day food record will be approximately 15 minutes.

Fecal Sample Collection

You will be asked to collect 6 fecal samples in total: 1 fecal sample (size of a dice) per day for the 3 consecutive days prior to your first and last study visits, according to the instructions in The Apple Study Handbook. You are advised to collect your samples at home using the materials provided. You have the option of either immediately bringing your collected samples directly to the HNRU to be frozen, or you may freeze your samples at home and bring them to the HNRU at your next study visit. Total time for each fecal sample collection will be approximately 5 minutes.

Study Visits

You will be asked to avoid alcohol, exercise and over-the-counter medications (including acetaminophen-containing products) for 48 hours prior to your study visits. You are encouraged to discuss with your physician the avoidance of any recommended over-the-counter medications. Also, you will be asked to eat a standard evening meal and then fast for 10-12 hours overnight before your morning appointment at the HNRU. You are advised to drink water during the fast, up to 1 hour before your study visit.

Prior to initiation of study visit activities, you will submit your 3-day food record and will be asked to complete a questionnaire detailing information about your diet, lifestyle patterns, and any recent health issues. Then, in a private area, a study coordinator will take anthropometric measures including your height, weight, waist circumference and blood pressure. Next, you will provide a fasting blood sample by finger-prick, conducted by a trained study coordinator, to immediately assess baseline blood glucose, insulin and triglyceride levels. Afterwards, a forearm vein catheter will be inserted for repeated intravenous (IV) blood sampling by a qualified phlebotomist. Once the catheter has been inserted, a fasting blood sample (12 mL) will be taken.

Acute + chronic phase participants

The total time commitment of the acute + chronic phases of the study is approximately 24 hours and 45 minutes over 7 weeks. If you volunteer to participate in the acute + chronic phases of the study, you will visit the HNRU for 3 separate study visits for 7 hours each, over the 7 weeks. You will complete the acute phase first, and then immediately start the chronic phase. At your study visit #1, after your fasting IV blood sample, you will be asked to consume a high-fat meal (whipping cream + water)
containing 1500 mg of acetaminophen*, and either 3 apples or no apples within 30 minutes. Then, repeated IV blood samples (12 mL each) will be collected every 20 minutes during the first 3 hours, and then each hour for the next 3 hours, for a total of 13 blood samples (156 mL total) during each 7-hour study visit. At your study visit #2, you will follow the same procedures and consume the high-fat meal, but with the opposite intervention (3 apples or no apples). From that point on, you will be included in the chronic phase of the study where you will be randomized to either the intervention group or the control group. The intervention group will eat 3 apples/day for 6 weeks, while the control group will eat no apples/day for 6 weeks. At your study visit #3, you will follow the same procedure as #1 and #2, but you will consume the high-fat meal with no apples. It is recommended that you avoid acetaminophen-containing products for 48 hours after your study visit to prevent over-consumption. It is also recommended that you avoid alcohol for 48 hours after your study visit to avoid liver damage.

**Chronic only phase participants**

The total time commitment of the chronic phase of the study is approximately 17 hours and 45 minutes over 6 weeks. If you volunteer to participate in the chronic phase of the study, you will be randomized to either the intervention group or the control group. The intervention group will eat 3 apples/day for 6 weeks, while the control group will eat no apples/day for 6 weeks. You will visit the HNRU for 2 separate study visits for 7 hours each, over the 6 weeks. At your study visits (#1 and #2), after your fasting IV blood sample (12 mL), you will be asked to consume a high-fat meal (whipping cream + water) containing 1500 mg of acetaminophen* within 30 minutes. Then, repeated IV blood samples (12 mL each) will be collected every 20 minutes during the first 3 hours, and then each hour for the next 3 hours, for a total of 13 blood samples (156 mL total) during each 7-hour study visit. It is recommended that you avoid acetaminophen-containing products for 48 hours after your study visit to prevent over-consumption. It is also recommended that you avoid alcohol for 48 hours after your study visit to avoid liver damage.

*Acetaminophen* absorption into the blood is an indicator of the rate that food leaves the stomach and enters the small intestine, a process termed gastric emptying. The rate of gastric emptying is related to the rate of dietary glucose and lipid absorption into the blood, and is also related to satiety, the feeling of fullness.

You will be asked to remain seated for the duration of each study visit with only short walks permitted to use the washroom. You will never be left unsupervised during the study. There will be magazines and movies available, and you are invited to bring your laptop, books, etc. from home to pass the time. After each study visit, you will be provided with a lunch consisting of a Subway sub and a juice box. Total time per study visit will be approximately 7 hours.
Check-in Visits

You will be asked to visit the HNRU on 2 additional separate occasions for brief check-in visits: 2 and 4 weeks after commencement of the chronic phase of the study. You do not need to follow any prior instructions, fast, or bring anything to your check-in visits. You will be greeted by the study coordinator who will take your height, weight, waist circumference and blood pressure in private, and you will be asked to complete a questionnaire detailing information about your diet, lifestyle patterns, and any recent health issues. Finally, you will pick-up enough apples to last until your next check-in visit (or not, if in control group). Total time for each check-in visit will be approximately 15 minutes.

The Apples

During the chronic phase of the study, if you are randomized to the intervention group, you will be asked to pick-up 42 Ontario-grown Gala apples every 2 weeks (study visit #1, and check-in visits #1 and #2). The apples must be stored in a refrigerator. The consumption of 3 apples/day is central to the study purpose; therefore, failure to pick-up your apples may result in your withdrawal form the study.

STUDY SAMPLE LABORATORY ANALYSIS

All blood and fecal samples will be labeled with your Participant ID number, never your name or any other identifying information. During both the acute and chronic components of the study, peripheral blood mononuclear cells, a type of immune cell, will be immediately isolated from blood samples taken at study visits #1, #2 and #3, and incubated to assess production of anti/pro-inflammatory mediators before and after the apple intervention. Blood samples will also be processed at the HNRU for analysis of acetaminophen, glucose, insulin, lipid, lipopolysaccharide (a gut-derived inflammatory stimulus), and anti/pro-inflammatory mediator levels, and will be stored indefinitely. Fecal samples will be analyzed for lipid digestion parameters and microbiota populations, and stored indefinitely.

STUDY RESULTS AND PUBLICATION

Results from this study may be published in scientific journals and/or presented at scientific conferences, but will be so as grouped data with no ability to link data back to any individual. Upon withdrawal from the study or study completion, you will be invited to complete a study exit questionnaire where you can indicate if you would like a summary of your individual and overall study results mailed to you. Importantly, results from this study will not be obtained from a licensed medical laboratory and thus should not be used for diagnostic purposes; if you have concerns about your results you are advised to see a physician.
POTENTIAL RISKS AND DISCOMFORTS

There are minimal risks associated with participation in this study.

At each of the 2-3 study visits, you will be asked to provide fasting and post-prandial IV blood samples, which may present some risk of discomfort, bruising, and a very small chance of infection. You are advised to drink water during the 10-12 hour fast prior to the study visit to remain well hydrated. Only a qualified, experienced phlebotomist will obtain IV blood samples, and, to minimize the risk of infection, the area of IV catheter insertion will be swabbed with ethanol and there will be no sharing of supplies between participants. You are encouraged to contact The Apple Study research team if redness or tenderness around the site of insertion persists after you leave the HNRU. Blood sampling may also cause dizziness, headache and/or mild nausea. You will be supervised at all times by a study coordinator and/or a medically trained technician at all visits to the HNRU throughout the study.

Also at each of the 2-3 study visits, you will be asked to consume a high-fat meal (whipping cream + water) containing 1500 mg of acetaminophen in order to measure the rate of gastric emptying. The 1500 mg dose is equivalent to three extra strength Tylenol pills, and is less than half of the maximum recommended daily intake (4000 mg). However, you are encouraged to consult your physician and notify The Apple Study research team if you experience any of the following potential side-effects: wheezing, rash, itching, increased sweating, nausea, vomiting, stomach pain, and loss of appetite. You will also be asked to avoid over-the-counter acetaminophen-containing products for 48 hours prior to your study visits, and for 24 hours after your study visits to avoid over-consumption of acetaminophen (>4000 mg in 24 h), which may lead to liver damage.

2 Extra strength Tylenol:

Medicinal ingredient: Acetaminophen (500 mg/pill)

Non-medicinal ingredients (alphabetical): cellulose, corn starch, hypromellose, magnesium stearate, polyethylene glycol, sodium starch glycolate.

3 Trade names of acetaminophen-containing products to avoid: Alka-Seltzer Plus Liquid Gels®, Dayquil®, Dimetapp®, Excedrin®, Midol®, Nyquil®, Robitussin®, Sinutab®, Sudafed®, Tylenol®, Tylenol Cold®

You will also be asked to provide fecal samples on 2 occasions during the study, which may cause personal discomfort and embarrassment. You will be provided with sample collection materials and clear instructions on how to properly collect and store samples. The research team will ensure that fecal samples are handled in a discrete and professional manner.
The research team will make every effort to ensure your safety and comfort during all visits to
the HNRU. In the unlikely event of a study-related injury, we will engage appropriate
evacuation response services to assist in your care.

POTENTIAL BENEFITS TO PARTICIPANTS AND/OR TO SOCIETY

You will benefit from participating in this study by learning about the relationship between
nutrition and health; with your approval, you will receive a summary of your individual and
overall study results to learn the effect of apple consumption on obesity-related disease risk
factors.

This research will contribute to an understanding of how bioactive-rich foods, such as apples,
can impact obesity-related chronic disease risk, and may lead to an increased consumer
awareness about the health benefits of apples. More generally, the knowledge gained from this
study may contribute to dietary recommendations for individuals to manage their risk of
developing obesity-related chronic disease. This research may also benefit the Ontario apple
industry by providing evidence to support apple health benefits.

COMPENSATION

You will be financially compensated for your participation in this study. You will receive $75 for
completing each study visit, $10 for each check-in visit, and a $50 bonus for completing the
entire study. Therefore, you may receive up to $295 for your participation in the acute +
chronic components of the study, and up to $220 for your participation in only the chronic
component. You will be required to provide your Social Insurance Number for compensation
purposes. The paperwork will be submitted to the University of Guelph Financial Services
department for processing and you should receive compensation within 4-6 weeks. If you
withdraw from the study before its completion, your compensation will be pro-rated
accordingly.

COST OF PARTICIPATION

There is no direct cost for participating in this study. However, you will be responsible for any
costs related to attending your scheduled study visits at the HNRU, such as gas money, public
transportation fees, parking fees, etc.

PARTICIPATION AND WITHDRAWAL

You can choose whether to participate in this study or not. You may refuse to answer any
questions that you do not want to answer and still remain in the study. You are free to
withdraw yourself, your samples and/or your data from the study at any time with no
consequences. Upon withdrawal from the study, you will be invited to complete a study exit
questionnaire where you can indicate if you would like your samples and/or data withdrawn
from the study as well, otherwise all samples and data collected up to the point of your
withdrawal may still be included in the study at the Principal Investigator’s discretion. If information becomes available that may be relevant to your willingness to continue participating in the trial, you will be informed in a timely manner. The Principal Investigator may withdraw you from this research if circumstances arise that warrant doing so.

CONFIDENTIALITY

Every effort will be made to ensure confidentiality of any identifying information that is obtained in connection with this study. You have been assigned a coded study number (Participant ID number), and all coded study documents will stored in a locked filing cabinet in a locked office, and compiled in a password-protected computer file. All coded samples will be stored in the Principal Investigator’s locked laboratory. Results from the study may be published or presented, but will be so as grouped data. The master list linking your name with your study code will locked in the Principal Investigator’s office, and will be shredded after the publication of results. At that time, all coded data will become anonymized, and all anonymized data (hard and electronic copies) and samples will be kept indefinitely for potential future use by the research team, investigating apple health benefits in obese adults. By following these procedures, your confidentiality as a participant will be maintained to the best of our ability.

If requested, direct access to your research records for this study will be granted to study monitors, auditors, the University of Guelph Research Ethics Board, and regulatory authorities for the verification of study procedures and/or data. Your confidentiality as a study participant will not be violated during this process, to the extent permitted by applicable laws and regulations. By signing this written informed consent form you are agreeing to authorize such access.

RIGHTS OF RESEARCH PARTICIPANTS

You do not waive any legal rights by agreeing to take part in this study. This project has been reviewed by the Research Ethics Board for compliance with federal guidelines for research involving human participants. If you have any questions regarding your rights and welfare as a research participant in this study (REB# 16JA013), please contact: Director, Research Ethics; University of Guelph; reb@uoguelph.ca; 519-824-4120 x56606.

Please indicate which components of The Apple Study you are volunteering to participate in.

_____ Acute + chronic apple consumption

_____ If the acute component of the study is at capacity or is complete, I volunteer for only chronic apple consumption instead
_____ Only chronic apple consumption

Please indicate if you allow the research team to use your anonymized data and blood and fecal samples for any future studies. All data and blood and fecal samples will be labeled with your Participant ID number with no direct link to your name or any other identifying information.

_____ Yes

_____ No

SIGNATURE OF RESEARCH PARTICIPANT

I have read the information provided for the study “The Apple Study: The effects of whole apple consumption on risk factors for chronic disease in obese adults” as described herein. My questions have been answered to my satisfaction, and I agree to participate in this study. I have been given a copy of this form.

__________________________________________________________
Participant Name (please print)       Signature       Date

__________________________________________________________
Address: Street, Town/City, Province, Postal Code

SIGNATURE OF WITNESS

__________________________________________________________
Witness Name (please print)       Signature       Date
**FIGURE 1: The Apple Study timeline.** The top timeline shows the weekly activities for participants included in the acute + chronic phases of the study, whereas the bottom timeline shows the weekly activities for participants included in only the chronic phase.

- **Complete 3-day food record**
- **Collect 3 consecutive fecal samples**
- **IV blood sample collection**
- **Chronic phase: eat 3 apples/day or no apples/day**
- **Check-in visit**
**TABLE 1: The Apple Study weekly activities.** Details about the weekly activities for participants included in the acute + chronic, and chronic only phases of the study.

<table>
<thead>
<tr>
<th>Acute + Chronic phase</th>
<th>Week</th>
<th>Chronic only phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Complete a 3-day food record</td>
<td>- 4</td>
<td>• Complete a 3-day food record</td>
</tr>
</tbody>
</table>

**Two-week run-in period**
- Follow dietary guidelines explained in The Apple Study Handbook
- Fecal sample collection: 3 samples over 3 consecutive days
- Complete a 3-day food record

**Study visit 1**
- Complete study visit questionnaire
- Anthropometric measurements
- Fasting blood sample by finger-prick
- Consume high-fat meal, with 3 apples or no apples
- IV blood sample collection: fasting; every 20 min. for 3 h after high-fat meal; every h for next 3 h (6 h total)

**Study visit 2**
- Complete study visit questionnaire
- Anthropometric measurements
- Fasting blood sample by finger-prick
- Consume high-fat meal, with 3 apples or no apples (opposite of study visit 1)
- IV blood sample collection: fasting; every 20 min. for 3 h after high-fat meal; every h for next 3 h (6 h total)

**Check-in visit 1**
- Complete check-in visit questionnaire
- Anthropometric measures
- Pick-up apples

**Check-in visit 1**
- Complete check-in visit questionnaire
- Anthropometric measures
- Pick-up apples
Appendix F. Pre-study Visit Questionnaire for Study 2

The Apple Study

The effects of whole apple consumption on risk factors for chronic metabolic diseases in overweight and obese adults

PRE-STUDY VISIT QUESTIONNAIRE

Study coordinator:

Date: Time:

Body measurements

A study coordinator will measure your:

Height (cm)
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (inches)</td>
<td></td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>1)</td>
</tr>
<tr>
<td></td>
<td>2)</td>
</tr>
</tbody>
</table>

1. How are you feeling today?

2. Are you experiencing any feelings of illness or discomfort other than those from fasting? If **YES**, please describe in this space.

**YES / NO**

3. Have there been any significant changes in your health or medication use since your last visit? If **YES**, please describe in this space.

**YES / NO**

4. In the past 10-12 hours, did you refrain from all food and drink, except water? **YES / NO**

5. In the past 12 hours, how many cups of water did you consume?
6. When was the last time that you consumed water?

7. In the past 48 hours, did you consume any alcohol?  **YES / NO**

8. In the past 48 hours, did you refrain from intense exercise?  **YES / NO**

9. In the past 48 hours, did you refrain from any over-the-counter medications (including acetaminophen-containing products like Tylenol)? If **NO**, please describe in this space.

   **YES / NO**

10. Are you following the dietary guidelines listed in The Apple Study Handbook?

11. Did you experience any problems avoiding/limiting the foods that we asked? If **YES**, please describe in this space.

   **YES / NO**

12. What did you eat for dinner last night? This includes any beverages and desert.

<table>
<thead>
<tr>
<th>Food/beverage</th>
<th>Amount</th>
</tr>
</thead>
</table>

239
Appendix G. Check-in Visit Questionnaire for Study 2

Apple Study

CHECK-IN VISIT QUESTIONNAIRE

<table>
<thead>
<tr>
<th>Study coordinator:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date:</td>
</tr>
<tr>
<td>Time:</td>
</tr>
</tbody>
</table>

Body measurements

A study coordinator will measure your:

<table>
<thead>
<tr>
<th>Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
</tbody>
</table>

| Waist circumference (inches) |
| Blood pressure (mmHg)       |
| 1)                           |
| 2)                           |

1. How are you feeling today?
2. Are you experiencing any feelings of illness or discomfort from following the dietary guidelines? If YES, please describe in this space.

YES / NO

3. Have there been any significant changes in your health or medication use since your last visit? If YES, please describe in this space.

YES / NO

4. Have you participated in any of the following since your last visit?
   
i) Weight training? YES / NO If YES, how often? __________________________

ii) Running/jogging? YES / NO If YES, how often? __________________________

iii) Aerobics? YES / NO If YES, how often? __________________________

iv) Team sports? YES / NO If YES, how often? __________________________

v) Other? YES / NO If YES, please describe in this space.

5. How many hours of sleep do you usually get per night?
6. Do you consume breakfast on a regular basis? | YES / NO

7. How many meals do you eat per day?

8. How many snacks do you eat per day?

9. Please indicate the amount of the following foods that you have consumed per day OR per week over the past 2 weeks. Use the serving size cheat sheet provided to estimate amounts.

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount/day</th>
<th>Amount/week</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fruits</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berries (e.g. blueberries, blackberries, cranberries, strawberries)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood oranges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plums</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pomegranates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red/black cherries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red/black grapes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other purple, red or blue fruit? Describe.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Vegetables

<table>
<thead>
<tr>
<th>Purple vegetables: asparagus, bok choy, carrots, corn, eggplant, olives, potatoes, tomatoes, etc.? Describe.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Red vegetables: cabbage, lettuce, olives, onion, pepper, potatoes, radish, swiss chard, tomatoes, etc.? Describe.</th>
</tr>
</thead>
</table>

## Grains & beans

<table>
<thead>
<tr>
<th>Beans: black or red kidney?</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Rice: black, red or wild?</th>
</tr>
</thead>
</table>

## Other

<table>
<thead>
<tr>
<th>Baked goods made with chocolate? Describe.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Question</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Baked goods made with any of the fruits listed above? Describe.</td>
</tr>
<tr>
<td>Candy with purple, red or blue colorants?</td>
</tr>
<tr>
<td>Cereals with purple, red or blue colorants? Describe.</td>
</tr>
<tr>
<td>Chocolate, or products containing chocolate? Describe.</td>
</tr>
<tr>
<td>Frozen deserts containing any of the fruits listed above? Describe.</td>
</tr>
</tbody>
</table>
Fruit juices containing any of the fruits listed above? Describe.

Products containing any of the fruits listed above? Describe.

**Beverages**

Cider: apple or grape?

Coffee (decaffeinated or caffeinated), of coffee-containing beverages (e.g. latte)? Describe.
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red wine?</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vegetable juices containing any of the vegetables listed above? Describe.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Comments?</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# The Apple Study

## SERVING SIZE CHEAT SHEET

<table>
<thead>
<tr>
<th>Tips on Estimating your Serving Sizes!</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ounces of meat is about the size and thickness of a deck of cards</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>1 cup of cold cereal is about equal to the size of your fist</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>½ c. cooked cereal, rice, pasta is about equal to a small computer mouse</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>1 medium fruit is about equal to 1 baseball</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>1 ounce of cheese is about the size of 4 stacked dice or 2 cheese slices</td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>½ cup of ice cream is about the size of a tennis ball</td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>1 cup of vegetables or mashed potatoes is about the size of your fist. 1 baked potato is also about the size of your fist.</td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
<tr>
<td>1 teaspoon of butter or peanut butter, or oil is about equal to the size of your thumb.</td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Appendix H. Study Exit Questionnaire for Study 2

**Apple Study**

**STUDY EXIT QUESTIONNAIRE**

Thank you for your interest in The Apple Study. The purpose of this questionnaire is to gather more information about your experience in the study; whether you completed all visits or not. Please feel free to NOT answer any questions that you are uncomfortable with answering. All information provided in this questionnaire will be kept confidential. Please feel free to ask a study coordinator any questions at any time. If you have any questions or concerns at any time, please feel free to contact:

<table>
<thead>
<tr>
<th>Danyelle Liddle, MSc</th>
<th>Lindsay Robinson, PhD</th>
<th>Amanda Wright, PhD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lead study coordinator</td>
<td>Principal Investigator</td>
<td>Faculty Co-investigator</td>
</tr>
<tr>
<td>PhD student, HHNS</td>
<td>Associate Professor, HHNS</td>
<td>Associate Professor, HHNS</td>
</tr>
<tr>
<td>Email: <a href="mailto:dliddle@uoguelph.ca">dliddle@uoguelph.ca</a></td>
<td>Email: <a href="mailto:lrobinso@uoguelph.ca">lrobinso@uoguelph.ca</a></td>
<td>Email: <a href="mailto:ajwright@uoguelph.ca">ajwright@uoguelph.ca</a></td>
</tr>
<tr>
<td>Phone: 519-824-4120 x 56967</td>
<td>Phone: 519-824-4120 x 52297</td>
<td>Phone: 519-824-4120 x 54697</td>
</tr>
</tbody>
</table>

1. Are you leaving the study before its completion? If YES, please share your reason(s) with us if you feel comfortable to do so.

2. Are you willing to allow your data already collected to be analyzed as part of the study results? YES / NO

3. Are you willing to allow your blood and fecal samples already collected to be analyzed as part of the study results? YES / NO
4. Do you have any specific feedback about what you enjoyed about being a participant in this study?

5. Do you have any specific feedback about what aspect(s) of being a participant in this study was most difficult?

6. Do you have any specific feedback about what might have improved your experience as a participant in this study?

7. Has participation in this study improved your willingness to incorporate more apples into your daily diet?

8. Do you have any other comments?
Thank you very much for your participation in The Apple Study. Your time, commitment and contributions are highly valuable and appreciated. We look forward to providing you with feedback on your individual study results.