

**Effect of co-consumption of enhanced protein concentration and modified casein:whey ratio milk with cereal on satiety, food intake and glycemic response and the elucidation of mechanisms through *in vitro* digestion**

by

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## ABSTRACT

### **Effect of co-consumption of enhanced protein concentration and modified casein:whey ratio milk with cereal on satiety, food intake and glycemic response and the elucidation of mechanisms through *in vitro* digestion**

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Exploring casein and whey proteins as a dietary intervention for managing Type 2 diabetes is of interest because of their capacity to modulate postprandial blood glucose (BG) and satiety. Yet, whether these proteins varying in concentrations and ratios co-ingested with high-carbohydrate cereal results in different effects, is unknown. The overall objective was to determine the effect of a novel formulation on glycemic and appetite response and to investigate the possible mechanism associated with gastric emptying (by indirect paracetamol concentration) via increased plasma branched-chain amino acids (BCAAs). A randomized, double-blinded, crossover human study was conducted to investigate effects on BG, appetite and subsequent caloric intake by healthy adults (n=32) following co-consumption of breakfast cereal and milk, with either 80:20 (normal) or 40:60 (modified) casein-to-whey protein ratios at normal (3.1wt%) or high protein (9.3wt%) concentration or control (water with permeate). Plasma AAs and paracetamol were further studied from subset participants (n=12). *In vitro* digestion experiments were conducted on the hydrolysis of the breakfasts, gastrointestinal viscosity and bioaccessibilities of sugars and AAs, and comparisons with *in vivo* responses.

Pre-lunch(0-120min) BG concentrations were lowered after milk consumption compared to control. High protein milks (9.3wt%) lowered BG and paracetamol, and plasma AAs increased, compared to 3.1wt% treatments. Also, 40:60 milks increased BCAA, compared to 80:20, but there was a modest effect of ratio on BG and paracetamol. Subsequent food intake was modestly affected by treatments in the

females, but not in overall participants. Post-lunch(120–200min) appetite was reduced by 9.3wt% compared to 3.1wt% protein concentration, as plasma AAs remained elevated. Higher *in vitro* gastrointestinal viscosity was observed for the 9.3wt%80:20 treatment. After simulated duodenal digestion, milk treatments had lower reducing sugars. Some *in vitro-in vivo* trends were observed between gastric viscosity and attenuated paracetamol, reducing sugar and BG, and release of free AAs. However, there was no trend between viscosity and sugar release. These results support that the observed postprandial attenuation of BG and post-lunch appetite were related to higher protein concentration of the dairy beverage, with some impact of higher whey proportion, through the possible mechanism of slowing gastric emptying and some amylolysis reduction.

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## LIST OF ABBREVIATIONS AND SYMBOLS

% = Percent

$\alpha$ -La = Alpha-Lactalbumin

$\beta$ -Lg = Beta-Lactoglobulin

3.1 wt% (80:20) = 3.1 protein concentration (weight %) 80% casein and 20% whey protein ratio

3.1 wt% (40:60) = 3.1 protein concentration (weight %) 40% casein and 60% whey protein ratio

5-HT = 5-Hydroxytryptamine

9.3 wt% (80:20) = 9.3 protein concentration (weight %) 80% casein and 20% whey protein ratio

9.3 wt% (40:60) = 9.3 protein concentration (weight %) 40% casein and 60% whey protein ratio

AAs = Amino Acids

AMPK = 5-AMP-activated protein kinase

ANOVA = Repeated Measures Analysis of Variance

AUC = Area Under the Curve

BCAA = Branched-Chain Amino Acids

BG = Blood Glucose

BMI = Body Mass Index

CCK = Cholecystokinin

Cmax = Peak Concentration

D = Duodenal Phase

DNS = Dinitrosalicylic acid colorimetric method

EAA = Essential Amino Acids

G = Gastric Phase

GC-FID = Gas Chromatography Flame Ionized Detection

GI = Glycemic Index

GL = Glycemic Load

GLP-1 = Glucagon like Peptide -1

GLUT = Glucose Transporter

HCl = Hydrochloric Acid

HGS = Human Gastric Simulator

iAUC = Incremental Area Under the Curve

ISO = International Organization Standardization

K<sub>2</sub>EDTA = Potassium Ethylene Diamine Tetracetic Acid

Kcal = Kilocalories

Kg = Kilogram

MTOR = Mammalian Target of Rapamycin

NaOH = Sodium Hydroxide

NEAA = Nonessential Amino Acids

O = Oral Phase

PYY = Peptide Tyrosine Tyrosine

RCF = Relative Centrifugal Force

REB = University of Guelph Human Research Ethics Board

RPM = Revolutions Per Minute

RDS = Rapidly Digestible Starch

RS = Resistant Starch

SCFA = Short Chain Fatty Acid

SD = Standard Deviation  
SDS = Slowly Digestible Starch  
SDS-PAGE = Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis  
SEM = Standard Error Mean  
SGF = Simulated Gastric Fluid  
SHIME = Simulator of Human Intestinal Microbial Ecosystem  
SIF = Simulated Intestinal Fluid  
SOP = Standard Operating Procedure  
SSF = Simulated Saliva Fluid  
TAA = Total Amino Acids  
tAUC = Total Area Under the Curve  
T1D = Type 1 Diabetes  
T2D = Type 2 Diabetes  
TFEQ = Three Factor Eating Questionnaire  
Tmax = Time of Peak Concentration  
TIM = TNO Gastro-Intestinal Models  
VAS = Visual Analogue Scale  
WPC = Whey Protein Concentrate  
WPI = Whey Protein Isolates  
SMP = Skim Milk Powder

## THESIS PURPOSE AND OBJECTIVES

The overall goal of the present work was to gain a better understanding of the mechanisms involved in attenuation of postprandial glycemic and appetite response from co-consumption of varying dairy protein amounts and casein/whey ratio with breakfast cereal. Specifically, in study #1 postprandial BG, appetite and subsequent meal food intake were measured in an acute meal crossover study where 32 healthy adult participants consumed breakfast cereal along with dairy beverage containing regular or high protein and regular or high whey:casein ratio. Study #2 investigated the food intake, gastric emptying (by paracetamol method) and plasma amino acid concentrations in a subset of participants. Lastly, study #3 adapted an *in vitro* digestion model to examine protein hydrolysis and sugar and amino acid release for the test meals and to investigate digestive mechanisms impacting the observed postprandial response. Furthermore, correlations between *in vivo* and *in vitro* results are included in study #3.

The specific objectives of **study #1** were:

- To formulate a dairy-based beverage with enhanced protein concentration and varying casein to whey protein ratio compared to commercial milk
- To test the effect of novel formulations on postprandial BG, appetite and subsequent food intake when co-consumed with a high-glycemic cereal as part of a breakfast meal in healthy young adults

The specific objectives of **study #2** were:

- To determine increased BCAA between treatments with higher proportion of casein or whey protein
- To investigate the effect of increased BCAA on postprandial BG response, gastric emptying (paracetamol concentration) and subsequent food intake in healthy adults
- To gain a better understanding of the mechanisms involved in attenuation of postprandial glycemic response through correlations between the plasma appearance of meal-derived amino acids (AAs) and gastric emptying

The specific objectives of **study #3** were:

- To investigate the proteolytic profiles of enhanced protein and/or modified protein ratio beverages during simulated gastro-duodenal digestion
- To investigate the relationships between *in vitro* chyme viscosity and gastric emptying and protein and starch hydrolysis with the plasma appearance of nutrients
- To gain a better understanding of the mechanisms involved in attenuation of postprandial glycemic responses by combining and comparing the *in vitro* digestion and human study results

## THESIS HYPOTHESES

- When consumed with a high glycemic breakfast cereal, milks containing modified 40:60 casein to whey protein ratios or enhanced protein concentrations or both, when compared to a protein-free control and commercial milk (3.1 wt%, concentration with 80:20 ratio), would be associated with reductions in postprandial BG, enhanced feelings of satiety, reductions in food intake at a subsequent meal, and reductions in BG following the subsequent meal, in a dose-dependent manner, related to the action of whey protein.
- Consumption of the relatively high (9.3 wt%) protein concentration and modified (40:60) ratio treatment will lead to higher plasma postprandial BCAA concentrations, reflecting whey's higher BCAA content, as well as slower gastric emptying and attenuations of BG.
- Due to different physicochemical properties of dairy proteins, the modified (40:60) protein ratio beverage will have a different proteolytic profile, and thus will decrease gastric viscosity, which would consequently increase hydrolysis of the breakfast meal and increase the bioaccessibility of nutrients during *in vitro* digestion compared to (80:20) protein ratio.
- Treatment trends from *in vitro* viscosity, free total AAs (TAA), D-glucose and reducing sugar are related to *in vivo* data (gastric emptying (paracetamol), plasma TAA and BG concentrations

## CHAPTER 1: LITERATURE REVIEW

### Introduction

Milk with cereal is a traditional and commonly-consumed breakfast meal. Milk is considered rich in macro- and micronutrients. Yet, some breakfast cereal products contain large amounts of starch and sugars and, when consumed, elicit an undesirably high glycemic response, potentially contributing to the development and mismanagement of diabetes. Diabetes is a chronic metabolic disease that is described as the inability of the body to metabolize glucose, resulting in an elevated BG level, with downstream implications. Type 2 Diabetes (T2D) is associated with lifestyle factors leading to the dysfunction to utilize insulin due to the loss of insulin sensitivity in the tissues or the failure of the pancreatic beta cells to produce enough insulin. Furthermore, obesity and overweight are associated risk factors for T2D but can be mediated through increased physical activity and lowered energy consumption. Dietary interventions that reduce appetite and food consumption could be considered a treatment strategy. Current literature suggests that whey proteins and particularly branched-chain amino acids (BCAA) are insulinemic and delay gastric emptying through stimulation of gastric hormones. However, the exact mechanisms of appetite and glycemic response attenuations by dairy protein are unclear.

This literature review will provide a brief overview of the major macronutrients in a bowl of milk and oat-based breakfast cereal, specifically, dairy proteins (casein and whey), starch and soluble fibre ( $\beta$ -glucan), followed by a discussion of digestion and absorption of the mentioned macronutrients in humans. A brief overview of *in vitro* digestion methods as potential replacement and complementary methods for human clinical trials follows. Next, a review of the prevalence of T2D and appropriateness of reduced appetite as a strategic intervention will be

provided. Finally, related research on the contributions of milk proteins as a dietary resource for the prevention and management of T2D and obesity is discussed.

### **1.1. Overview of dairy proteins**

#### *1.1.1. Cow's milk composition*

Bovine (dairy cow's) milk is synthesized in the alveolus of the mammary gland in the udder after the birth of a calf. The composition of dairy cow's milk contains approximately 87.3% water, 3.2% protein, 3.7% fat, and 4.6% carbohydrates (mainly lactose) (Table 1.1) (Fox and McSweeney, 1998). Regarding micronutrients, milk notably contains 0.65% calcium and other minerals such as phosphorus, citrate, magnesium, potassium, sodium, zinc, chlorine, iron, copper and sulfate. Vitamin A, C, D, thiamine, riboflavin are present in milk (Goff, 2015), as well as numerous hormones and enzymes. Within North America, the consumption of liquid milk in 2014 was 258 per capita consumption of the population (million tonnes)(IDF, 2016). The recommended daily intake of milk beverage was 2 servings (500 mL of milk) for adult males and females between the ages of 19 – 50 years old, and the recent food guide lists unsweetened lower fat milk as a healthy drink option (Health Canada, 2011, 2019).

#### *1.1.2. Proteins structure and physical properties*

At the most basic level, protein is formed through the joining of individual AAs by peptide bonding between the amino (-NH<sub>2</sub>) and (-COOH) carboxyl terminal. There are generally four levels of protein structures depending on the type of protein. Briefly, the formation of the peptide chain is referred to as the **primary structure**, and a sequence longer than 50 AA is referred as a polypeptide chain. The **secondary structure** depends on the confirmation of the peptide strands into two main types,  $\alpha$ -helix and  $\beta$ -sheet. Moreover, the **tertiary structure** creates the overall three-dimensional shape of the entire protein molecules. Finally, the **quaternary structure** is made up of protein subunits which are the previously described polypeptide conformations. Protein subunits interact with each other and arrange themselves to

form a larger aggregate protein complex, stabilized by hydrogen bonding, disulfide bridges and salt bridges (Particle Sciences, 2009). Thus, structural conformation of each protein influences the physiochemical properties.

**Table 1. 1** Basic composition, mineral and vitamin contents of cow and human milks

Macronutrient	Bovine <sup>1</sup>	Human <sup>1</sup>
Protein (g)	3.4	1.0
Casein (g)	2.8	0.4
Whey (g)	0.7	0.6
Fat (g)	3.7	3.8
Carbohydrates (g)	4.6	7.0
Lactose (g)	0.7	0.2
Energy (kcal)	66	72
<b>Mineral (mg)</b>		
Sodium	58	15
Chloride	100	60
Potassium	152	55
Magnesium	12	4
Calcium	122	33
Phosphate	119	43
Iron	0.08	0.20
Copper	0.06	0.06
Manganese	0.02	0.07
Selenium	0.96	1.52
Zinc	0.53	0.38
Iodine ( $\mu$ g)	0.021	0.007
<b>Vitamin</b>		
Vitamin A (IU)	126	190
Vitamin D (IU)	2.0	1.4
Thiamine (mg)	0.045	0.017
Riboflavin (mg)	0.16	0.02
Niacin (mg)	0.08	0.17
Pantothenic acid (mg)	0.32	0.20
Vitamin B <sub>6</sub> (mg)	0.042	0.011
Folic acid ( $\mu$ g)	5.0	5.5
Biotin ( $\mu$ g)	2.0	0.4
Vitamin B <sub>12</sub> ( $\mu$ g)	0.357	0.03
Vitamin C (mg)	0.94	5.00

<sup>1</sup>Mean values per 100 g

<sup>2</sup>Modified from (Jensen, 1995; Park et al., 2008)

### 1.1.3. Casein; structure and physical properties

Within cow's milk there two types of proteins: casein and whey, with an approximate

distribution of 80% and 20%, respectively (Table 1.2). In terms of protein structure, casein

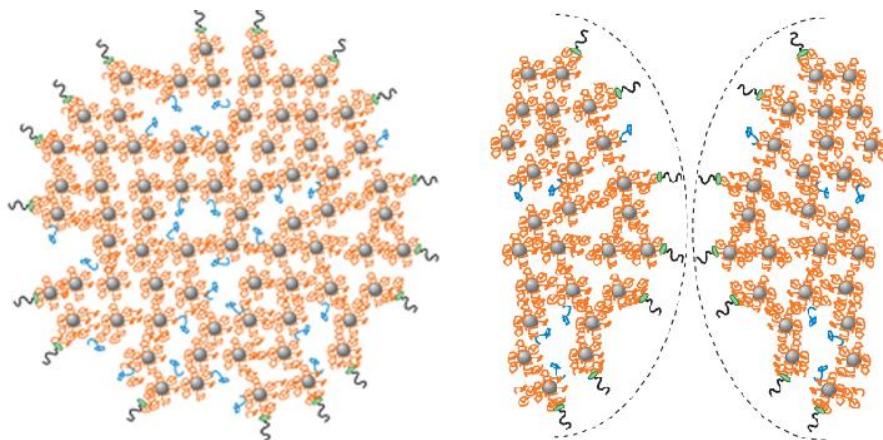
**Table 1. 2** Prevalence of casein and whey protein fractions and physical properties involved forming the secondary structure

Properties	Casein Proteins <sup>1</sup>			Whey Proteins <sup>1</sup>		
	% of total protein	78.8		19.4		
Molecular Weight	$\alpha_{s1}$ - 23,612	$\alpha_{s2}$ - 25,228	$\beta$ - 23,980	$\kappa$ - 19,005	$\alpha$ -La 18,362	$\beta$ -Lg 14,174
Residues	199	207	209	169	162	123
Concentration in milk (g/L)	12-15	3-4	9-11	2-4	3.0	0.7
Phosphate residues	8-9	10-13	4-5	1-2	0	0
$\frac{1}{2}$ Cystine	0	2	0	2	5	8
Sugars	0	0	0	Yes	0	0
Prolyl residue per molecule	17	10	35	20	8	2
Secondary Structure	Low	Low	Low	Low	High	High

<sup>1</sup>Modified from Fox (2009) and Goff (2015)

differs from whey protein due to the high number of proline residues (17% of all residues) which form bends that inhibit the close-packed secondary structure. Also, casein contains no disulfide bridges due to the low content of AAs with sulfur side-chains (methionine (0.8%) and cysteine (1.7%)), resulting in a lack of tertiary structure. There are four types of caseins ( $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$ - and  $\kappa$ -caseins), which in total equate to 80% of all bovine proteins. The distribution of the different types of proteins are presented in Table 1.2. In the presence of calcium phosphates, the four types of caseins form together into an aggregate of 150 – 200 nm called micelles (Dagleish and Corredig, 2012). The casein micelle structure resembles a sphere.  $\alpha_{s1}$ ,  $\alpha_{s2}$  proteins are found throughout the structure and  $\beta$ -caseins are found in the inner parts of the sphere. This arrangement of  $\alpha$ - and  $\beta$ - caseins relates to the micelle hydration properties which are associated with the polarity of the AAs present.  $\beta$ -casein, for example, is the most hydrophobic casein because of its high content of hydrophobic AAs such as proline. The  $\kappa$ -casein is found on the surfaces of the micelles and macropeptides, a hairy layer estimated to be 5 – 10 nm thick, which

extends out of the surface and stabilizes against aggregation through steric repulsion between other aggregates (Horne, 2009; Dalgleish and Corredig, 2012). A widely accepted theory about the micelle structure is an open spherical formation comprised of aggregates of protein around calcium phosphate nanoclusters (Little and Holt, 2004). According to this model, the interactions between caseins and micellar calcium phosphate lead to the formation of ‘nanoclusters’, as caseins allow interactions between several ‘nanoclusters’ via calcium bridges. This phenomenon causes the aggregation of nanoclusters to form an open three-dimensional network, i.e. the casein micelle. Figure 1.1 illustrates the ‘open structure’ model of the casein micelle structure and the hairy layers. Thus, the protein structure and the arrangement of outer hydrophobic AA residues of casein results lower solubility compared to whey protein (Anema, 2009).

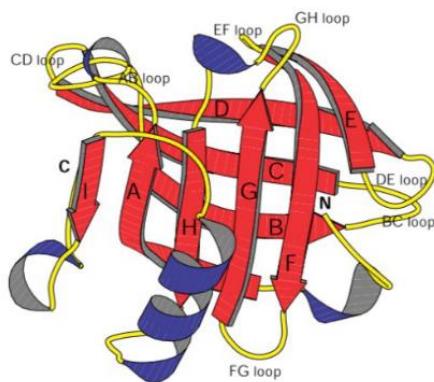


**Figure 1. 1** Schematic section of a micelle showing the  $\alpha_s$ - and  $\beta$ -caseins (orange) attached and linked to the calcium phosphate nanoclusters (grey spheres). The para- $\kappa$ -casein (green) and the caseinomacropeptide chains (black) on the outermost parts of the surface (right). Interacting native micelles are sterically stabilized by macropeptide hairs with the zone of action of the steric effect indicated by dashed lines (left). Reproduced from Dalgleish and Corredig (2012).

#### 1.1.4. Whey; structure and physical properties

Compared to casein, whey proteins exist in the globular quaternary structures due to a strong secondary structure (Fox, 2009). There are two main types of whey proteins,  $\beta$ -lactoglobulins and  $\alpha$ -lactalbumins, along with bovine serum albumin and immunoglobulins.

$\beta$ -lactoglobulin comprises 58% of the whey protein and includes two internal disulfide bonds and one free thiol (sulphydryl) group. The chain conformation of one subunit of  $\beta$ -lactoglobulin is shown in Figure 1.2. The secondary structure of  $\beta$ -lactoglobulin contains 8  $\beta$  barrel (hydrophobic center) and an  $\alpha$ -helix located on the surface of the molecule. The other prevalent protein in whey is  $\alpha$ -lactalbumin, which comprises 13% of the total whey protein. The  $\alpha$ -lactalbumin contains four disulfide linkages and no phosphate group (Kilara, 2008). Unlike casein, whey proteins are more water soluble and have an increased capacity to hold water.



**Figure 1. 2** Ribbon diagram of a single subunit of  $\beta$ -lactoglobulin reproduced from Brownlow et al., (1997). The extended loop between  $\beta$  strands A and B is involved in hydrogen-bonding interactions with the same loop in the other monomer. Disulphide bridges are formed between strand D and close the C-terminus. Strands B, C, D, F and G are joined together by  $\beta$  turns. The three-turn  $\alpha$  helix I is followed by the final  $\beta$  strand, I, which is in the same  $\beta$  sheet as strands, E, F, G, H and the C terminal.

#### 1.1.5. AA classifications

As mentioned earlier, AAs are important for the formation of protein structure. The R side chain is what gives the AAs its distinctive properties and leads the ultimate conformation of the protein. The R side chains confer different chemical, physical and structural properties. There are 20 common AAs that can be classified as acidic, basic or neutral based on its chemical structure (polar or non-polar). Also, AAs can be classified based on nutritional value, generally, either as non-essential (NEAA) or essential (EAA) amino acids. EAA cannot be synthesized in the body and must be obtained through diet and thus can be a potential biomarker (Cynober,

2013). There are 9 EAA (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) and 11 NEAA (alanine, asparagine, cysteine, glutamine, glycine, proline, serine, tyrosine, arginine, aspartic acid, glutamic acid).

#### *1.1.6. Branched-chain amino acids (BCAA)*

Notably, whey protein contains a greater amount of BCAA compared to casein: leucine, isoleucine, and valine. Leucine represents 12% of the AA found in whey protein. BCAA participates in critical metabolic processes such as protein synthesis and energy substrates. Also, it has been suggested that BCAA have a direct and indirect effect on glucose metabolism. BCAA directly stimulate pancreatic insulin release and intracellular signalling the uptake of glucose in muscle and adipose tissues (Bornet et al., 2007). Specifically, leucine is a known regulator of insulinotropic enzymes such as glucagon like protein-1 (GLP-1) (Cynober, 2013). Also, it has been hypothesized that leucine plays a role in reducing dietary intake by inhibiting the activity of 5-AMP-activated protein kinase (AMPK) and activating the mammalian target of rapamycin (mTOR) enzymes (Nishitani et al., 2005).

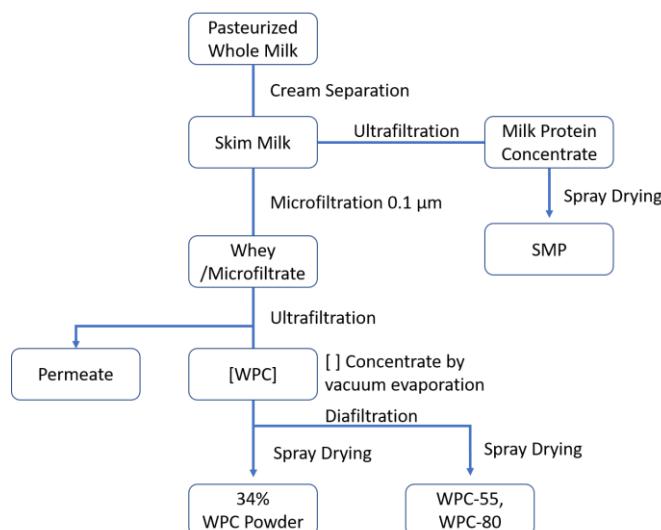
#### *1.1.7. Protein quality*

Proteins are judged based on their ‘quality’, which refers to the digestibility and quantity of EAA to meet the requirement of protein metabolism for human consumption (D’Mello, 2012). The balance of AA and nitrogen is essential for protein synthesis in humans for growth and maintenance of tissues, enzymes, hormones, cells, and immune system. Overall, casein and whey proteins combined provide all the EAA (Fox and McSweeney, 1998).

#### *1.1.8. Production of Protein Powders*

Pasteurized liquid milk can be commercially processed into protein powders (Figure 1.3). First, fat is separated from liquid by centrifugation. Whey for protein powder production is then clarified and filtered using ultrafiltration. Ultrafiltration-diafiltration removes lactose and minerals in the permeate. An agitator can be employed to minimize lactose crystallization. The

final whey protein solution is concentrated by vacuum evaporation and then spray dried at a temperature below 60°C (Kilara, 2008). Whey protein concentrate 35 (WPC 35) has 36.2% crude protein and 46.5% lactose. Similarly, skim milk powder can be produced through vacuum evaporation of skim milk and then spray drying (Kilara, 2008). Production of milk protein concentrates utilizes ultrafiltration-diafiltration to reduce the lactose and mineral content and concentrate the protein. Protein powders are commercially available as isolates or concentrates and can be added to other food products either as additive ingredients or to sold separately as nutritional supplements.



**Figure 1. 3** Simplified schematic of process for the manufacture of permeate, skim milk powder (SMP) and whey protein concentrates (WPC) adapted from Kilara (2008)

## 1.2. Whole grain cereal

### 1.2.1. Definition and recommended intake of whole grain cereal

Breakfast cereal is commonly made from whole grain cereal because of the associated health benefits. As per Health Canada's (2013) definition, a whole grain product contains all three parts of the kernel: bran, endosperm and the germ. Furthermore, whole grain foods should contain all the essential parts and naturally-occurring nutrients of the entire grain seed. If the grain has been processed (such as cracked, crushed, rolled, extruded, and/or cooked), the food product should deliver the same nutrients (vitamin, minerals, antioxidants, enzymes) that are

found in the original grain seed (Whole Grain Council, 2015). Generally, breakfast cereal contains 25% whole grain (Fardet, 2010) and the remainder may contain oil, starches, sugar and food additives. Adult females and males between the ages of 19 – 50 years old are recommended to consume 6-7 and 8 servings, respectively, of grain products. Moreover, it is recommended that at least half of daily grain consumption be whole grain (Health Canada, 2011), as consumption of whole grain has been associated with a lower risk of developing various chronic diseases because it is rich in fibre and bioactive compounds (Fardet, 2010).

#### *1.2.2. Definition of starches*

The endosperm of a whole grain contains the main digestible carbohydrate, starch granule (85%)(Fardet, 2010). Starch is the remainder content after the removal of fibre and protein. It is comprised of a linear chain of glucose polymers of  $\alpha$ -(1,4) linked by anhydroglucose residues called amylose, and a branched  $\alpha$ -(1,6) linkage molecule, amylopectin. Three main categories of starches are often discussed, defined by the measurement of glucose released using glucose oxidase after hydrolysis by amyloses. These are rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starches (RS) (Englyst et al., 1999). RDS and SDS are defined as the amount of glucose released after hydrolysis at the temperature of 37°C for a period of 20 and 120 min, respectively (Singh et al., 2010).

#### *1.2.3. Resistant starches*

RS are those fractions of starches that are not digested in the small intestine because the glucose molecules are bonded by  $\beta$ -1,4 linkages. RS can be calculated as the total starch minus amount of glucose released within 120 min of *in vitro* digestion (RS = Total Starch – (RDS+SDS)) (Englyst et al., 1992). Briefly, RS1 types are physically inaccessible to  $\alpha$ -amylase and  $\alpha$ -amyloglucosidase enzymes because of intact cell walls. RS2 are raw starch granules protected from conformation or structure, while RS3 are physically recrystallized and retrograded amylose. Finally, RS4 are chemically esterified or cross-bonded (Raigond et al.,

2015). After bypassing digestion in the small intestine, RS are fermented in the colon by microflora producing an increased amount of short chain fatty acid (SCFA)(the importance of SCFA is reviewed later) (Raigond et al., 2015).

#### *1.2.4. Fibre*

Fibre is the other main carbohydrate component of whole grain. Dietary fibre is found within the edible parts of the plants such as in the aleurone and pericarp layer of whole grains (Fardet, 2010). Examples of dietary fibres include non-starch polysaccharides, oligosaccharides, and lignin. There are four categories of dietary fibres based on source, chemical composition, digestibility, and beneficial biological and physiological effects. In terms of chemical structure, the monomeric units of dietary fibre are connected by  $\beta$ -linkages. Thus, dietary fibre is immune to human digestive enzymes that are limited to hydrolyzing  $\alpha$ -linkages. As a result, dietary fibre is resistant to digestion and absorption in the small intestine, but can undergo complete or partial fermentation in the large intestine (Fardet, 2010). In addition, dietary fibre is chemically characterized as insoluble or soluble in hot aqueous buffer solution.

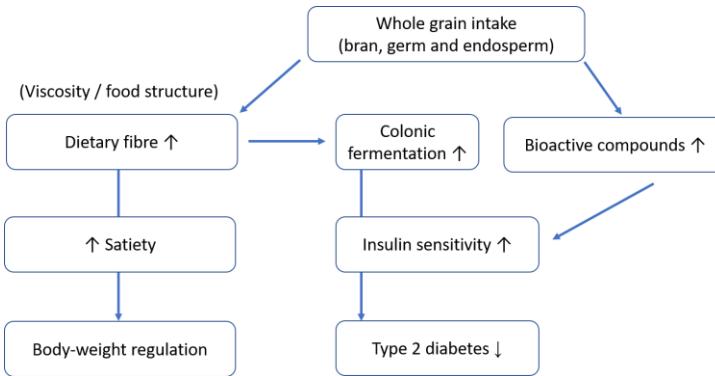
Oats contain about one-third soluble fibres while the rest is insoluble (Fiszman and Varela, 2013). Insoluble fibres such as cellulose, hemicellulose, RS and chitin, do not dissolve into a hot aqueous buffering solution (El Khoury et al., 2012). After consumption, insoluble fibres can improve bowel health by promoting laxation and increasing stool weight (Dikeman and Fahey Jr., 2006). Alternatively, soluble fibre has water holding capacity, and forms a gel during digestion because of physical entanglements among the polysaccharide constituents within the fluid or solution (Dikeman and Fahey Jr., 2006). Examples of soluble fibres include pectin,  $\beta$ -glucan, galactomannan gums, and mucilages. Soluble fibres have been associated with a decrease in plasma insulin and glucose response, which contributes to the prevention and management of diabetes, and obesity (El Khoury et al., 2012).

### *1.2.5. β-glucan and glycemic management*

A flow chart describing the effect of whole grain consumption on T2D and obesity is in

Figure 1.4.  $\beta$  (1-3)(1-4) glucan ( $\beta$ -glucan) is a soluble fibre that can be found in the aleuronic layer of whole grain oat and barley, and is non-digestible due to the interchain of D-glucose monomers joined by  $\beta$ -glycosidic bonds.  $\beta$ -glucan is particularly soluble if the molecule is highly branched and lower in the degree of polymerization (<100). After consumption,  $\beta$ -glucan forms a viscous gel in the gut (Hu et al., 2015). The increased viscosity creates a distension of the gastric antrum that delays gastric emptying and prolongs the transport of nutrients to the small intestine as well as the time course of post-absorptive signals (Dikeman and Fahey Jr., 2006; Fiszman and Varela, 2013; Mäkeläinen et al., 2007). Furthermore, increased viscosity slows enzyme efficacy and glucose diffusion in the small intestine for absorption (Fiszman and Varela, 2013) and provides a feeling of satiety (El Khoury et al., 2012). Digesta viscosity is the physical characteristic that is considered the main mechanism by which  $\beta$ -glucan alters glycemic response (Jenkins et al., 1978) and could lead to the sensation of gastric fullness and increased satiety (Bornet et al., 2007). Thus, a high molecular weight, a linear and unbranched structure, and a higher degree of crosslinking would result in a higher viscosity in the gut. However, food form, dose amount, storage and processing conditions can affect the molecular weight of  $\beta$ -glucan in food products. Furthermore, soluble fibre creates bulk and a high volume by water holding capacity (absorbing large amounts of water) increases gastric distension, which could lead to an increase in the feeling of satiety (El Khoury et al., 2012; Fiszman & Varela, 2013). In the colon, similar to RS, soluble fibres are highly fermentable by microorganisms (El Khoury et al., 2012). Soluble fibres have been suggested as a resource for endogenous production of distal gut hormones (Bornet et al., 2007) and increase insulin-responsive glucose transporter, reduce

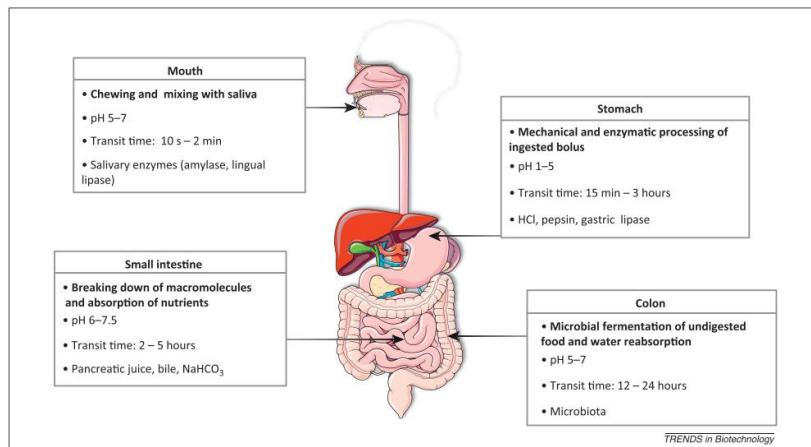
ghrelin via increasing circulating peptide tyrosine tyrosine (PYY) gastric hormone (El Khoury et al., 2012). More information regarding SCFA in the satiety cascade section to follow.



**Figure 1.4** Brief schematic of whole grain intake on body weight and T2D adapted from Fardet (2010)

### 1.3. Digestion and absorption of protein and carbohydrate

The average internal body temperature in human is 37.0°C and is constant throughout digestion in the mouth, stomach, small and large intestines. A general overview of human digestion is provided in Figure 1.5.



**Figure 1.5** Regional specificity of the human gastrointestinal tract (Guerra et al., 2012)

#### 1.3.1. Oral phase

When solid foods (such as cereal) enter the mouth, food particles are broken down by mastication, i.e. the tearing and grinding mechanical action of the teeth. Mastication of solid particles depends on the food composition, volume, texture, the number of chewing cycles, bite force, teeth conditions, degree of hunger and personal chewing habits (Minekus et al., 2014).

Also, a cohesive bolus is formed through lubrication with saliva. Human saliva contains 0.3% of protein (immunoglobulin A,  $\alpha$ -amylase, lysozyme, lactoferrin and mucosal glycoproteins (mucins)) as well as electrolytes such as sodium, potassium, calcium, magnesium, phosphate and bicarbonate. The  $\alpha$ -amylase enzyme present in saliva is active at pH 6.8 and cleaves the hydrated starch molecules in the middle of the interchain at the  $\alpha$ -(1,4) bond (Butterworth et al., 2011). This releases maltose, maltotriose and branched  $\alpha$ -limit dextrans, which are large oligosaccharides. Particles in the final bolus are less than 2 mm and well lubricated and soft enough to be swallowed down the esophagus (Minekus et al., 2014).

### 1.3.2. *Gastric phase*

The stomach is divided into three regions: the proximal stomach (cardia, fundus, and proximal body), the distal stomach (distal body and antrum) and the pylorus. The proximal stomach stores ingested food, initiates contact between bolus and gastric juices (water, hydrochloric acid (HCl), mucus, and inactive proteolytic enzymes), regulates intragastric pressure and propels chyme (Hasler, 2009). The fundus region exhibits two contractile patterns: a slow sustained contraction that lasts up to 6 min and a more rapid phasic contraction that lasts up to 30s. The main role of the slow contraction is to move food from the top to the bottom of the stomach towards the pylorus. The distal body of the stomach is where the grinding of solid food occurs with the mixing of gastric juices. Food is propelled into the antrum distally, and then repelled back into the proximal stomach, producing a mixing and grinding action (Hasler, 2009). The frequency of the contractions of the antrum are approximately  $3 \text{ min}^{-1}$  and the pressure generated by the peristaltic contraction are 300 millibar. These forces crush food particles and decrease the particle size leading to the disintegration of the solid food (Kong and Singh, 2008).

After 5-10 mins following protein and carbohydrate consumption, the intermittent phasic contractions begin. A solid meal usually stays within the stomach for 3 to 4 hr depending on the

volume, composition and physical state of the food. A homogenous solid meal digests faster than a heterogenous meal, but a liquid meal digests the fastest (Minekus et al., 2014). Ingestion of a food may increase gastric pH up to 4.5 – 5.8 and the stomach secretes additional acid to lower and maintain the pH as low as 3.1 (Dressman, 1986). Dairy proteins exhibit a capacity to buffer (resist) the pH change of the surrounding environment due to the amount of acid or base residues in the overall AA profile. The maximum buffering capacity of whey protein is between pH 3 and 4, whereas for casein protein is between pH 5 and 5.5 because of higher amounts of phosphoserine and histidine residues (Srilaorkul et al., 1989; Salaün et al., 2005; Kailasapathy et al., 1996). In addition to stomach contraction, chyme mixed with HCl is secreted by the parietal cells in the stomach. Viscous mucus is secreted from the epithelial lining of the stomach to protect and provide lubrication from peristaltic (continual wave of involuntary contraction along the linings of the stomach) propulsion.

#### *1.3.2.1. Gastric digestion of whole grain cereal*

When the bolus reaches the stomach,  $\alpha$ -amylase is inactivated by stomach acid and becomes well mixed with gastric acid and digestive enzymes. There may be some starch hydrolysis by HCl at pH 2.6 producing smaller polysaccharides (Dona et al., 2010). Notably, soluble fibre begins to absorb water and increase digesta viscosity.

#### *1.3.2.2. Gastric digestion of protein*

In the acidic environment, precursor proteolytic enzymes are converted into active forms. Precursor pepsinogen enzyme is secreted from the chief cells and autocatalytically converted to the active form of pepsin (Table 1.3) (Ganapathy et al., 2009). Briefly, protonation of the cationic AA at pH 3 reveals the active site and removes the amino-terminal region, activating the enzyme (Ganapathy et al., 2009). Pepsin tends to hydrolyze peptide bonds in the middle of the chain (endoproteases) particularly between aromatic AA (phenylalanine, tyrosine, and leucine),

liberating larger peptide fragments. Pepsin enzymes further the casein aggregation initiated by the acidic environment (Goff, 2015).

**Table 1. 3 Characteristics of proteases and peptidases (Ganapathy et al., 2009)**

Precursor form (inactive form)	Activator	Active Form	Site of peptide hydrolysis Endo/Exoproteases
<b>Stomach</b>			
Pepsinogen	Acid pH Autoactivation	Pepsin	Endoprotease Aromatic (PHE, TRP, TYR) and BCAA (LEU)
<b>Duodenum</b>			
Trypsinogen	Enteropeptidase Autoactivation	Trypsin	Endoprotease, Cationic AAs (LYS and ARG)
Chymotrypsinogen	Trypsin	Chymotrypsin	Endoprotease (Carboxyl Terminus), Aromatic AAs (PHE, TYR, TRP)
Proelastase	Trypsin	Elastase	Endoprotease (Carboxyl Terminus), Aliphatic AAs (Gly, Ala, Val, Leu, iLe)
Procarboxypeptidase A	Trypsin	Carboxypeptidase A	Exoproteases (Carboxyl Terminus), Neutral AAs
Procarboxypeptidase B	Trypsin	Carboxypeptidase B	Exoproteases (Carboxyl Terminus), Cationic AAs
<b>Brush Border</b>			
		Aminopeptidase N	Exopeptidase (amino termini) AAs at the terminus is a neutral AAs
		Aminopeptidase A	Exopeptidase (amino termini) AAs at the terminus is an anionic AAs
		Dipeptidylcarboxypeptidase	Exopeptidase that hydrolyzes the peptide bond adjacent to the carboxyl-terminal peptide bond
		Dipeptidylaminopeptidase	Exopeptidase, but it hydrolyzes the peptide bond adjacent to the amino-terminal peptide bone.

Casein and whey protein react differently once they enter the acidic environment. HCl denatures proteins and reveals sites for pepsin enzyme to hydrolyze. Particularly, when casein enters an acidic environment near its isoelectric point of pH 4.8, the solubility of calcium phosphate increases. When this occurs, micelles destabilize, precipitate and  $\kappa$ -casein is released. Also, steric repulsion decreases leading to entropy driven hydrophobic interactions between

protein particles and aggregation (Corredig et al., 2011). However, the aggregate gel network formed is weak due to presence of hydration and some separation by the  $\kappa$ -casein layer (Li and Dalgleish, 2006). Aggregation affects the accessibility of proteolytic enzymes to proteins (Widmaier et al., 2011). Whey protein, on the other hand, remains soluble in the acidic environment leading to a phase separation from the casein aggregates. Specifically,  $\beta$ -lactoglobulin is a compact globular protein that limits the accessibility to enzymes and has a high resistance to pepsin (Guo et al., 1995; Mat et al., 2018).  $\alpha$ -lactalbumin can be hydrolyzed by pepsin into large peptide fragments corresponding to the unfolding of  $\alpha$ -lactalbumin in an acidic environment (Guo et al., 1995). The digestion kinetics of casein and whey have been characterized as “slow” and “fast”, respectively. Whey protein induces a short and earlier increase of plasma AA, whereas casein induces a prolonged but slower and lowered release of AA due to clotting, prolonged gastric digestion and slower gastric emptying (Boirie et al., 1997; Dangin et al., 2001; Hall et al., 2003).

**1.3.2.3. Protein-polysaccharide ( $\beta$ -glucan) interaction during gastric digestion**  
Weak protein-polysaccharide interactions may occur at the gastric stage depending on the pH, ionic strength, ratio of protein to polysaccharide and charge density of protein and polysaccharides (Corredig et al., 2011; Ye, 2008). But generally, high molecular weight  $\beta$ -glucan is considered a non-interacting polysaccharide with casein. This may result in separation of the mixture in two layers: one rich in dairy proteins and the other in polysaccharides. Droplet-like structures form when the volume fraction of the dairy protein is higher than the continuous phase (similar to those found in oil in water emulsions). The continuous phase is the viscous gastric juices with soluble fibre (Corredig et al., 2011). However, there are more complexities, including stomach motility and enzymes, affecting the protein-polysaccharide interactions that need to be elucidated.

#### *1.3.2.4. Gastric emptying of chyme*

Eventually, the meal is gradually emptied into the duodenum through the sphincter-like pylorus. The pylorus selectively empties chyme particles no larger than 2 mm along with gastric juices, but restricts larger particles (Guerra et al., 2012). The kinetics of gastric emptying control the rate of nutrient appearance in the intestine, which affects subsequent digestion in that compartment. With consumption of a solid-liquid mixed meal, phase separation occurs in the stomach. Solid food particles are retained for digestion, but liquid is able to leave the stomach (Hasler, 2009). Different physiological mechanisms have been attributed to the gastric emptying of solid and liquid components. The motor activity of the distal stomach empties the solid components, whereas the emptying of liquids is controlled by the pressure gradient across the gastroduodenal junction (Heading et al., 1976). Also, gastric emptying depends on the nutrient and physical composition of the food content. Large meal volume has been shown to increase the rate of gastric emptying (increased gastric pressure), whereas increased viscosity, energy density and fibre content delay gastric emptying. In addition, nutrient-induced feedback on vagal efferent outflow and endocrine hormones influence antral contraction, contraction of the pylorus, and decrease proximal gastric tone.

#### *1.3.3. Small intestine phase*

Through the pylorus, chyme reaches the small intestine, which has three sections: duodenum, jejunum and ileum. The epithelial lining increases the surface area for absorption by increasing finger-like projections called the villi, and on the villi are microvilli (Ganapathy et al., 2009). Nutrients released during digestion pass through the epithelial cell lining of the small intestine and are transferred to the blood and participate in postprandial metabolism. The intestine moves in peristalsis and segmentation movements, due to the outer longitudinal layer and inner circular layer, to transport the chyme and allow mixing with digestive enzymes.

Peristalsis is characterized as a wave of relaxation followed by a ring of contraction motions that moves chyme along the intestine (Pandol et al., 2009). Also, the segmentation contractions occur in intervening area that divides the lumen into segments across the intestines, slowing down transit to further enhance absorption (Thomas, 2006).

Sodium bicarbonate is released from the pancreas and enters through ducts to the gut lumen. The intraluminal acid is neutralized and forms a protective mucus layer for epithelial cells of the small intestine (Keely et al., 2009). Sodium bicarbonate increases the pH to 6 – 7.5 to balance the acidic environment, depending on the meal type and gastric emptying rate. Furthermore, pancreatic enzymes (proteases, amylases and lipases) enter the duodenum of the small intestine through the hepatopancreatic duct. Also, bile is produced from the liver and stored in the gallbladder until release into the duodenum, for lipid digestion by emulsifying dietary fats into droplets. The bile salts are recycled and reabsorbed in the ileum. Protein and starch are hydrolyzed within the jejunum and the proximal ileum under the influence of pancreatic enzymes.

#### *1.3.3.1. Digestion and absorption of starch and sugars*

Digestion of starch in the small intestine occurs from pancreatic and brush border enzymes containing  $\alpha$ -amylase, mucosal maltase-glucoamylase, sucrase-isomaltase and  $\alpha$ -amyloglucosidases that hydrolyzes the  $\alpha$ -(1,6) bonds (Table 1.3). Thus, starch is hydrolyzed into smaller oligosaccharides, maltose and glucose molecules because both  $\alpha$ -(1,4) and  $\alpha$ -(1,6) bonds are hydrolyzed (Warren et al., 2015). One factor that influences the appearance of BG depends on the amount of starch and disaccharide hydrolysed into monomers. Digestion continues until only monomers can diffuse across the basolateral membrane of enterocytes by facilitated glucose transporters (GLUT). Specifically, glucose transporter-4 (GLUT-4) is regulated by insulin. Insulin binds to receptors on cell membranes and activates GLUT4 to assist

glucose passing in adipose tissue and muscle cells. Also, GLUT1 and GLUT2 transporters are not receptive to insulin. GLUT1 facilitates glucose into liver, muscle and adipose cells and GLUT2 specifically into liver cells.

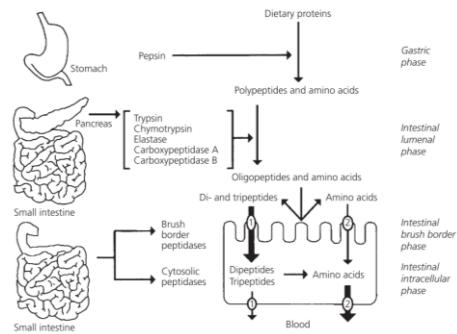
Milk sugar, primarily lactose, a disaccharide, is hydrolyzed by brush border  $\beta$ -galactosidase (lactase) enzyme to yield glucose and galactose. Symptoms of incomplete digestion leads to gastrointestinal discomfort, flatulence, and diarrhea (Sibley, 2009). In older populations and certain ethnicities (e.g. African and Asian), lactose intolerance is common due to reduced lactase gene expression (Fox & McSweeney, 1998), consumers may decide to avoid dairy consumption all together. However, some dairy products include the lactase enzyme, providing conscientious adjustments to consumer's needs.

#### *1.3.3.2. Digestion of protein and absorption of peptides and AAs*

Pepsin is inactivated in response to increased pH in the small intestine. Pancreatic proteases and peptidases are produced in their inactive precursors, then activated by brush border enteropeptidase and autoactivation (Table 1.3). Trypsin, chymotrypsin and elastase are endoproteases that hydrolyze the internal peptide bonds. Trypsin generates cationic AAs at the carboxyl termini, and chymotrypsin and elastase generate neutral AAs at the carboxyl termini. Carboxypeptidase A and B hydrolyze peptide bonds at the carboxyl terminal of the peptide chain. Carboxypeptidase A favours neutral AAs and B favours cationic AAs (Ganapathy et al., 2009). Further digestions of oligopeptides by brush border enzymes produce a mixture of oligopeptides, dipeptides, tripeptides and free AAs (Table 1.3). The absorption of protein occurs mainly in the proximal jejunum and ileum for transport into the bloodstream (Ganapathy et al., 2009).

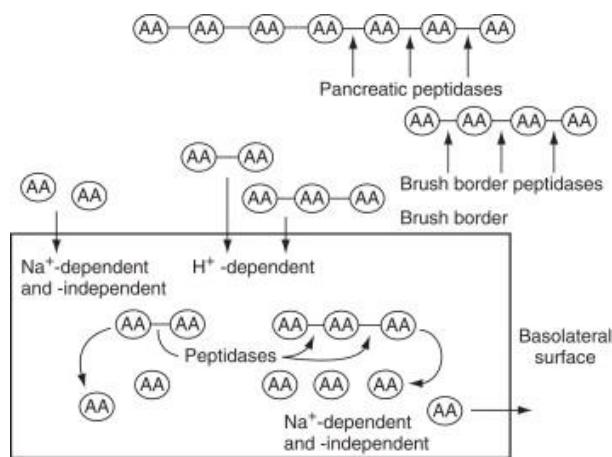
Di- and tripeptides are absorbed into the enterocytes across the brush border membrane mediated by specific active H<sup>+</sup> transporters (Figure 1.6) (Ganapathy et al., 2009), whereas free

AAs are absorbed through passive and active transport against  $\text{Na}^+$  gradient into the portal circulation (Figure 1.7). The liver metabolizes AAs and utilizes the AAs to synthesize circulating



**Figure 1. 6** Digestion of dietary proteins and absorption of digestion products in the gastrointestinal tract (Ganapathy et al., 2009)

proteins, for the production of enzymes and muscle tissues (Cynober, 2013). Notably, BCAA are exempted from hepatic metabolism because of low amounts of mitochondrial transferase transporters in the liver (BCAT2 and BCATm) and have an increased appearance in the blood over non BCAA (Harper et al., 1984). Thus, BCAA could be considered a good biomarker of protein digestion and absorption.



**Figure 1. 7** AAs transport across the brush border barrier (Ganapathy et al., 2009)

#### *1.3.4. Large intestine phase*

The large intestine is divided into 3 sections: cecum, colon, and rectum. The circumferential circular muscular layer contains three overlying longitudinal strips  $120^{\circ}$  apart that store and propel the feces until it reaches the anus. At the end of the colon the relaxation of the sphincters facilitates elimination of the feces. Inside the colon are anaerobic bacteria living in a density of  $10^{11}$  per gram that ferment unabsorbed nutrients. Gut microorganisms are acquired during birth from exposure to the vagina, feces, skin, and breast milk (Ley and Gordon, 2009), although they are altered throughout the human lifecycle due to influences of diet, antibiotic use, and health states (Nolan et al., 2011).

##### *1.3.4.1. Fermentation and absorption in the large intestine*

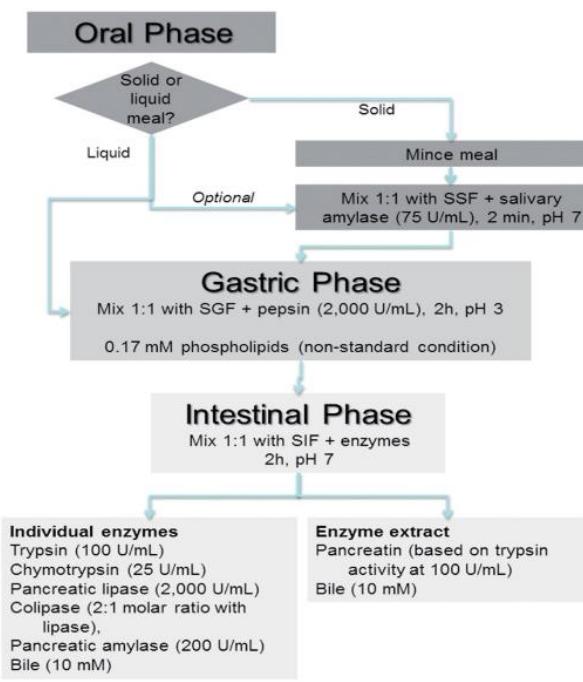
Resistant starch and dietary fibre that are not able to be digested or remaining peptides, free AAs, and sugars in the small intestine, eventually reach the colon for fermentation by living microflora. The end products of fermentation are hydrogen, carbon dioxide, methane and SCFA (acetate, propionate, and butyrate), which are absorbed by colonocytes as a source of energy (Boisen and Eggum, 2011). Specifically, propionate is primarily removed by the liver after entering the portal vein and affects glucose metabolism by increasing hepatic glycolysis and decreasing glucose production in the liver (Fardet, 2010). Furthermore, SCFA production increases secretion of PYY and other regulatory peptides (GLP-1, Cholecystokinin (CCK), 5-hydroxytryptophan (5-HT)) that stimulate the vagal nerves in the gut and intestinal smooth muscle tone, delaying gastric emptying (El Khoury et al., 2012). However, some nutrients (for example AAs and sugars) may bypass fermentation and are excreted in the urine and fecal matter (Cynober, 2013).

### **1.4. Overview of *in vitro* digestion techniques**

#### *1.4.1. Description of *in vitro* digestion*

*In vitro* digestion methods were developed to avoid the difficulties and expenses of human clinical trials and to elucidate underlying mechanisms. Specifically, *in vitro* digestion

experiments allow digesta sampling, which is often too invasive in human studies. Hence, *in vitro* digestion can provide information on nutrient bioaccessibility (Kong & Singh, 2009) and assess changes in physicochemical properties such as water solubility, viscosity and digestibility of food products (Minekus et al., 2014). Moreover, *in vitro* digestion methods can be useful for filling in gaps in literature, by examining mechanisms by which the co-ingestion of foods alters postprandial response. Generally, mimicking digestion *in vitro* generally requires two categories of factors to consider



**Figure 1.8** Overview and flow diagram of a simulated *in vitro* digestion method. SSF, SGF and SIF are simulated salivary fluid, simulated gastric fluid and simulated intestinal fluid, respectively. Enzyme activities are in units per mL of final digestion mixture at each corresponding digestion phase (Minekus et al., 2014; Fang, 2015).

1) mechanical reduction of food products and 2) enzymatic hydrolysis of macromolecules (Guerra et al., 2012). The design takes into consideration of simulated salivary, gastric and intestinal fluids with electrolyte stock solution, minerals, acid, base, and water and enzymes purified from plant, animal and fungal sources (Minekus et al., 2014). A schematic outline of *in*

*vitro* protocol with standardized time, enzyme composition and pH values was proposed by Minekus et al. (2014) as shown in Figure 1.8. Depending on the research question or food product, slightly different digestion model variables may be applied. Thus, the overall strategy of *in vitro* digestion method is to mimic physiological conditions.

#### *1.4.2. General types of digestion models*

*In vitro* digestion models vary depending on the field of research and food products. Broadly speaking, there are generally two approaches to digestive modeling: static and dynamic. In a static model, *in vitro* gastric and intestinal digestion occurs consecutively in a mono chamber. The peristaltic motions are simulated using a shaking water-bath with glass beads (Minekus et al., 2014) or a beaker equipped with rotating paddle (Fang et al., 2016a). This approach is simple and has utility to target particular research questions. Dynamic *in vitro* digestion is bi- or multi-chambered and computer controlled to reproduce the temperature, change pH levels in associated digestive stages and provide gastric emptying and dialysis of digestive products (Guerra et al., 2012). Also, there is a progressive flow of acidification or neutralization, bile, pancreatic juices and digestive enzymes. The Human Gastric Simulator (HGS) is a specific example of an *in vitro* digestion model focused on food degradation in the stomach. Usually made from latex material, the artificial stomach is surrounded by a mechanical system to mimic grinding in amplitude, intensity and frequency (Guerra et al., 2012). Another system is the Simulator of Human Intestinal Microbial Ecosystem (SHIME), which integrates the small and large intestines to study the interaction of food with microbiota (Molly et al., 1993). Yet another *in vitro* example, that provides a dynamic system, is the TIM-1 (TNO gastrointestinal model 1), which includes the stomach and small intestine, and TIM-2 (TNO gastrointestinal model of the colon 2), which adds colon simulation and microbiota. These systems combine the multi-compartmentalization and dynamism as previously stated, as well as

transit time, peristaltic mixing and chyme transport, digestive secretion, passive absorption of water and small molecules through a dialysis system.

#### *1.4.3. Challenges with in vitro digestion models*

Despite the huge potential for *in vitro* digestion model to compliment clinical studies, there are still some limitations to consider. *In vitro* digestion is a simplified approach for answering specific research question, but the simplistic method may be an indefinite reproduction of the more complex *in vivo* conditions (Minekus et al., 2014). The human digestive system is continually sensing and changing shear and grinding forces that have an impact on the digestion kinetics and release of nutrients. Within the field of *in vitro* digestion models, there is huge range of model parameters and this has caused challenges for consensus. For example, there are variations between chemical composition of the digestive solution, source and enzyme concentrations. Also, variations exist in terms of type of mechanical stresses and fluid dynamics (Minekus et al., 2014). Finally, different durations have been used for each phase (oral, gastric and intestinal). The need for standardization was recognized by Minekus et al. (2014), and Woolnough et al. (2010) for carbohydrate digestion, specifically. However, the standardization of *in vitro* digestion parameters has not been achieved yet, although some flexibilities need to be considered for certain food product on a case by case basis.

#### *1.4.4. Usage of in vitro digestion experiments for dairy food products*

There have been various *in vitro* digestion models used for studies of dairy food products. Hur et al. (2011) surveyed 7 *in vitro* digestion models related to dairy foods. Particularly, Almaas et al. (2006) observed the digestion of bovine and caprine milk, using two step human gastric and duodenal digestion. Also, Chatterton et al. (2004) observed the digestion of milk protein ingredients by incubating with human digestive juice for 1 hr. Aside from the studies cited by Hur et al. (2011), Dupont et al. (2010) observed the effect of dairy milk ( $\beta$ -Lg and  $\beta$ -casein) and ovalbumin on phosphatidylcholine concentration in an orbital shaking incubator for 2 hr.

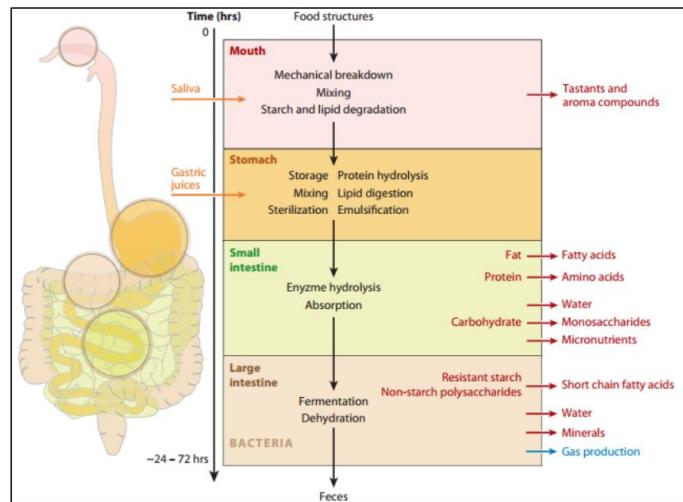
However, there are still gaps in literature as there has not been a study on the disintegration of co-ingestion of varying proteins and limited application to investigate mechanisms involved with the co-ingestion of proteins and carbohydrate-rich foods.

The disintegration of food solids is an important concept for understanding the breakdown of food during simulated digestion and nutrient release (i.e. bioaccessibility). Disintegration kinetics relates the speed of food particle break down into small fragments, and the dissolution of nutrients to dissolve into digestive juices (Kong and Singh, 2008). “Bioaccessibility” is dependent on food composition and matrix (Turgeon and Rioux, 2011; Versantvoort et al., 2005). The first step of disintegration is the mastication during the oral phase for particle size reduction to an average of 2 mm. It is often mimicked by using commercial kitchen utensils such as choppers, grinders, and/or blenders mixed with salivary enzyme for the minimum duration of 2 min (Minekus et al., 2014). Many *in vitro* digestion models omit the oral phase if the physical state of the food product is liquid (Mat et al., 2016; Minekus et al., 2014). Alternatively, some studies includes the oral phase and collect human saliva from participants or employ the use of human participants to create a bolus (Minekus et al., 2014; Woolnough et al., 2010).

For the gastric phase, gastric motility is mimicked by using a stirrer, shaker, or impeller to create a homogenous chyme during exposure to acid and enzymes. The recommended duration for the gastric phase is 2 hr (Minekus et al., 2014). Greater disintegration of protein occurs during the gastric phase depending mainly on the efficiency of pepsin activity (Guo et al., 2014; Ye et al., 2016), resulting in an increase in pH due to the buffering capacity. Thus, the pH has been maintained through increased secretion of HCl (Mat et al., 2016). Disintegration of solid foods could affect the gastric retention and the disintegration kinetics can help to predict the postprandial nutrient release (Kong and Singh, 2008). The determination of free AAs and

glucose helps to estimate the food matrix composition, structural and textural changes and rate of nutrient release (Turgeon and Rioux, 2011). A summarizing scheme of food matrix digestibility (i.e., disintegration, bioaccessibility and bioavailability (the fraction of ingested nutrient that reaches the systemic circulation for utilization)) through gastrointestinal digestion is shown in Figure 1.9.

Within a static digestion model, gastric and intestinal phases occur in the same mono chamber and there is no transit. Some studies simulate gastric emptying by stopping the digestion process and taking out the gastric content at a fixed interval. Solid food disintegration is measured using the sieving method to simulate gastric emptying (Kong and Singh, 2008). In dynamic models, gastric emptying is regulated by a valve that allows the smallest particles to leave the stomach, while the larger ones are retained for longer because of reflux in the top chamber for further digestion.



**Figure 1.9** Schematic of the transit of food structure during digestion reproduced from Norton et al. (2014)

#### 1.4.5. Analysis related to gastric viscosity

Rheology is defined as the study of flow behavior and deformation when matter is subjected to a deforming stress (Steffe, 1996). Rheology is important for the characterization of

visco-elastic solutions but can also be utilized to determine structural conformation of a solution under simulated digestion, whereas the same *in vivo* measurement would be invasive. The use of rheological analysis can help to identify the strength of the polysaccharide-protein network when the viscosity of gastrointestinal fluids is determined. As mentioned above, digestate viscosity has been associated with inductions of satiety. In addition, Rioux and Turgeon (2012) have utilized a rheometer to simulate gastrointestinal motility, calculate matrix disintegration and nutrient bioaccessibility. For example, higher viscosity and delayed matrix disintegration were observed for yogurts with higher casein compared to whey protein ratio, but there was no difference between bioaccessibility of AAs (Rioux and Turgeon, 2012). Despite the potential, one challenge of measuring *in vitro* gastrointestinal viscosity is that phases of human digestion have different shear rates and a standard shear rate has not been specific for *in vitro* research (Dikeman and Fahey Jr., 2006).

#### *1.4.6. Analysis related to AAs*

The ninhydrin colorimetric analysis for primary amino groups was first introduced by Siegfried Ruhemann (1910). The mechanism of reaction entails the reaction of two molecules of ninhydrin for each molecule of AA to form a colored chromophore referred to as “Ruhemann’s purple”, CO<sub>2</sub> and an aldehyde. The typical purple-blue color is produced for most AAs, whereas the color produced by reaction with proline is yellow (Friedman, 2004). The use of the ninhydrin assay of amino groups in protein is not always consistent and does not always correspond to predicted amounts due to slower or incomplete reactions (Friedman, 2004). Yet, successful applications have been documented to quantify the amount of rennet whey added to raw milk (acidic ninhydrin)(Fukuda et al., 2004) and for estimating peptides and free AAs (cadmium chloride)(Doi et al., 1981).

#### *1.4.7. Analysis related to starch digestion*

Studies on *in vitro* carbohydrate digestion were first introduced by Southgate (1969) and the method was furthered by Englyst et al. (1982). The Englyst method uses controlled enzymatic hydrolysis to measure starch fractions in foods and has become the foundation for many *in vitro* digestion models (Englyst et al., 1992). In addition, the log of slope analysis of digestibility curve was developed by Edwards et al. (2014) to model data from the *in vitro* digestion of starch granules using the first-order kinetics. A more recent technical development is the use of a shaking water bath with dialysis to simulate gastric digestion and glucose diffusion (Fabek and Goff, 2015). Following digestion, colorimetric methods have been utilized to measure liberated reducing sugar and glucose concentrations. A commonly accepted method of analysis for measuring reducing sugar is the dinitrosalicylic acid (DNS) colorimetric method (Miller, 1959). The method tests for the presence of free carbonyl group. Another widely accepted method for measuring D-glucose is glucose oxidase-peroxidase (GOPOD) which measures the quantity of D-glucose production after reacting with glucose oxidase-peroxidase.

#### *1.4.8. In vitro-in vivo correlations*

The ability to relate *in vitro* results with *in vivo* response is important for validating *in vitro* models. Anderson et al. (2010) found strong positive associations between *in vitro* starch digestibility determined by the Englyst method with *in vivo* glycemic response and food intake in healthy young men. Similarly, a significant relationship was observed when liberated sugars and oligosaccharides were correlated with *in vivo* BG from a clinical trial (Jenkins et al., 1982). A strong correlation was obtained when *in vitro* results of AA digestibility was used to estimate AA availability (Boisen and Eggum, 2011). Logan et al. (2015) also related apparent viscosities of pectin fibres after simulated digestion with human subjective satiety responses, although, the correlation was not strong. However, despite the useful potential for the role of *in vitro* digestion in predicting *in vivo*, there are still challenges and mixed results. There is a lack of consensus

regarding which forms of measurement are best to use (e.g. AUC, mean values, or specific time points) and standardization between *in vitro* digestion methods. Even further, inconsistency between *in vitro-in vivo* comparisons hinders drawing conclusion from correlation studies.

## **1.5. Type 2 Diabetes (T2D)**

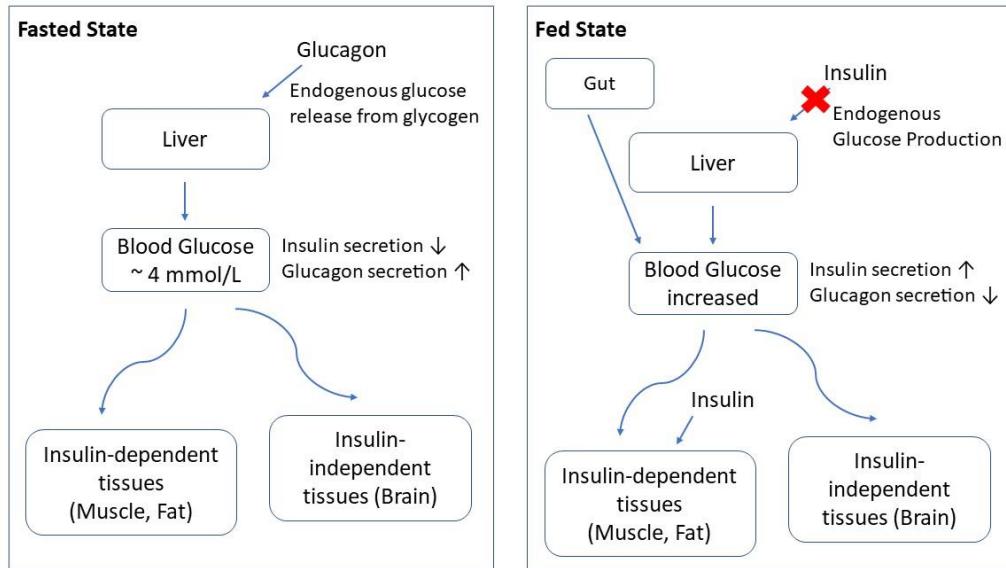
### *1.5.1. The global and Canadian impact of diabetes*

Diabetes is a chronic disease that has serious implications on public health. Globally, it was estimated that 422 million adults were living with diabetes in 2014, and diabetes was directly related to 1.5 million deaths in 2012 (World Health Organization, 2016). Cases of diabetes are expected to grow, worldwide, by 35% by 2040 (International Diabetes Federation, 2015). In Canada (2015), 3.34 million (8.9%) of the Canadian population has been diagnosed with diabetes (Canadian Diabetes Association, 2015). Overall, 90% of the adult diabetes cases are T2D and 10% are T1D and gestational diabetes (Public Health Agency of Canada, 2011). The global financial cost related to diabetes annually is \$827 billion USD (Seuring et al., 2015) while in Canada alone it was estimated to be \$17 billion USD in 2015 (International Diabetes Federation, 2015).

### *1.5.2. Categories of diabetes*

There are three main types of diabetes: gestational (which occurs during pregnancy) and T1D and T2D relating to insulin hormone production. In Type 1 diabetes (T1D, insulin-dependent or childhood-onset diabetes), the pancreas is unable to produce any insulin hormone due to an interaction between genes and/or environmental factors. Symptoms of T1D include excessive urination and thirst because the body is trying to remove excess glucose. Also, there is associated hunger, weight loss and fatigue (World Health Organization, 1999). T2D (non-insulin-dependent or adult-onset diabetes) is the most prevalent form of diabetes and it is defined as the inability of the pancreas to sustain and/or the body cannot utilize insulin. T2D is caused by a combination of genetic disposition, lifestyle, and excessive high caloric food consumption.

### 1.5.3. T2D pathobiology, insulin and pancreatic beta-cells



**Figure 1. 10** Overview of normal glucose homeostasis adapted from Nolan et al. (2011)

When a healthy individual is in a fasted state, glucagon is released from  $\alpha$ -cells while insulin is suppressed, so as to produce endogenous glucose from glycogen in the liver into the blood. Whereas during the fed state,  $\beta$ -cells are sensitive to the glucose concentration in the blood via intracellular metabolism and release insulin, while glucagon is suppressed (Figure 10). With T2D, the complication is usually associated with the following two processes. First, insulin receptors in muscle cells become less sensitive to insulin, leading to inadequate activity of GLUT4 transporters to translocate glucose through the plasma membrane (Nolan et al., 2011). Second, impaired insulin production occurs when the  $\beta$ -cells in the islets of the pancreas are unable to produce enough hormones to regulate the excess amount of BG from overconsumption (International Diabetes Federation, 2015). Both the insulin resistance and deficiency lead to elevated levels of BG. Insulin hormone is required to transport glucose from the bloodstream into critical tissues, such as muscles and the brain, to be used as an energy source (Nolan et al., 2011). Left untreated, excessive glucose (fasting BG  $\geq 7.0$  mmol/L) remain in the blood

(hyperglycemia) and will lead to reduced blood flow (World Health Organization, 2016). This could lead to further complications including cardiovascular issues, kidney failure, vision loss, nerve damage, infection and need for amputations of extremities (World Health Organization, 2016).

#### *1.5.4. Measurement of glycemic response*

Post-prandial glycemic response is the change in BG concentration following the consumption of available carbohydrate containing foods (Health Canada, 2013a). Study of postprandial glycemic response evaluates a person's glycemic control over time, which provides a key understanding of glucose movement for utilization in the body. Also, the information gained on glycemic response after consumption of various foods is beneficial for minimizing hyperglycemia. Glycemic response is commonly measured after consumption of 50 g of available carbohydrates, calculated after the subtraction of dietary fibre from total carbohydrate. White bread or glucose drinks have often been utilized to represent this carbohydrate load. To obtain plasma glucose, generally, venous forearm or capillary fingerprick is used instead of the invasive procedure of obtaining arterial blood. Capillary blood is expected to be more consistent but at a greater glucose concentration than venous glucose measurement (Brouns et al., 2005). Blood sampling time points are usually between 15 to 30 min intervals. Particularly, for healthy participants, Brouns et al. (2005) recommends sampling in the fasted state and at 15, 30, 45, 60, 90 and 120 min after consumption of the test meal. The glycemic index (GI) is a classification system that was developed for identifying foods which are favourable for maintaining a low glycemic response (Wolever & Jenkins, 1986). It was introduced to assess glycemic responses of different foods based on the ingestion of a standard amount of available carbohydrate. The GI is a tool that rates post-consumption of a single carbohydrates-rich ingredient on the elevation of glycemic response, between a scale of 0 to 100 (Wolever et al., 1991). There is a great interest

in targeting attenuation of glycemic response through different dietary strategies, either through increasing consumption of low GI carbohydrates or by identifying other food ingredients or technologies that can be applied to modulate glycemic response when co-consumed in a carbohydrate-rich meal.

## **1.6. Satiety**

### *1.6.1. Definitions, relationship with T2D and weight management*

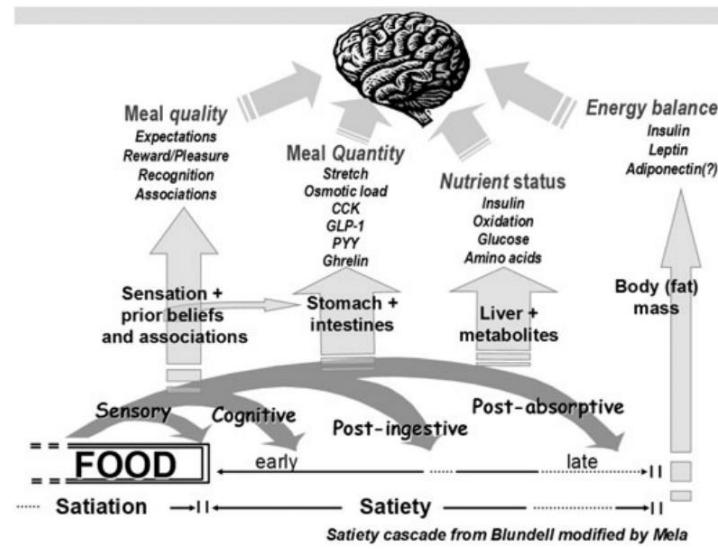
Ingesting foods high in fat and sugar accompanied by physical inactivity lead to an accumulation of excess body weight due to increased adipose tissue, which is an associated risk factor for T2D (International Diabetes Federation, 2015). Weight gain occurs due to an excess intake of energy, which leads to an increase in adipose tissue stores. Increased adipose in visceral tissues can cause hormonal dysregulation, inflammation and increased secretion of inflammatory cytokines, leading to stress and damage to multiple tissues. In addition, chronic glucose toxicity from frequent overconsumption elevates BG and could cause irreversible damage to pancreatic  $\alpha$  and  $\beta$  cells, leading to impaired glucagon and insulin production and secretion.

‘Overweight’ and ‘obesity’ are two categories of excess body weight defined by higher waist circumferences and body mass index (BMI). BMI is calculated by dividing the weight (kg) by a person’s height squared ( $m^2$ ). A person over the age of 18 years with a  $BMI \geq 25 \text{ kg/m}^2$  is considered overweight and  $\geq 30 \text{ kg/m}^2$  is considered obese (Vazquez et al., 2007). Waist size that is greater than 34 inches for women or greater than 40 inches for men are also considered to be normal upper limits (Katz and Friedman, 2008). Reductions in food intake can be supported by reducing feelings of appetite, although the underlying causes of obesity/overweight and overeating, generally, involve extremely complex psychosocial factors (Mukherjee et al., 2017) which are beyond the scope of this thesis. Generally, appetite refers to the desire to eat food and the opposite of satiety. ‘Satiety’ is defined as the feeling of fullness and inhibition of hunger that occurs after food consumption (Blundell and Halford, 1994). Also ‘satiation’ is defined as the

process that leads to the termination of eating. Satiation is important because it controls meal portion size and can prevent overconsumption even if food is readily available (Blundell et al., 2010). Thus, increased feeling of fullness along with increased physical activity is an effective strategy for weight management and lowers risk for developing T2D.

### *1.6.2. Satiety cascade*

The ‘Satiety Cascade’ from Blundell et al. (2010) provides a framework for the cephalic phase responses from sensing food to after ingestion (Figure 11). To simplify, there are four stages of modulating satiety and satiation: sensory, cognitive, post-ingestive, and post-absorptive. Briefly, food intake is anticipated and the stimulation from smell and visual cues increase gastric acids and digestive enzymes production. The hedonic ‘pleasantness’ of the foods increase or reduce the intake of foods. While eating, cognitive signals provide early satiation due to the meal size habit. Also, post-ingestive satiety signals travel from the gastrointestinal tract by gastric hormones (CCK, PYY, GLP-1 and ghrelin) to cross the blood-brain barrier and directly transmit gastric emptying inhibition signals (Bellisle, 2008). Many of the gut peptides act on gastrointestinal receptors that relay messages to the brain via the afferent vagus nerve. Furthermore, CCK, PYY and GLP-1 are hormones that directly transmit signals to inhibit gut motility, slow gastric emptying and decreases appetite (Luhovyy et al., 2007; Akhavan et al., 2014). Ghrelin is the only known orexigenic hormone in the gut and is an appetite stimulant. Ghrelin is at peak concentration immediately prior to food consumption and then declines quickly after the meal (Luhovyy et al., 2007). Post-absorptive phase rises after the nutrients have crossed the intestinal wall and into the systemic circulation and maintain satiety as the brain is informed about the amount of nutrient ingested and provides feedback to stop food intake. Although the cascade is presented in a linear manner, physiological systems and their relationship to each other are more complicated.



**Figure 1.11** The Satiety Cascade (Bellisle et al., 2008)

### 1.6.3. Factors affecting satiety

Previous research has indicated several intrinsic and extrinsic factors that modulate satiety. Identical energy products may not be associated with equal satiation effect as nutrient content seems to have a stronger association with reduced food intake. Protein is considered more satiating than carbohydrates (Veldhorst et al., 2008) due to the longer inhibition of ghrelin after ingestion of protein (milk, soy and gluten protein) than after carbohydrate ingestion (Bowen et al., 2006). Also, physical properties of the meal are associated with feelings of fullness. Higher food volume, energy density and increased viscosity have been associated with greater satiation than lower volume, energy density and low-viscous meal (Bellisle, 2008). In regards to extrinsic factors, social cues, environmental settings, psychological, palatability (how pleasant the food is), and economic cost will influence food intake (Katz and Friedman, 2008).

Meal timing is another factor that affects satiety. Awareness of time (e.g. time of day) can influence meal initiation and termination related to habitual eating patterns (Blundell et al., 2010). Eating at a certain time of day can affect the total consumption for the rest of the day. Particularly, the morning breakfast meal is important because intake in the morning is more

satiating and can reduce the total food consumption for the day compared to late night eating, which tends to lack satiating value and can result in greater overall energy intake (de Castro, 2004). In short-term satiety studies, timing influences the relationship from the initial ingestion of the test meal and subsequent food intake, also referred to as the “second meal effect” (Blundell et al., 1996). Specifically, there is potential for the test meal to elicit a sensation of fullness that can delay the onset and/or lower food intake at the subsequent meal. However, any effect of the preload can diminish after a certain duration (meal interval) and can influence the outcome of the subsequent food intake. Between different research studies, meal interval varied between < 30 min to hours, depending on the research question. Thus, there is great interest in finding a breakfast-related food product that can suppress the feeling of hunger prior to and following the next meal.

#### *1.6.4. Satiety and satiation measurements*

Satiation and satiety are assessed in two different ways. Feelings of satiety are usually measured by a self-reporting questionnaire. Since satiety measurement is subjective for each person, the assessment method needs to be reliable, easy to use and interpretable. Typically, a visual analogue scale (VAS), a 100 or 150 mm line scale with anchors for ranges on each end, is employed. There are four questions: 1) How hungry are you? (Not at all – Extremely); 2) How full are you? (Not at all – Extremely); 3) How strong is your desire to eat? (Very weak – Very strong); 4) How much do you think you could eat right now? (Nothing at all – A very large amount) (Blundell et al., 2010). A sample of a VAS scale is shown in Appendix G: Study Handbook. Thus, with the use of these four scales, the mean appetite score can be calculated. For the purposes of this thesis, ‘appetite’ is defined as the average score of increase in hunger, desire to eat, prospective consumption and a decrease in fullness (Kung et al., 2018). The method for assessing satiation is by measuring food intake, usually a cover buffet style (*ad libitum*) meal of

one or a variety of food products. Consideration for choice of food product is based on palatability and acceptability to the participant, although it has been suggested that palatability only affects satiation but not satiety (De Graaf, 1999). Meal size is another factor that determines satiety and *ad libitum* consumption and a set of factors including the time of day (or night), the sensory characteristics of the food presented (palatability, variety), and food restraint. There is some report of significant correlations between the size of a meal and the pre-prandial or post-prandial intervals in some human studies (Bellisle, 2008). The timing of the meal interval between breakfast and lunch is also influential for energy intake. There are inconsistencies between the reporting of the feeling of satiety and with energy intake at the subsequent meal (Blundell et al., 2010).

#### *1.6.5. Summary of relationship between diabetes and body weight*

T2D is associated with lifestyle factors leading to a dysfunction to properly manage BG. One strategic method to lower the risk of T2D is through body weight management. Weight management can be achieved through lifestyle modulation by increasing physical activity along with reducing appetite limiting caloric consumption. Furthermore, a dietary intervention that can be co-ingested with a carbohydrate-rich meal and attenuate glycemic response and appetite, could strategically assist in managing glycemia, with benefits beyond weight management.

### **1.7. Dairy protein in modulating glycemic response and satiety**

#### *1.7.1. Current statuses of satiety and postprandial glycemia health claims*

Currently, there are no Canadian health claims associated with dairy proteins, while the only health claim associated with β-glucan is reducing cholesterol, a risk factor for heart disease. However, there are two guidance documents “Satiety Health Claims on Food” and “Food Health Claims Related to Post-Prandial Glycaemia” from Health Canada (2012, 2013a) to set the criteria for the submission of scientific evidence for function claims on foods. The following is a review of associated dairy protein-related satiety and postprandial glycemia human clinical research.

### *1.7.2. Dietary intervention studies of varying protein source*

The glucostatic theory suggests an association between the rise of BG with reducing appetite and energy intake (Bornet et al., 2007; Mayer, 1953). Thus, satiety and glycemia studies are often conducted together to elucidate a relationship. Our current understanding of protein-induced satiety and glycemic response suggests that a protein-enriched diet is known as the most satiating macronutrient compared to carbohydrate and fat (Anderson and Moore, 2004). However, the source of protein may be a possible determining factor in suppressing ghrelin and hunger response (Anderson et al., 2011; Veldhorst et al., 2008). In a 2004 study, energy intake was compared between 45-50 g isolates of whey, soy, egg albumen or 400 mL water (control) in healthy men (Anderson et al., 2004). Whey and soy protein, but not egg albumen, suppressed pizza lunch intake at 1 hr after treatment ingestion. Consistent suppression of pizza intake after 2 hr was observed after consumption of whey protein compared to the control. More recently, Law et al. (2017a) compared the subjective appetite, postprandial glucose and insulin between 250 mL of dairy (1% fat milk and yogurt) and non-dairy (almond, soy and water) beverages along with breakfast cereal (Honey Nut Cheerios) in healthy young adults. Consumption of milk and yogurt led to the highest insulin concentration. Milk and soy reduced postprandial glucose compared to the control. Food intake was lower after milk compared with all treatments except yogurt beverage. Also, all beverages increased satiety compared to water with cereal. Overall, there seems to some evidence of reduced appetite and glycemic response by dairy based proteins.

### *1.7.3. Dietary intervention studies of dairy proteins*

Even within dairy proteins, there seems to be varying effect on satiety and glycemia related to the difference in physical characteristic between whey and casein. The “fast” digestion kinetic of whey protein, early rise in blood concentration and AA composition may be accompanied by an early suppression of appetite, compared to casein (Lambers et al., 2013; Boirie et al., 1997; Hall et al., 2003). This relationship between AAs and appetite was first

termed the Aminostatic theory by Mellinkoff et al. (1956). In a 2003 study, Hall et al. further demonstrated that casein and whey proteins exert different effects on appetite, and CCK and GLP-1 release. More descriptively, AA concentration peaks rapidly, but for a short duration (between 40 min to 2 hr), after consumption of whey protein. Casein, in contrast, sustains a plateau for 7 hr (Anderson et al., 2011). The stimulation of gastric hormone release (increased GLP-1, CCK, insulin and decreased ghrelin), in particular, may be due to the higher composition of BCAA found in whey compared to casein protein, as these have been associated with gastric hormones (Aldrich et al., 2011). Nilsson et al. (2004) evaluated the effect of animal and vegetable protein sources on glycemia and gastric hormones in healthy males and females. Milk powder and whey had lower postprandial glucose AUC than the bread control. Whey resulted in higher insulin AUC and pronounced leucine, valine and isoleucine response among other AAs. Also, there was a correlation between postprandial insulin response and leucine, valine, lysine and isoleucine. However, there was no GLP-1 difference between test meals (Nilsson et al., 2004). In addition, increasing dairy protein amount and BCAA seems to have a dose-dependent effect on satiety and glycemic management. Veldhorst et al. (2009) observed that a higher protein dose in a breakfast containing 25% of energy from casein was more satiating and lowered glucose and insulin compared to 10% of energy breakfast in healthy participants. These results were accompanied by a prolonged elevation of BCAA and TAA concentrations. However, there was no difference in GLP-1 and ghrelin or a reduction in subsequent energy intake. In another study, 10 – 40 g of whey protein in 300 mL of water reduced post-lunch BG and insulin AUC in a dose-dependent manner in healthy males and females. BCAA seems to be stimulating insulin release and secretion (Akhavan et al., 2010). Thus, there is some evidence of appetite and energy intake suppression and stimulation of gastric hormones elicited by BCAA release, but the results remain inconsistent.

#### *1.7.4. Dietary intervention of dairy proteins on gastric emptying*

The mechanism underlying reductions in glycemic response and enhanced satiety may be due to hormonal feedback related to delayed gastric emptying (Ganapathy et al., 2009). Gastric emptying rate can be assessed through scintigraphy, orocaecal transit time, magnetic resonance imaging (MRI) and more commonly, the indirect paracetamol absorption technique for liquids (Willems et al., 2001). To conduct the test, paracetamol (acetaminophen) (1000 to 1500 mg) is orally administered. Since paracetamol is poorly absorbed by the stomach but is rapidly absorbed in the duodenum, the rate-limiting step in the appearance of paracetamol in the blood is measured (Willems et al., 2001) and the rate of appearance of paracetamol in the blood correlates with emptying of the stomach's liquid contents. Regarding dairy proteins, whey in particular seems to promptly delay gastric emptying due to the early peak in AA concentration. Akhavan et al. (2014) observed that glucose attenuation was not only a response to insulin secretion, but perhaps delayed gastric emptying associated with the influence of GLP-1 and PYY. In a 2014 study, 10 and 20 g of whey protein, or glucose with 300 mL water (control) were compared in healthy males. Whey protein delayed pre-lunch gastric emptying compared to 10 g of glucose and the control. Also, whey protein lowered post-lunch insulin, but increased GLP-1 and PYY (Akhavan et al., 2014). In another study, Gunnerud et al. (2012) compared the consumption of different milk meals with white bread (control) in healthy male and female participants. Higher whey meal products (human milk and reconstituted whey) resulted in earlier increase of AAs and GLP-1 (hormone that delays gastric emptying), compared to normal milk and reconstituted casein. However, there are inconsistent results regarding the effect of protein on gastric emptying. Calbet and Holst (2004) measured the gastric volume and AA absorption between 60 g of whey or casein in water in healthy males. They observed a slower absorption of AA by casein, but similar GLP-1 and PYY responses to both proteins. They concluded that gastric

emptying rates and GLP-1 and PYY responses are independent of AA composition differences. Also, Hoefle et al. (2015) did not observe any difference in gastric emptying, although there was a reduction in postprandial glucose and a peak in insulin by 50 g of whey and casein co-ingested with maltodextrin<sup>19</sup> (MD19) compared to water with MD19 (control). Thus, it remains unclear if protein and BCAA composition have a direct effect on the gastric emptying rate through gastric hormones.

#### *1.7.5. Dietary intervention studies of modified dairy protein ratio*

Currently, there are limited studies that explore the co-consumption of casein and whey protein and the modification of protein ratio. Of the most relevant, Doyon et al. (2015) studied the modification of casein to whey ratio and enrichment with dietary fibre in a yogurt snack (isoproteinemic at approximately 4.5 g) in healthy men. High whey yogurt (1.5 casein:1 whey) lowered energy intake (-812 kJ) compared to the control yogurt (2.8 casein:1 whey), but there was no impact on appetite or BG.

#### *1.7.6. Summary of clinical studies*

In summary, human clinical studies seem to suggest that the effect of dairy protein on the attenuation of BG is due to the secretion of insulin and delayed gastric emptying. In addition, delayed gastric emptying seems to enhance satiety and lower food intake. However, the exact mechanism remains unclear. There are inconsistent treatment differences when comparing the effects of whey versus casein protein. Furthermore, it is unclear if BCAA is the underlying nutrient behind the effect of whey protein on incretin and gastric hormones.

### **1.8. Conclusion**

Dairy products, particularly their protein components, seem to attenuate glycemic response and reduce appetite. Thus, increasing dairy protein consumption can be a potential dietary intervention for the prevention and management of T2D. A survey of literature suggests that whey protein as the “fast” digested protein leads to an early rise of AA and stimulation of

incretin and gastric hormones. Yet, there are still gaps in literature that need to be investigated. There are limited studies that compare differences in the effect of casein and whey on glycemia and appetite management. Often treatment is tested with only one form of protein isolate/concentrate powder combined with water. This is unrealistic to a meal matrix and is not representative of commonly-consumed dairy products that usually include both types of protein. Also, there needs to be more investigation to determine if the mechanism of action is dependent on amino acid (BCAA) composition, and nutrients that induce gastric emptying by gastric hormone feedback. Furthermore, there are other mechanisms that affect the rate of gastric emptying that have not been explored. Thus, more research on the digestion kinetics from co-ingestion of casein and whey proteins in a meal form and elucidation of mechanisms is necessary.

Understanding more about the mechanism behind co-ingestion of proteins in a meal can assist in the formulation of food products that are more effective in reducing BG and appetite. For instance, the digestion kinetics between whey and casein as “fast” and “slow” is known. Thus, we can modify the protein concentration and ratio to see if we can affect glycemic or satiety responses. In conclusion, protein is a macronutrient that affects many functions in the body, including digestive handling and postprandial response. Further exploration of the mechanisms involved can lead to the development of functional ingredients to enhance existing food products for prevention and management of metabolic disease risk, including overweight/obesity and T2D.

## **CHAPTER 2: EFFECT OF MILK PROTEIN INTAKE AND CASEIN-TO-WHEY RATIO IN BREAKFAST MEALS ON THE POSTPRANDIAL GLUCOSE, SATIETY RATINGS, AND SUBSEQUENT MEAL INTAKE**

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*B. Kung was the principal author for the preparation and writing of the manuscript and major role in the experimental design.*

### **2.1. ABSTRACT**

Whey and casein proteins differentially affect postprandial BG and satiety mechanisms, with relevance for T2D and obesity. Therefore, the purpose of this work was to investigate the effect of the casein-to-whey protein ratio and total protein concentration of milks consumed with cereal on postprandial BG, appetite ratings, and subsequent food intake in a randomized, controlled, double-blinded study with healthy young adults ( $n = 32$ ,  $23.4 \pm 3.1$  yr, body mass index =  $22.2 \pm 2.5$  kg/m<sup>2</sup>). Fasted participants consumed milk (250 mL) with either 80:20 or 40:60 (modified) casein-to-whey protein ratios at commercially normal (3.1 wt%) or high protein (9.3 wt%) concentration, or control (water with whey permeate), each along with 2 servings of oat-based breakfast cereal. BG concentrations were determined from finger prick blood samples and appetite was assessed using visual analog scales. Participants consumed a measured *ad libitum* pizza lunch at 120 min and BG determination and appetite assessment continued following the lunch meal (140–200 min) to observe the second meal effect. Pre-lunch (0–120 min) incremental area under the curve (iAUC) and mean change from baseline BG were reduced with consumption of all milk treatments relative to control. However, we found no differences

between all treatments on pre-lunch appetite change from baseline and total area under the curve (tAUC) or lunch meal food intake. In terms of protein concentration results, high protein (9.3 wt%) treatments contrasted to normal protein (3.1 wt%) treatments lowered BG change from baseline and iAUC, and post-lunch appetite change from baseline and tAUC. Protein ratio showed a modest effect in that modified (40:60) protein ratio lowered pre-lunch BG change from baseline but not iAUC, and normal (80:20) protein ratio lowered pre-lunch appetite change from baseline but not tAUC. Therefore, high carbohydrate breakfast meals with increased protein concentration (9.3 wt%) could be a dietary strategy for the attenuation of BG and improved satiety ratings after the second meal.

## **2.2. INTRODUCTION**

Metabolic diseases are on the rise globally, with T2D and obesity as leading concerns in human health. Obesity is now recognized as a risk factor for T2D and can occur concurrently (Eckel et al., 2005). Thus, there is impetus to develop dietary strategies for the risk reduction and management of obesity and diabetes to empower consumers to improve their personal health. The role of the breakfast meal and composition has received relatively little attention beyond the high glycemic index attributed to many high-carbohydrate foods consumed at breakfast. However, these foods are often consumed with a source of protein, including dairy and eggs, which modify the postprandial glycemic response. Frequent consumption of cow milk and dairy products has been associated with lower risk of obesity and T2D (Anderson et al., 2011). Meal studies have specifically shown that milk consumed with a high-glycemic, ready-to-eat breakfast cereal consumed by young adults (Law et al., 2017b) or cheese and yogurt consumed with toast and jam by older adults (Law et al., 2017a) markedly lower postprandial glycemia compared with the carbohydrate alone.

The lower postprandial glycemia occurring when dairy is consumed with carbohydrates may be explained by dairy casein and whey proteins, which are present in an 80:20 ratio and have different AA compositions, physical structures, and physiological properties. Casein is higher in methionine, phenylalanine, proline, and histidine; whey proteins have higher amounts of lysine, threonine, tryptophan, leucine, and isoleucine. Based on the rise of their plasma AA concentrations rapidly digested proteins and found to pass relatively quickly through the stomach. In contrast, caseins are considered to be slowly digested (Dangin et al., 2002; Hall et al., 2003) due to forming clotted casein aggregates that delay gastric emptying (Boirie et al., 1997). Consumption of whey protein increases early feelings of satiety and reduces food intake, whereas the effect of casein occurs later (Anderson et al., 2004). For example, consumption of 45 to 50 g of whey protein decreased food intake more than casein at a meal interval of 30 to 90 min (Anderson and Moore, 2004), but casein consumption reduced food intake more than whey protein at 180 min (Anderson et al., 2011). Differences in the digestion kinetics of whey and casein proteins facilitate the stimulation of gastric hormones that delay gastric emptying, thus increasing feelings of fullness and attenuation of food particle breakdown and release in the small intestine. Relative to whey, casein digests and releases AA slowly, leading to a delayed stimulation of gastric hormones (Benelam, 2009).

Although the physiological characteristics of whey and casein are well described, the physiological significance of their ratio and concentration in milk are not. Of note, human milk has a ratio of casein to whey approximating 40:60 and a protein content of 1 wt% (Fox and McSweeney, 1998). Current infant milk formulas often approximate this ratio (Klein et al., 2002), but are higher in protein (2–2.5%, Martin et al., 2016). Although not recommended in the first year for infants, both the ratio and protein concentration change to 80:20 and 3.1 wt%, respectively, with the introduction of cow milk.

In addition to the potential for dairy proteins to affect postprandial metabolism, evidence has shown that daily protein intake should be spread equally over 3 main meals, indicating a need to include more protein with the breakfast meal (Mamerow et al., 2014). Therefore, the purpose of our work was to test the effect of novel milk formulations with altered amounts and proportions of whey and casein proteins on postprandial BG, feelings of satiety, and subsequent food intake when consumed with a high-glycemic carbohydrate breakfast cereal in healthy young adults. Milk products containing similar protein content as commercial milk (3.1 wt% protein) or increased to 9.3 wt%, both formulated to contain either a 80:20 or 40:60 casein-to-whey ratio, were consumed with breakfast cereal containing a total of 76.7 g of available carbohydrates. We hypothesized that the milk products containing modified 40:60 casein-to-whey protein ratios or enhanced protein concentrations or both, when compared with the control and commercial milk (3.1 wt% concentration and 80:20 ratio), would be associated with reductions in post-lunch BG, enhanced feelings of satiety, reductions in food intake at a subsequent meal, and reductions in BG following the subsequent meal.

## **2.3. MATERIALS AND METHODS**

### *2.3.1. Treatment preparation*

Breakfast drinks (250 mL) based on skim milk (Neilson Dairy–Saputo Dairy Products Canada G.P., St-Laurent, Quebec) or control (water with whey permeate) were formulated and cold-mixed together using whey permeate (DariSweet 200, #215503), skim milk powder (Low Temp, #202001), and whey protein concentrate (Prodel 35, #33703; all from Parmalat Canada, London, ON, Canada) to increase the concentration of commercial milk protein (3.1 wt%) 3-fold (9.3 wt%) and to modify the casein-to-whey protein ratio from (normal) 80:20 to high whey (i.e., 40:60) while holding the lactose (34.7 g) contents constant (Table 2.1). Treatments and the water and permeate control were combined with 58 g of oat-based cereal (Honey Nut Cheerios,

General Mills, Mississauga, Canada) to form the breakfast meal, totaling 76.7 g of available carbohydrates from cereal (42 g) and from lactose (34.7 g; Table 2.2).

**Table 2. 1** Composition of breakfast drinks served with Cheerios (General Mills, Mississauga, Canada) to participants

Treatment <sup>1</sup> (250 mL)	Water (mL)	Skim Milk Liquid (mL)	Permeate Powder (g)	Skim Milk Powder (g)	Whey Protein Concentrate Powder (g)
Control	250	-	47.3	-	-
3.1% MP (80:20)	-	250	31.9	-	-
3.1% MP (40:60)	125	125	31.6	-	12.5
9.3% MP (80:20)	-	250	-	46.8	-
9.3% MP (40:60)	-	250	-	11.8	37.7

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey; normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

**Table 2. 2** Combined composition of milk treatment (250 mL) with breakfast cereal (58 g)

Treatment <sup>1</sup> (250 mL)	Calories (kcal)	Available carbohydrates (g)	Total fats (g)	Casein protein (g)	Whey protein (g)	Total protein (g)
Control	403.8	76.7	2.8	0	0.3	4.6
3.1% MP (80:20)	433.1	76.7	3.2	6.2	1.6	12.4
3.1% MP (40:60)	435.6	76.7	3.0	3.1	4.8	12.4
9.3% MP (80:20)	485.6	76.7	3.4	18.6	4.7	27.9
9.3% MP (40:60)	499.1	76.7	4.0	9.3	14.1	27.9

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey; normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

### 2.3.2. Study design and protocol

Healthy young male and female adult participants were recruited from the University of Guelph and surrounding community. Participants met the inclusion criteria of 18 to 30 yr old, healthy, body mass index between 20 and 24.9 kg/m<sup>2</sup>, regular milk consumers (>2 servings/week), typical breakfast consumers (before 0900 h, 5 d/week), and fasting BG <5.5 mmol/L (but not <3.5 mmol/L). Exclusion criteria consisted of the presence of any medical condition, including gastrointestinal disorders, regular medication use (excluding hormonal contraceptives), any food allergy or anaphylactic allergy or both, smoking, and recreational drug use. Elite or training athletes were excluded, along with individuals trying to lose weight or

whose body weight had changed >5 kg within the previous 6 mo. Participants were excluded if they scored >16 on the cognitive restraint scale on the three-factor eating questionnaire (Stunkard and Messick, 1985). Additionally, persons regularly consuming a high number of caffeinated (>4 drinks/day) or alcoholic drinks (>14 drinks/wk), taking protein supplements, or who were pregnant or breastfeeding were excluded. The study was approved by the University of Guelph Human Research Ethics Board (#14JN004) and was registered with clinicaltrials.gov (NCT02471092) and all participants provided written informed consent.

A double-blinded, controlled, crossover randomized design was used. Healthy men and women (16 males and 16 females) randomly consumed 1 of each of the 5 allocated 3-digit number coded treatments at study visits separated by 1 wk. On study visit days, participants arrived at the Human Nutraceutical Research Unit at the University of Guelph after a 12h overnight fast. Water was allowed up to 1 h before the start of each session. Upon arrival, participants filled out a questionnaire on sleep habits and stress factors. Participants who indicated feelings of illness, atypical sleep, or stress were asked to reschedule. Satiety was measured using a 100-mm visual analog scale self-reported paper questionnaire with hunger, fullness, desire to eat, and prospective food consumption horizontal anchored scales (i.e., not at all hungry versus extremely hungry; Blundell et al., 2010). Baseline finger prick blood samples for glucose concentration were obtained in duplicate and analyzed using a glucose meter (HemoCue Glucose 201+ Analyzer, HemoCue, Angelholm, Sweden). Participants, in isolation, then consumed the treatment or control (250 mL), served in a large bowl with 2 servings (58 g) of cereal (Honey Nut Cheerios, General Mills) and a glass of 100 mL of cool water, at a constant pace within 5 min. We added 1.5 g of crushed acetaminophen (Tylenol, McNeil Consumer Healthcare, Markham, ON, Canada) to each 250-mL serving of breakfast for the purposes of measuring gastric emptying (Chapter 3). Appetite was assessed before and immediately

following breakfast consumption and before finger prick blood samples, and then at 15- to 30-min intervals thereafter (i.e., 0, 15, 30, 45, 60, 90, 120, 140, 155, 170, 185, and 200 min). A subset of 6 males and 6 females provided blood samples taken by an indwelling venous catheter to allow sufficient blood volume for gastric hormones, acetaminophen, and AA analyses, which will be reported elsewhere. At 120 min, participants, in isolation, were served an *ad libitum* pizza lunch and instructed to eat until comfortably full. The 4-cheese 13-cm pizzas (McCain Foods, Florenceville, NB, Canada) were prepared by baking in an oven at 425°F for 10 min. Pizzas were cut into quarter slices and 4 pizzas were served on individual plates (approximately 81 g/plate) on each tray and served with a 500-mL glass of cool water. Participants were given 6 to 7 min with each tray, and up to 3 trays were presented during a maximum total lunch period of 20 min. The pizza and water were weighed before and after serving to determine food and energy intakes, which were calculated based on the product label nutritional information. BG and satiety measurements continued for 1 h after the pizza lunch (i.e., at 140, 155, 170, 185, and 200 min). Participants were also asked to complete questionnaires related to palatability of the breakfast and lunch meals and gastrointestinal symptoms (abdominal discomfort, bloating, cramping, rumbling, flatulence, bowel movement, bowel consistency, problems with defecation function, bowel movement urgency, and overall gastrointestinal function) during the 24-h period following each study visit using an anchored 100-mm horizontal visual analog line scale (Veenstra et al., 2010).

### 2.3.3. Data and statistical analyses

The sample size of 32 was determined on the basis of previous studies (Law et al., 2017a), which showed that 26 participants would be required to detect a 150-kcal difference in treatment effects on food intake and a 10% difference in subjective appetite ratings and *ad libitum* energy intake and considering a 15% attrition rate. Appetite score was calculated based

on the average of individual scales [i.e., (hunger + (100 – fullness) + desire to eat + prospective food consumption)/4]. Pre-lunch changes from baseline were calculated from 0 (immediately before breakfast consumption) to 120 min and post-lunch changes from 120 (before lunch meal consumption) to 200 min. Postprandial glycemia and appetite scores were also explored based on area under the curve (**AUC**) results. An AUC over 120 min is a standard measure for the glycemic index and recommended by Health Canada for postprandial glycemia health claims (Health Canada, 2013a). Incremental area under the curve (**iAUC**) for BG and total area under the curve (**tAUC**) for appetite were calculated using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA). Last, energy intake was calculated by multiplying the weight of the consumed pizza (grams) by the calories per serving, as provided by the manufacturer.

For the statistical analysis, all milk treatments were first compared with the whey permeate control. Specifically, differences in BG and appetite scores change from baseline (pre- and post-lunch) were investigated using a repeated-measures PROC MIXED 3-way (treatment, time, and sex) ANOVA, followed by Tukey-Kramer testing to compare treatments to the control using SAS version 9.3 (SAS Institutes, Cary, NC). Results were pooled for males and females, unless a significant sex effect or sex and treatment interaction or both were found, in which case a 2-way ANOVA (treatment and time) followed by Tukey's *post hoc* testing was performed to compare the effect of treatment. When an interaction was found, a 2-way ANOVA (treatment and sex for pooled participants) or a 1-way ANOVA (treatment for males and females) followed by Tukey-Kramer testing was performed to identify the effect of treatment at each time point. BG iAUC, appetite tAUC, and energy intake were all investigated using a repeated-measures PROC MIXED 2-way (treatment and sex) ANOVA, followed by Tukey-Kramer testing. Area under the curve and energy intake results were pooled for males and females unless a significant sex effect or interaction or both was found, in which case a one-way ANOVA (treatment)

followed by Tukey's *post hoc* testing was used to identify differences between treatments within each sex.

Orthogonal contrast statements were also used to group pairs of treatments (not including the whey permeate control) according to (1) protein concentration [mean of 3.1%, wt (80:20) + 3.1%, wt (40:60) vs. mean of 9.3%, wt (80:20) + 9.3%, wt (40:60)] and (2) protein ratio [mean of 3.1%, wt (80:20) + 9.3%, wt (80:20) vs. mean of 3.1%, wt (40:60) + 9.3%, wt (40:60)], to specifically compare the overall effect of protein concentration and ratio for all endpoints.

Linear regression and Pearson correlation coefficient were used to explore associations between appetite change from baseline (mm) and food intake (kcal), with all 4 treatments and the control, significance set at P< 0.05. Data are presented as mean ± standard error of the means.

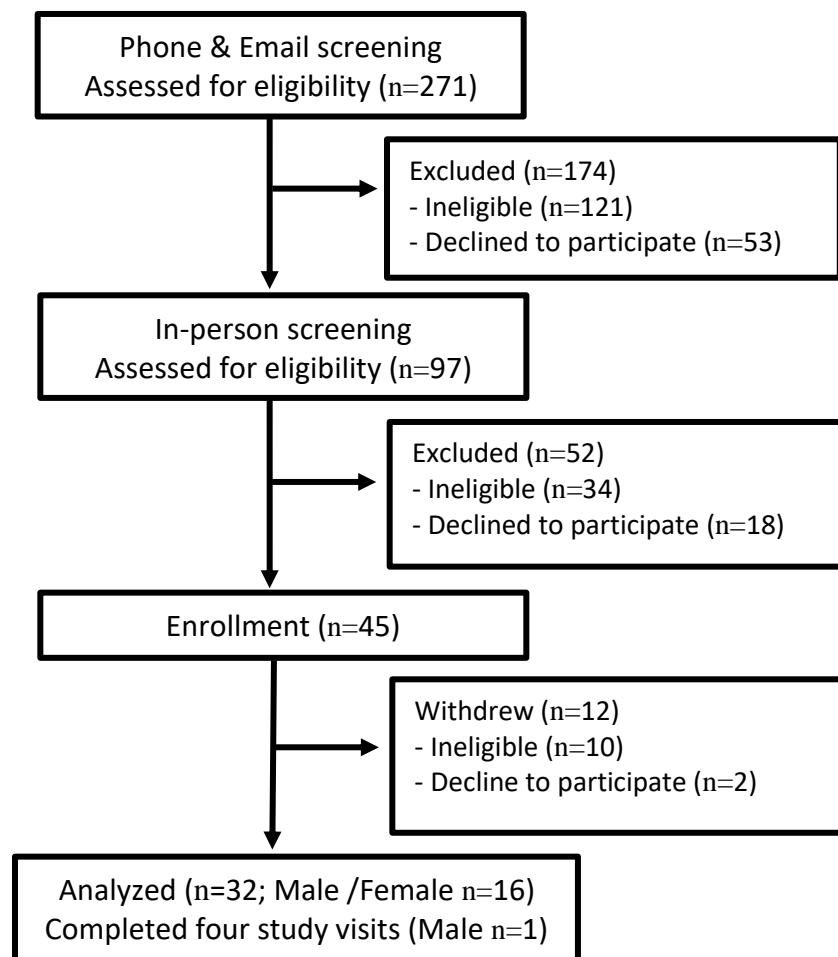
Gastrointestinal questionnaire results were analyzed with a one-way ANOVA when normally distributed or the Kruskal-Wallis test for nonparametric distribution. When significance was found, all 4 treatments and the control were analyzed by Tukey's *post hoc* test or the Kruskal-Wallis paired test. Normally distributed data are presented as mean ± standard error of the means and nonparametric data are presented as median followed by interquartile range (25 to 75%).

## 2.4. RESULTS

Participant characteristics at the time of study enrolment are presented in Table 2.3. In total, 271 participants were phone screened, 97 were invited for an in-person eligibility assessment, and 45 were enrolled in the study (Figure 2.1). Participants were excluded from the study due to scheduling conflict, fasting glucose >5.5 mmol/L on the day of study visit, or reasons unrelated to the study. Data for a total of 32 participants, including 1 male participant who only completed 4 study visits, were analyzed.

**Table 2. 3** Baseline characteristics for participants collected during in-person screening

	All Participants (n=32)		Male Participants (n=16)		Female Participants (n=16)	
	Mean	SD	Mean	SD	Mean	SD
Age (y)	23.4	3.1	24.5	3.2	22.4	2.7
Height (m)	1.7	0.1	1.8	0.1	1.6	0.1
Weight (kg)	66.8	10.8	73.9	8.2	59.6	8.1
BMI ( $\text{kg}/\text{m}^2$ )	22.2	2.5	22.8	2.3	21.6	2.6
Systolic/Diastolic	121.2	14.1	129.8	11.5	112.7	11.0
Blood Pressure (mmHg)	/ 72.3	/ 9.5	/ 72.8	/ 9.4	/ 71.7	/ 9.9
Fasting BG (mmol/L)	4.9	0.4	4.3	0.4	4.8	0.4

**Figure 2. 1** Consolidated standards of reporting trials (CONSORT) flow diagram  
2.4.1. *BG change from baseline and iAUC*

Change from baseline (0 min for pre-lunch and 120 min for post-lunch responses) values

are reported in Figure 2.2. Pre-lunch (0–120 min) mean BG change from baseline concentration

showed a significant effect of treatment ( $P<0.01$ ), time ( $P<0.01$ ), and treatment  $\times$  time ( $P<0.01$ ) and treatment  $\times$  sex ( $P<0.05$ ) interactions were observed, but we found no sex effect ( $P=0.76$ ; Table 2.4). All milk treatments differed relative to the water with whey permeate control ( $P<0.01$ ). Pre-lunch BG change from baseline concentration peaked at 30 min for all treatments. Furthermore, when treatments were contrasted by protein concentration or ratio, high-protein treatments (9.3 wt%) were associated with greater attenuations of BG change from baseline by  $-1.01 \pm 0.12$  mmol/L compared with the normal protein level (3.1 wt%) treatments ( $P<0.01$ ).

**Table 2. 4** Mean ( $\pm$ SEM) BG (mmol/L) from baseline during pre-lunch (0-120 min) and post-lunch (120-200 min) periods

	Treatments <sup>1</sup>					P-value <sup>2</sup>	Concentration P-value <sup>3</sup>	Ratio P-value <sup>4</sup>
	Control	3.1% (80:20)	3.1% (40:60)	9.3% (80:20)	9.3% (40:60)			
<b>Pre-lunch (0-120 min, mmol/L)</b>								
All (n=32)	$2.3 \pm 0.1^a$	$1.9 \pm 0.1^b$	$1.7 \pm 0.1^b$	$1.4 \pm 0.1^c$	$1.3 \pm 0.1^c$	<0.01	<0.01	0.03
Males (n=16)	$2.2 \pm 0.2^a$	$2.0 \pm 0.1^{ab}$	$1.8 \pm 0.1^{bc}$	$1.5 \pm 0.1^{cd}$	$1.3 \pm 0.1^d$	<0.01	<0.01	0.02
Females (n=16)	$2.5 \pm 0.2^a$	$1.8 \pm 0.1^b$	$1.7 \pm 0.1^b$	$1.3 \pm 0.1^c$	$1.3 \pm 0.1^c$	<0.01	<0.01	0.47
<b>Post-lunch (120-200 min, mmol/L)</b>								
All (n=32)	$0.4 \pm 0.1$	$0.4 \pm 0.1$	$0.5 \pm 0.1$	$0.3 \pm 0.1$	$0.3 \pm 0.1$	0.49	0.10	0.69
Males (n=16)	$0.9 \pm 0.1^a$	$0.7 \pm 0.1^a$	$0.6 \pm 0.1^{ab}$	$0.5 \pm 0.1^b$	$0.4 \pm 0.1^b$	<0.01	0.01	0.16
Females (n=16)	$-0.2 \pm 0.1^a$	$0.1 \pm 0.1^{ab}$	$0.3 \pm 0.1^b$	$0.2 \pm 0.1^b$	$0.3 \pm 0.1^b$	<0.01	0.64	0.03

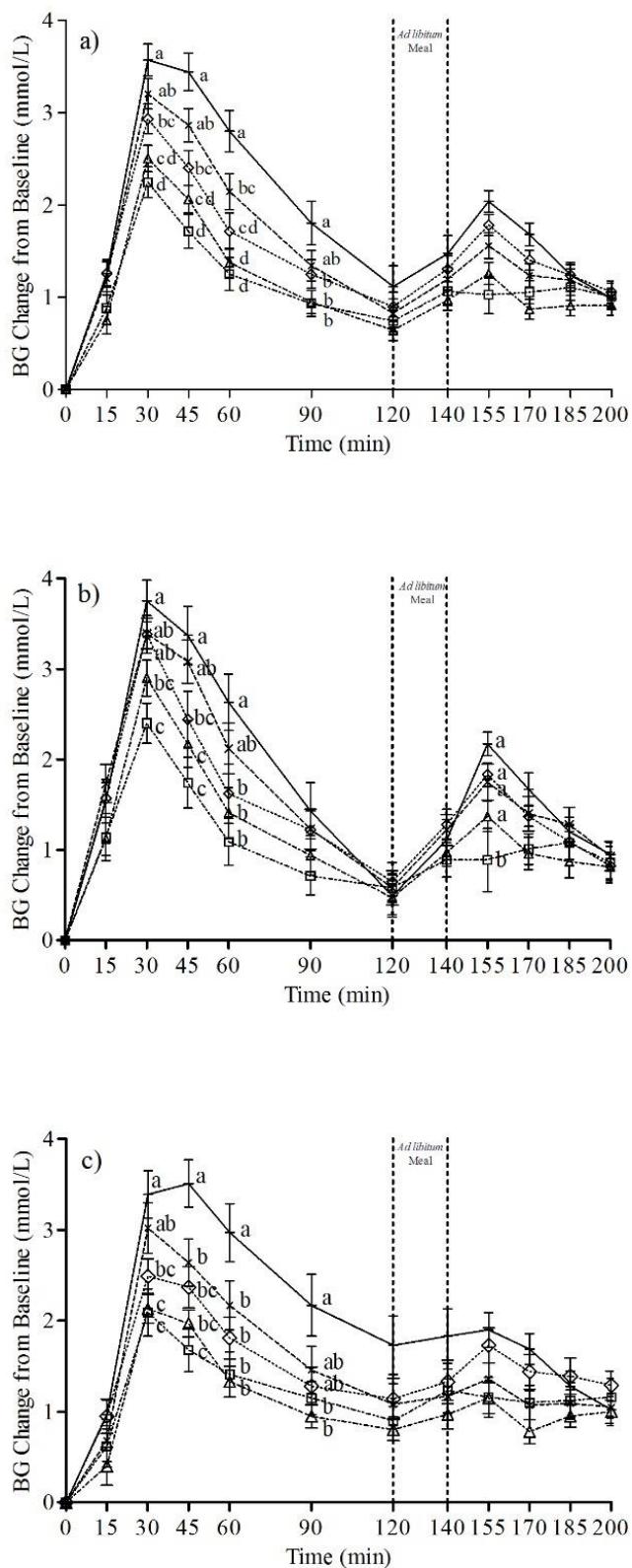
<sup>a-d</sup>Means within a row with different superscripts differ according to adjusted Tukey-Kramer ( $P<0.05$ ).

<sup>1</sup> Control (water and whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>2</sup> ANOVA P-value for treatment effect

<sup>3</sup> Contrast between high and low protein concentration (mean 9.3 wt% (80:20) + 9.3 wt% (40:60) to mean 3.1 wt% (80:20) + 3.1 wt% (40:60))

<sup>4</sup> Contrast between modified and normal ratio (mean 9.3 wt% (40:60) + 3.1 wt% (40:60) to mean 9.3 wt% (80:20) + 9.3 wt% (80:20))



**Figure 2. 2** Mean ( $\pm$  SEM) blood glucose change from baseline for a) all participants (n=32), b) male participants (n=16), c) female participants (n=16) from 0-120 min after the consumption of breakfast meal, followed by pizza lunch (120-200 min) for control (+), 3.1% milk protein (MP) (80 casein:20 whey protein) (X), 3.1% MP (40 casein:60 whey protein) ( $\diamond$ ), 9.3% MP (80 casein:20 whey protein) ( $\triangle$ ), and 9.3% MP (40 casein:60 whey protein) ( $\square$ ). <sup>a-d</sup> Different letters at each measured time are different between treatment according to adjusted Tukey-Kramer ( $P < 0.05$ ).

Likewise, regardless of protein concentration, modified protein ratio (40:60) was associated with greater attenuations of BG by  $-0.26 \pm 0.12$  mmol/L compared with the normal ratio (80:20) treatments over the 120-min pre-lunch period ( $P=0.03$ ). At specific time points, according to the contrasts, high (9.3 wt%) protein concentration attenuated BG change from baseline from 15 to 90 min ( $P<0.01$ ), whereas the modified (40:60) ratio attenuated BG change from baseline at 30 ( $P<0.04$ ) and 45 min ( $P=0.01$ ) in pooled participants. Differences found in treatment  $\times$  sex interactions ( $P<0.05$ ) may be related to the significant contrast results between modified (40:60) and normal (80:20) protein ratios in male ( $P=0.02$ ), but not female, participants ( $P=0.47$ ). Following the pizza lunch meal, BG change from baseline was not affected by treatment ( $P=0.49$ ) and we found no significant treatment  $\times$  time interaction ( $P=0.13$ ); however, we noted significant effects of time ( $P<0.01$ ) and sex ( $P<0.01$ ) and a treatment  $\times$  sex interaction ( $P<0.01$ ; Figure 2.2). BG post-lunch change from baseline was not different when contrasted between high and normal protein concentrations ( $P=0.10$ ) or modified and normal ratio ( $P=0.69$ ). In male participants, BG post-lunch change from baseline concentration was attenuated by high (9.3 wt%) compared with low (3.1 wt%) protein concentrations ( $-0.43 \pm 0.16$  mmol/L,  $P=0.01$ ), but we found no difference between modified (40:60) and normal (80:20) protein ratio ( $P=0.16$ ). However, the BG post-lunch change from baseline contrast trends were reversed in female participants, whereby milk treatments were not different by protein concentrations ( $P=0.64$ ), but modified (40:60) ratio increased BG compared with the normal (80:20) ratio by  $0.31 \pm 0.14$  mmol/L ( $P=0.03$ ; Table 2.4).

**Table 2. 5** Mean ( $\pm$ SEM) incremental area under the curve (iAUC) for BG (mmol/L·min) from pre-lunch (0-120 min) and post-lunch (120-200 min) periods

	Treatment <sup>1</sup>					P-Value <sup>2</sup>	Concentration p-value <sup>3</sup>	Ratio p-value <sup>4</sup>
	Control	3.1% (80:20)	3.1% (40:60)	9.3% (80:20)	9.3% (40:60)			
<b>Pre-lunch (0-120 min, mmol/L·min)</b>								
All (n=32)	258.9 $\pm$ 17.0 <sup>a</sup>	211.4 $\pm$ 13.9 <sup>b</sup>	190.2 $\pm$ 13.0 <sup>bc</sup>	149.6 $\pm$ 10.4 <sup>cd</sup>	143.0 $\pm$ 12.3 <sup>d</sup>	<0.01	<0.01	0.23
Males (n=16)	241.7 $\pm$ 22.4 <sup>a</sup>	217.3 $\pm$ 16.6 <sup>a</sup>	196.8 $\pm$ 19.1 <sup>ab</sup>	161.1 $\pm$ 19.1 <sup>bc</sup>	138.3 $\pm$ 17.6 <sup>c</sup>	<0.01	<0.01	0.16
Females (n=16)	276.0 $\pm$ 25.5 <sup>a</sup>	205.5 $\pm$ 22.9 <sup>b</sup>	183.5 $\pm$ 18.2 <sup>b</sup>	138.8 $\pm$ 9.2 <sup>b</sup>	147.7 $\pm$ 17.7 <sup>b</sup>	0.02	<0.01	0.70
<b>Post-lunch (120-200 min, mmol/L·min)</b>								
All (n=32)	46.7 $\pm$ 7.7	40.08 $\pm$ 8.11	41.6 $\pm$ 6.4	32.3 $\pm$ 6.1	31.5 $\pm$ 5.2	0.24	0.11	0.99
Males (n=16)	72.8 $\pm$ 10.7 <sup>a</sup>	60.5 $\pm$ 13.0 <sup>ab</sup>	54.3 $\pm$ 9.6 <sup>ab</sup>	43.1 $\pm$ 9.2 <sup>ab</sup>	36.7 $\pm$ 6.5 <sup>b</sup>	0.04	0.06	0.45
Females (n=16)	20.6 $\pm$ 6.4	19.6 $\pm$ 6.9	28.9 $\pm$ 7.5	22.2 $\pm$ 7.5	26.3 $\pm$ 8.2	0.79	1.00	0.28

<sup>a-d</sup>Means within a row with different superscripts differ according to adjusted Tukey-Kramer ( $P < 0.05$ )

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>2</sup> ANOVA treatment effect P-value

<sup>3</sup> Contrast between high and low protein concentration (mean 9.3 wt% (80:20) + 9.3 wt% (40:60) to mean 3.1 wt% (80:20) + 3.1 wt% (40:60))

<sup>4</sup> Contrast between modified and normal ratio (mean 9.3 wt% (40:60) + 3.1 wt% (40:60) to mean 9.3 wt% (80:20) + 9.3 wt% (80:20))

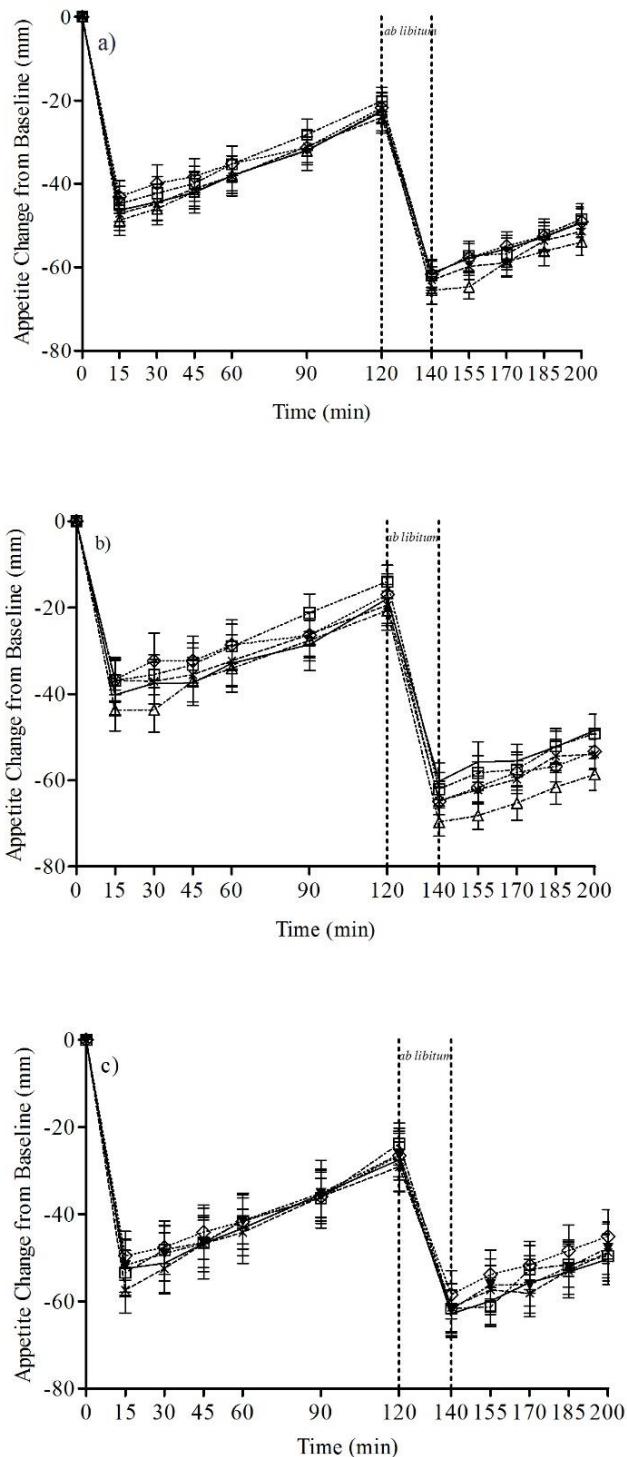
Pre-lunch BG iAUC showed a significant treatment effect ( $P < 0.01$ ), but we observed neither an effect of sex ( $P = 0.99$ ) nor a treatment  $\times$  sex interaction ( $P = 0.42$ ; Table 2.5). When comparing among all treatments, milk treatments had lower BG iAUC values relative to the control ( $P < 0.05$ ). Furthermore, when a high protein concentration was contrasted with a low protein concentration, high protein attenuated BG iAUC ( $-110.05 \pm 22.43$  mmol/L · min,  $P < 0.01$ ), mirroring the change from baseline trend; however, we found no difference between modified and normal protein ratio ( $P = 0.23$ ) in pooled participants. Overall, high protein (9.3 wt%) consumption consistently resulted in greater suppression of pre-lunch BG.

Post-lunch BG iAUC values were not affected by treatment ( $P=0.24$ ) and we found no treatment  $\times$  sex interaction ( $P = 0.22$ ), but we did note a significant effect of sex ( $P<0.01$ ). The sex effect was likely related to significant treatment effects in male participants, but not in female participants ( $P=0.04$  and  $P=0.79$ , respectively). For male participants, the high protein (9.3 wt%) modified (40:60) treatment resulted in lower iAUC values ( $36.7 \pm 6.5 \text{ mmol/L} \cdot \text{min}$ ) compared with control ( $72.8 \pm 10.7 \text{ mmol/L} \cdot \text{min}$ ; adjusted Tukey-Kramer,  $P=0.03$ ), but we observed no effect of treatment when high (9.3 wt%) protein was contrasted with low (3.1 wt%) protein concentrations ( $P = 0.06$ ) or between modified (40:60) and normal (80:20) ratio ( $P=0.45$ ; Table 2.5). We found no treatment effect in post-lunch BG iAUC in female participants ( $P=0.79$ ).

#### *2.4.2. Subjective appetite change from baseline and tAUC*

Pre-lunch appetite change from baseline showed a significant effect of time ( $P<0.01$ ), but not of treatment ( $P=0.15$ ) or sex ( $P=0.06$ ), and we noted no treatment  $\times$  time ( $P=1.00$ ) or treatment  $\times$  sex ( $P=0.38$ ) interactions; thus, data for the sexes were pooled (Figure 2.3). Normal (80:20) ratio resulted in lower appetite when contrasted with the modified (40:60) protein ratio ( $-5.97 \pm 2.38 \text{ mm}$ ,  $P=0.01$ ), and we found no difference between high (9.3 wt%) and low (3.1 wt%) protein concentrations ( $P=0.97$ ; Table 2.6). These contrasting results observed in pooled participants may have been driven by males, specifically as normal (80:20) versus modified (40:60) ratio showed lower appetite ( $-8.9 \pm 3.2 \text{ mm}$ ,  $P<0.01$ ); although we observed no difference between high (9.3 wt%) and low (3.1 wt%) protein concentrations ( $P=0.49$ ). Higher appetite with modified protein ratio treatments was not found at specific time points in male and pooled participants. Within female participants, we observed no treatment effect when treatments were compared with each other or in contrast groups ( $P>0.05$ ).

In terms of post-lunch appetite, main effects of time ( $P<0.01$ ), treatment ( $P<0.01$ ), and sex ( $P=0.01$ ) and a treatment  $\times$  sex interaction ( $P=0.04$ ), but no treatment  $\times$  time interactions ( $P=1.0$ ), were observed (Figure 2.3). Post-lunch appetite change from baseline was attenuated with the 9.3 wt% (80:20) treatment ( $-37.6 \pm 1.7$  mm) relative to the whey permeate water control ( $-32.7 \pm 1.6$  mm;  $P<0.05$ ; Table 2.6). Female post-lunch appetite change from baseline showed effects of treatment ( $P=0.02$ ) and time ( $P<0.01$ ), but no treatment  $\times$  time interaction ( $P=1.0$ ). Similarly, male participants post-lunch appetite change from baseline showed effects of treatment ( $P=0.02$ ) and time ( $P<0.01$ ), but no treatment  $\times$  time interaction ( $P=1.0$ ). When expressed as a mean change from baseline over the post-lunch period for the 5 breakfasts, including the control, males had a lower subjective appetite score ( $-40.9 \pm 17.8$  mm) than females ( $-27.8 \pm 1.0$  mm). However, in both sexes and comparing all 5 breakfast meals, the 9.3 wt% normal (80:20) treatment led to the greatest suppression of appetite from baseline. This was true in males ( $-44.4 \pm 2.2$  mm) relative to nondairy protein water control ( $-36.6 \pm 2.5$  mm;  $P<0.01$ ), but not commercial milk (3.1 wt% 80:20;  $P=0.35$ ), and in females ( $-31.2 \pm 2.5$  mm), relative to the commercial milk formula ( $-25.4 \pm 2.5$  mm;  $P<0.05$ ), but not to the control ( $-28.9 \pm 2.0$  mm;  $P=0.87$ ). In pooled participants, significant differences were found when high (9.3 wt%) protein concentration was contrasted to low (3.1 wt%) protein concentration ( $-7.18 \pm 2.26$  mm;  $P<0.01$ ), but we found no difference between modified (40:60) and normal (80:20) protein ratio ( $P=0.79$ ). Similar findings were mirrored by high (9.3 wt%) protein concentration treatments lowered appetite compared with low (3.1 wt%) protein concentration ( $-10.19 \pm 3.13$  mm,  $P<0.01$ ), but we noted no difference between ratios ( $P=0.59$ ) in female participants, which may have driven the results in the pooled contrast. We found no difference when high (9.3 wt%) was contrasted to low (3.1 wt%) protein ( $P=0.20$ ) and between modified (40:60) and normal (80:20) ratio ( $P=0.87$ ) in male post-lunch change from baseline.



**Figure 2. 3** Mean ( $\pm$ SEM) ratings of appetite change from baseline for a) all participants (n=32), b) male participants (n=16), c) female participants (n=16) for control (+), 3.1% milk protein (MP) (80 casein:20 whey protein) (X), 3.1% MP (40 casein:60 whey protein) ( $\diamond$ ), 9.3% MP (80 casein:20 whey protein) ( $\triangle$ ), and 9.3 MP (40 casein:60 whey protein) ( $\square$ ) for cumulative (0-200 min), pre-lunch (0-120 min) and post-lunch (120-200 min). Statistical significance at P<0.05.

Pre-lunch appetite tAUC was not affected by treatment ( $P=0.96$ ), but was affected by sex ( $P=0.01$ ), and we observed no treatment  $\times$  sex interaction ( $P=0.64$ ). Likewise, we found no difference when high protein (9.3 wt%) was contrasted to low (3.1 wt%) protein concentration ( $P=0.48$ ) nor when modified (40:60) and normal (80:20) protein ratio ( $P=0.77$ ) was contrasted. Sex differences may explain this, as males had higher appetite tAUC values than females ( $5,596.4 \pm 170.7$  and  $4,038.8 \pm 238.8$  mm·min, respectively).

**Table 2. 6** Mean ( $\pm$ SEM) appetite change from baseline (mm on 100-mm satiety scale) during pre-lunch (0-120 min) and post-lunch (120-200 min) periods

	Treatment <sup>1</sup>					P-Value <sup>2</sup>	Concentration p-value <sup>3</sup>	Ratio p-value <sup>4</sup>
	Control	3.1% (80:20) <sup>2</sup>	3.1% (40:60) <sup>3</sup>	9.3% (80:20) <sup>4</sup>	9.3% (40:60) <sup>5</sup>			
<b>Pre-lunch (0-120 min, mm)</b>								
All (n=32)	-37.5 $\pm$ 3.0	-38.2 $\pm$ 3.0	-35.0 $\pm$ 3.0	-38.0 $\pm$ 3.0	-35.2 $\pm$ 1.7	0.15	0.97	0.01
Males (n=16)	-32.2 $\pm$ 3.8	-32.0 $\pm$ 3.8	-28.9 $\pm$ 3.8	-34.5 $\pm$ 3.8	-28.6 $\pm$ 3.8	0.74	0.49	<0.01
Females (n=16)	-42.8 $\pm$ 4.7	-44.3 $\pm$ 4.7	-40.9 $\pm$ 4.7	-41.4 $\pm$ 4.7	-41.8 $\pm$ 4.7	0.39	0.56	0.39
<b>Post-lunch (120-200 min, mm)</b>								
All (n=32)	-32.7 $\pm$ 1.6 <sup>a</sup>	-32.8 $\pm$ 1.7 <sup>a</sup>	-33.6 $\pm$ 1.3 <sup>ab</sup>	-37.6 $\pm$ 1.7 <sup>b</sup>	-35.1 $\pm$ 1.6 <sup>ab</sup>	0.01	<0.01	0.79
Males (n=16)	-36.6 $\pm$ 2.5 <sup>a</sup>	-39.7 $\pm$ 1.9 <sup>ab</sup>	-42.2 $\pm$ 1.4 <sup>ab</sup>	-44.4 $\pm$ 2.2 <sup>b</sup>	-42.0 $\pm$ 2.0 <sup>ab</sup>	0.02	0.20	0.87
Females (n=16)	-28.9 $\pm$ 2.0 <sup>ab</sup>	-25.4 $\pm$ 2.5 <sup>a</sup>	-25.1 $\pm$ 1.8 <sup>ab</sup>	-31.2 $\pm$ 2.5 <sup>b</sup>	-28.3 $\pm$ 2.2 <sup>ab</sup>	0.02	<0.01	0.59

<sup>a-d</sup>Means within a row with different superscripts differ according to adjusted Tukey-Kramer ( $P<0.05$ ).

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

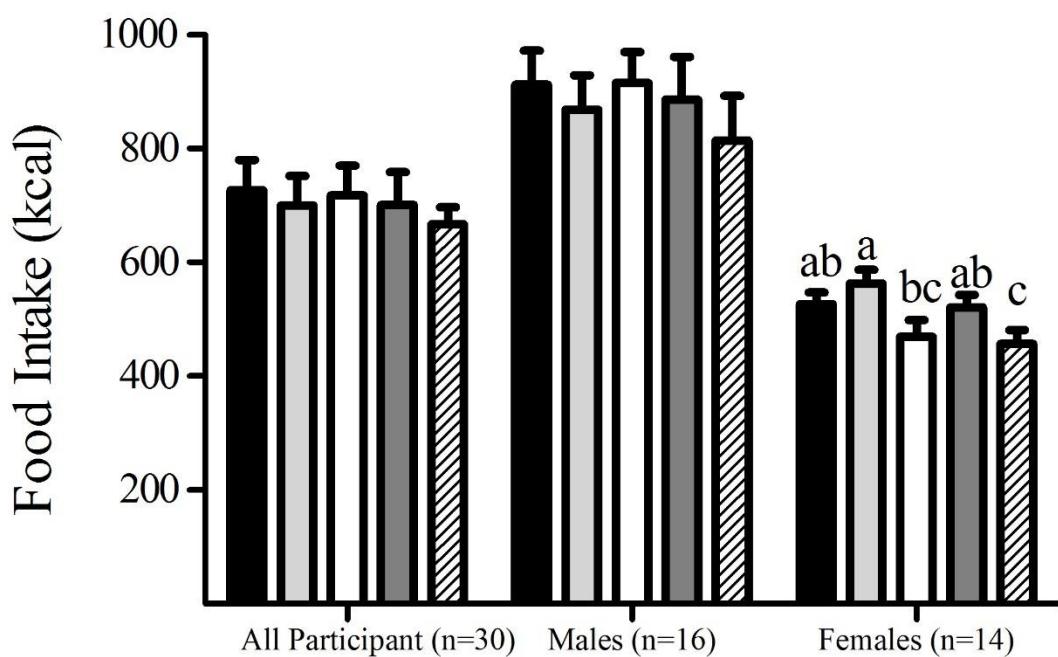
<sup>2</sup> ANOVA treatment effect P-value

<sup>3</sup> Contrast between high and low protein concentration (mean 9.3 wt% (80:20) + 9.3 wt% (40:60) to mean 3.1 wt% (80:20) + 3.1 wt% (40:60))

<sup>4</sup> Contrast between modified and normal ratio (mean 9.3 wt% (40:60) + 3.1 wt% (40:60) to mean 9.3 wt% (80:20) + 9.3 wt% (80:20))

Post-lunch appetite tAUC values were not associated with treatment ( $P=0.22$ ) or sex ( $P=0.37$ ) and we found no treatment  $\times$  sex interaction ( $P=0.70$ ). However, contrasts between high (9.3 wt%) protein concentration treatments indicated lower appetite tAUC compared with low (3.1 wt%) protein concentration ( $-466.24 \pm 199.02$  mm·min,  $P=0.02$ ), mirroring the above change from baseline contrast trend in pooled participants, and no difference between normal (80:20) and modified (40:60) ratio ( $P=0.69$ ).

#### 2.4.3. Food intake



**Figure 2.4** Mean ( $\pm$ SEM) pizza lunch energy intake (kcal) for all participants ( $n=30$ ,  $P=0.16$ ), male participants ( $n=16$ ,  $P=0.40$ ), and female participants ( $n=12$ ,  $P=0.01$ ) for control (black), 3.1% (MP 80 casein:20 whey; light gray); 3.1% MP (40 casein: 60 whey protein; white), 9.3% (80 casein: 20 whey protein; dark gray) and (40 casein: 60 whey protein; striped) at 120 min. Statistical significance at  $P<0.05$ .

At the lunch meal, male participants consumed an average  $879.1 \pm 29.12$  kcal of the cheese pizza whereas female participants consumed  $503.7 \pm 20.7$  kcal. Two female participants' energy intake results were omitted due to low average calorie consumption at the lunch meal

(<150 kcal), which is not representative of lunch caloric consumption 2 h after breakfast (Akhavan et al., 2010). We found no significant effect of treatment observed within pooled participants ( $P=0.16$ ) and no overall treatment  $\times$  sex interaction ( $P=0.46$ ), although we did note an effect of sex ( $P<0.01$ ; Figure 2.4). There were no differences when high (9.3 wt%) protein concentration was contrasted to low (3.1 wt%) protein concentration ( $P=0.53$ ) or when modified (40:60) was contrasted to normal (80:20) protein ratio ( $P=0.12$ ) in pooled participants. We observed no treatment effect within males ( $P=0.40$ ); however, female participants showed a treatment effect on food intake ( $P<0.01$ ). The modified protein ratio 40:60 treatments resulted in fewer calories consumed contrasted to the 80:20 ratio treatments ( $-157.52 \pm 29.37$  kcal,  $P=0.01$ ), but we found no difference between high (9.3 wt%) and low (3.1 wt%) protein concentration ( $P=0.08$ ).

#### *2.4.4. Appetite and food intake correlation*

Pre-lunch appetite change from baseline (mm) and food intake (kcal) were correlated for all 4 treatments and control beverage and in all participants ( $P<0.05$ ,  $R^2=0.04$ ), but not in either male ( $P=0.64$ ,  $R^2=0.0$ ) or female ( $P=0.75$ ,  $R^2=0.0$ ) groups (Figure 2.5). Post-lunch food intake (kcal) and appetite (mm) were inversely correlated for all ( $P<0.01$ ,  $R^2=0.24$ ), male ( $P<0.01$ ,  $R^2=0.14$ ), and female participants ( $P<0.01$ ,  $R^2=0.23$ ; Figure 2.5). Two female participants with low lunch meal consumption (<150 kcal) were not included in the correlation.

#### *2.4.5. Palatability and gastrointestinal questionnaire*

Overall, palatability of the breakfast significantly differed ( $P < 0.05$ ) between the control and milk treatments, with the 9.3 wt% (80:20) treatment being most preferred ( $60.3 \pm 3.9$  mm) and the whey permeate control being rated the least palatable ( $37.3 \pm 4.1$  mm). The inclusion of 1.5 g of crushed acetaminophen (Tylenol, McNeil Consumer Healthcare) to each 250- mL serving of treatment, for the purposes of measuring gastric emptying (data not shown), may have

affected palatability. Palatability of the pizza did not differ between study visits (mean score 73.5 ± 1.22 mm; P=0.87).

Gastrointestinal function was recorded by participants 24 h following each study visit on a 100-mm scale. These were analyzed for differences between treatments and control and also compared with the gastrointestinal function data collected from participants at the time of prescreening (referred to as baseline). We found no differences in overall gastrointestinal function (P=0.74) between all 4 treatments and the control. However, some slight differences were seen when comparing gastrointestinal function 24 h after the study visit to that at the time of pre-screening. Problems with abdominal discomfort (scored from none to worst possible, 0–100 mm) increased after consumption of treatment meals and control compared with baseline (P=0.04), particularly the 3.1% [wt, 80:20; median 0 (interquartile range 0–19); P<0.01] and 3.1% [wt, 40:60; 0 (0–29); P<0.01] treatments. Cramping (scored from none to worst possible, 0–100 mm) increased after the consumption of 3 treatments and control [0 (0–9); P<0.05], except for the 9.3 wt% (40:60) treatment [0 (0–0); P=0.20], compared with baseline value of 0 (0–0). Flatulence (scored from none to worst possible, 0–100 mm) increased after consumption of the all treatments and control [9 (0–30)] compared with baseline value of 0 (0–12.5; P=0.04). Problems with defecation (scored from none to worst possible, 0–100 mm) increased after consumption of all treatments and control [7.5 (2–23)] compared with baseline value of 1 (0–3; P<0.01). Bowel urgency (scored from not urgent to worst possible, 0–100 mm) increased with all treatments and control [25 (13–48)] compared with baseline value of 5.5 (1–26.5; P=0.02). Last, overall gastric function (scored from perfect to worst possible, 0–100 mm) increased in all treatments and control [22 (13–40)] compared with baseline value of 10 (4–17; P<0.01).

## **2.5. DISCUSSION**

Consumption of milk products with enhanced protein concentration (9.3wt%), when compared with commercial milk (3.1 wt%) concentration, reduced postprandial BG and lowered post-lunch satiety, but had no effect on pre-lunch feelings of satiety, food intake at the pizza lunch meal, or post-lunch meal BG concentrations. Modifying the casein-to-whey ratio from 80:20 to 40:60 had a modest effect on lowering pre-lunch BG, regardless of protein concentration, whereas the normal casein-to-whey (80:20) ratio had lower pre-lunch Appetite than the treatments with proportionally more whey.

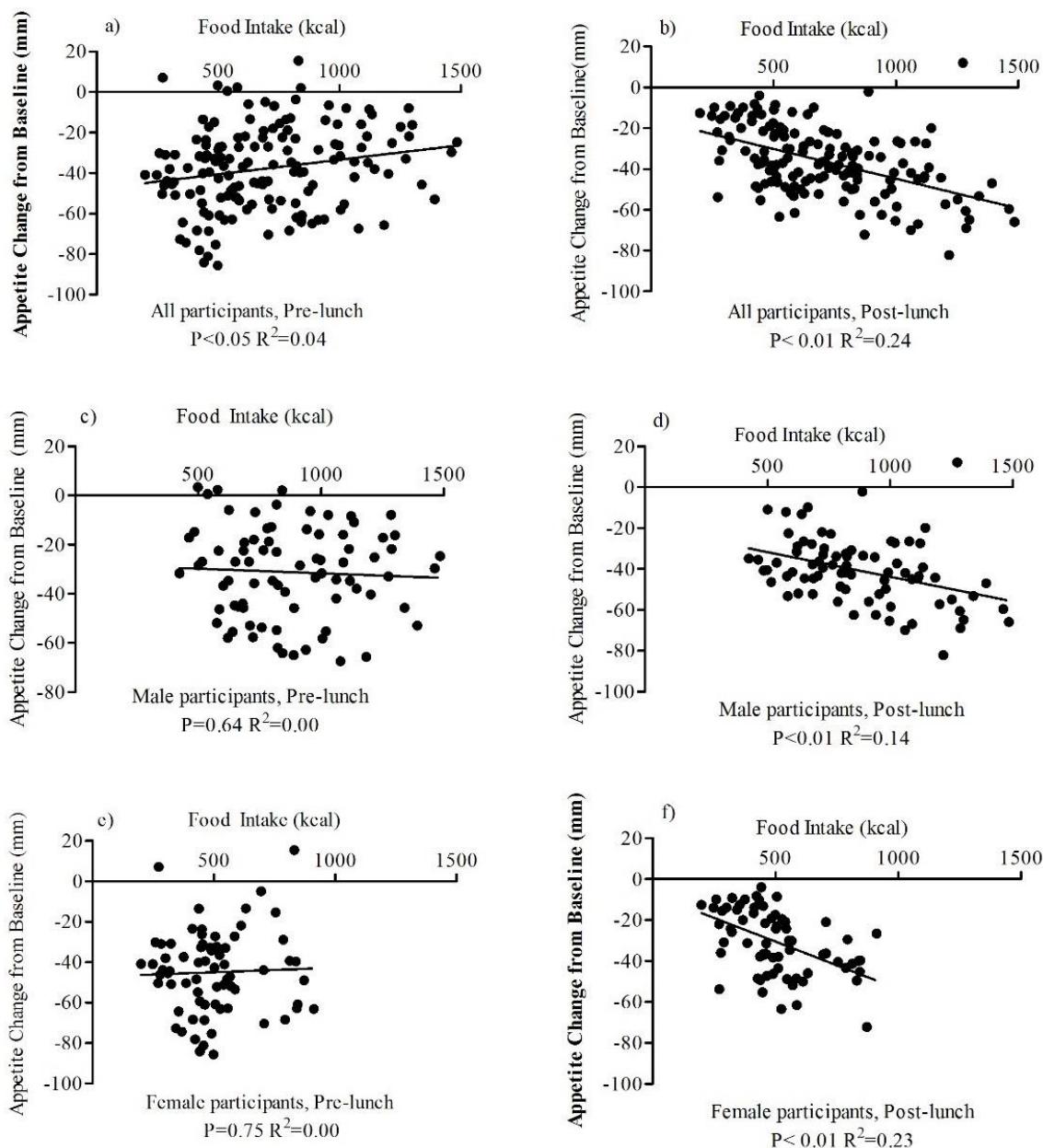
The pre-lunch (0–120 min) results support our hypothesis that the high protein (9.3 wt%) modified ratio (40:60) milk treatment with breakfast cereal would result in the lowest postprandial BG concentrations and iAUC values relative to the protein-free control. The greater reduction in BG with high-protein beverages is consistent with previous studies (Anderson et al., 2011; Panahi et al., 2013). The high-protein milks with 27.9 g of protein resulted in the lowest mean postprandial BG concentration compared with the low-protein milks containing 12.4 g. This result is comparable to Akhavan et al. (2010), who showed dose-dependent BG attenuation following consumption of 20, 30, and 40 g of whey protein, compared with 10 g, until 140 min. The lack of differences between the normal (80:20) and modified (40:60) protein ratio on BG iAUC was consistent with another study that compared a control casein-to-whey protein (2.8:1; 4.51 g of total protein) ratio against modified casein-to-whey protein (1.5:1) ratio in yogurt (4.49 g of total protein) in healthy men (Doyon et al., 2015). The increased whey protein concentration and modified ratio treatment combined with cereal meant that the treatment with the most whey protein (14.1 g of whey protein) had the lowest iAUC BG. This inverse relationship may relate to the fact that whey protein is insulinemic, resulting in a stronger influence on BG compared with casein (Luhovyy et al., 2007; Nilsson et al., 2004). However, the reduction of BG in the current

study by complete milk proteins is consistent with evidence of insulin-independent actions, including delayed gastric emptying and stimulation of the release of glucoregulatory hormones (Panahi et al., 2013; Akhavan et al., 2014, Chapter 3, Appendix L).

We anticipated that the higher-protein milks would promote feelings of satiety and decrease subsequent food intake. However, pre-lunch appetite changes from baseline and food intake were not different from control and across the milk treatments. The lack of effect of the differences in protein content of the beverages on appetite and food intake was surprising, but may be explained by several factors, including the high energy content of the treatments when combined with 2 servings of cereal, the time between the treatments and the meal, the differences in palatability of the treatments, the low food intakes of the female participants compared with male participants, and the high lactose content of the beverages. In our study, the breakfast meal of 2 servings of Honey Nut Cheerios (58 g total) and 250 mL of beverage provided an energy content varying from 400 to 500 kcal. The high energy content was clearly a factor, as the reduction in appetite was similar after the treatment meal compared with the control and remained below baseline to 120 min. This reduction in appetite to 120 min is consistent with a previous study that found chocolate milk or infant formula with energy contents of 340 and 370 kcal, respectively, but not milk with 200 kcal, suppressed appetite for 90 min but not up to 120 min. Food intake at a meal consumed at 30 min, but not 120 min later, reflected differences in appetite suppression (Panahi et al., 2013). Thus, in the present study, the time to the subsequent meal may not have been sufficient for differences in the composition of the treatments to be reflected beyond the effects of their energy content. The palatability of the beverages or the pizza meal did not appear to be a factor, as it was not correlated with either appetite ratings or food intake. Overall, gastrointestinal symptoms measured up to 24 h post-treatment were not different among treatments and control, but were higher compared with baseline ratings collected at

screening. The lactose content of the treatments perhaps offers an explanation for this, even though the participants all habitually consumed dairy products. The total lactose content of the beverages was 34.7 g, which is 3-fold the amount in a typical 250-mL serving of milk. High protein with normal lactose levels could have been achieved with the use of high-protein milk or whey protein concentrates rather than skim powder and whey protein concentrate-35, as was used in the current study. Also, on each study day participants received 12 finger pricks over the course of 3 h. Physical discomfort and stress from finger prick blood sampling can have an influence on glycemic response and antagonize insulin (Brouns et al., 2005), although it is standard for this type of protocol. Steps were taken to seat participants in a comfortable environment and the inclusion of acetaminophen in the study protocol may have partially mitigated pain as a contributing factor to stress.

Sex affected food intake ( $P<0.05$ ), as females weighed less than males but consumed the same treatment meals. Females appeared fuller after consuming all the treatment meals and maintained a suppressed appetite over time compared with males. This may have led to the opposite effects observed in post-lunch BG, as we observed no treatment effect but suppression of appetite was found. The difference in the contrasts of post-lunch BG change from baseline between sexes may be due to varying food intake, as female participants consumed less than male participants. Appetite suppression during the post-lunch period (120–200 min) by the 9.3 wt% (80:20) treatment was observed in pooled and female, but not male, participants. It was previously suggested that 9 g of combined milk protein should provide observable significance for whey to show an early satisfying effect and casein at a later duration (Anderson et al., 2011). In the current study, we were able to validate the satiating effect of casein in the post-lunch duration, but not the satiating effect of whey during the pre-lunch period. The suppression of appetite with 9.3 (wt%) protein (80:20) suggests that the aggregation and slow-digesting nature



**Figure 2.5** Correlations between food intake (kcal) and appetite change from baseline (mm·min) for 4 treatments and the control for pre-lunch (0–120 min) and post-lunch (120–200 min) periods for (a-b) all participants ( $n=30$ ), (c-d) males ( $n=16$ ), and (e-f) females ( $n=14$ )

of casein may have prolonged the feeling of fullness from the pizza meal. Thus, post-lunch correlation between food intake (kcal) and pre-lunch appetite change from baseline (mm) for all 4 treatments and the control showed an inverse relationship for all ( $P < 0.05$ ,  $R^2 = 0.24$ ), for male

( $P<0.05$ ,  $R^2=0.14$ ), and for female participants ( $P<0.05$ ,  $R^2=0.23$ ). However, an inverse correlation was only found in the pre-lunch period for pooled participants ( $P<0.05$ ,  $R^2=0.04$ ), and not within only male ( $P=0.64$ ,  $R^2=0.04$ ) or within only female ( $P=0.75$ ,  $R^2=0.0$ ) participants (Figure 2.5).

## **2.6. CONCLUSIONS**

Milk consumed with breakfast cereal reduced postprandial BG concentration compared with a water control, and high dairy protein concentration (9.3 wt%) reduced postprandial BG concentration compared with normal dairy protein concentration (3.1 wt%). The high (9.3 wt%) protein concentration treatments also reduced appetite after the second meal compared with the low (3.1 wt%) protein equivalent. A modest effect of modified (40:60) protein ratio was observed in terms of lower pre-lunch BG change from baseline and normal (80:20) protein ratio lowering pre-lunch appetite change from baseline. However, protein concentration and casein-to-whey ratio did not show effects on food intake at a subsequent meal in pooled participants. Thus, the results can be used to inform future development of milk-based beverages to focus on increase protein concentration to attenuate postprandial glucose and appetite suppression.

## **2.7. ACKNOWLEDGMENTS**

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Researchers maintain independence in conducting their studies, own their data, and report the outcomes, regardless of the results. The decision to publish the findings rests solely with the researchers.

## **2.8. BRIDGE TO CHAPTER 3**

As reviewed in Chapter 1, T2D is a serious metabolic disease and dietary interventions along with increased levels of exercise could potentially mitigate impaired glucose response. Chapter 2 considers the addition of dairy proteins as part of a high carbohydrate breakfast meal in 32 male and female participants. It indicates that a higher concentration of dairy proteins and modified ratio with enhanced whey results in the largest attenuation of BG during the pre-lunch duration. Thus, a closer examination into the effect of casein and whey ingestion is warranted. Therefore, Chapter 3 focuses on data from 12 subset participants (6 males and 6 females) from whom greater volumes of blood were obtained for further analysis. Specifically, the appearance of AAs was quantified, and the correlation of AA digestion kinetics with paracetamol appearance in the blood, as an indirect assessment of gastric emptying, was studied as a potential mechanism for glucose management. Of note, blood samples were also analyzed by collaborators for gastric hormones such as insulin, C-Peptide, PYY, ghrelin and GLP-1 and is found in Appendix L. The emphasis of this thesis was to discern the role of whey proteins on postprandial glycemia and satiety ratings, focusing on the mechanisms of carbohydrate and protein digestibility using gastric emptying, plasma AA appearance, and *in vitro* digestion measurements.

**CHAPTER 3: PLASMA AMINO ACID RESPONSES FOLLOWING MILK CONSUMPTION CONTAINING ENHANCED PROTEIN CONCENTRATION AND MODIFIED CASEIN-TO-WHEY RATIO WITH BREAKFAST CEREAL IN HEALTHY YOUNG ADULTS**

*In Preparation*

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### **3.1. ABSTRACT**

Increasing the total protein content and reducing the casein:whey ratio in milks consumed with breakfast cereal has been shown to reduce BG concentrations. However, the effect on plasma AAs and gastric emptying (by indirect paracetamol method) has not been reported. In a randomized, controlled, doubled-blind crossover study with healthy young adults (n=12, 6M/6F, 22.8 ± 3.0 y, BMI 23.2 ± 2.7 kg/m<sup>2</sup>), participants consumed milk (250 mL) with commercial (3.1 wt%) or high protein (9.3 wt%) concentration in which the casein:whey ratios were either 80:20 (normal) or 40:60 (modified) or control (water with whey permeate). Milks were served with 2 servings (58 g) of oat-based breakfast cereal. Blood for analysis of free AAs (total amino acids (TAA), branched-chain amino acids (BCAA), essential amino acids (EAA) and nonessential amino acids (NEAA)) and paracetamol were obtained by indwelling venous catheter for 2 hr (0 – 120 min). Participants consumed a weighed *ad libitum* pizza lunch at 120 min and blood sampling continued following the lunch meal (140 – 200 min). Pre-lunch, high (9.3 wt%) protein milk increased all AA groups (TAA, BCAA, EAA and NEAA) when contrasted to normal (3.1 wt%) protein concentration ( $P<0.01$ ). Also, the low casein to whey ratio (40:60) milks increased BCAA and EAA iAUC compared to the normal (80:20) ratio ( $P<0.01$ ), but not TAA and NEAA ( $P=0.05$  and  $P=0.18$ , respectively) in the pre-lunch duration. Blood paracetamol pre-lunch iAUC

(P=0.03) was lower after ingestion of the high (9.3 wt%) protein milks, but there was only an effect of modified ratio at 30 min (P=0.01). All pre-lunch (0-120 min) AAs were inversely correlated with paracetamol change from baseline concentration (P<0.05). However, food intake was not affected by treatment (P=0.72). This provides a novel observation into the post-lunch meal period. In the post-lunch duration (120-200 min), high (9.3 wt%) protein milks continued to increase TAA, BCAA, EAA and NEAA iAUC compared to those with low (3.1 wt%) protein concentration (P<0.05). Normal (80:20) ratio increased TAA compared to modified (40:60) protein ratio at 200 min (P=0.02), and 9.3 wt% 40:60 treatment lowered paracetamol concentration compared to the control (P<0.01). Therefore, pre-lunch plasma AA concentrations were increased by the high (9.3 wt%) protein milks and plasma BCAA and EAA, specifically, by the milks with higher proportion of whey. The increased presence of BCAA and EAA were accentuated by slower gastric emptying and may provide an explanation for the attenuation of BG observed in Chapter 2.

### **3.2. INTRODUCTION**

T2D is a chronic metabolic disease that occurs when the body cannot properly produce and or use insulin to manage BG (World Health Organization, 2016). Over 10 million Canadians are currently affected by the disease (Canadian Diabetes Association, 2015) and many more are at risk. The growing prevalence of T2D rationalizes dietary strategies for prevention and/or management. In particular, previous research into dairy bovine proteins has demonstrated their BG-lowering effects, which occur by stimulation of insulin and incretin hormones (McGregor and Poppitt, 2013; Anderson et al., 2011; Law et al., 2017a and 2017b). Also, there are suggestions that consumption of a high protein diet lowers subsequent food intake (Akhavan et al., 2010; Anderson et al., 2011). Our own findings demonstrated that high (9.3 wt%) protein

concentration significantly attenuated postprandial BG compared to low (3.1 wt%) protein concentration but no difference in subsequent food intake in pooled 32 participants (Kung et al., 2018, Chapter 2). Furthermore, other studies have suggested delayed gastric emptying of stomach chyme as playing an alternate role in glucose management by serving to delay the uptake of carbohydrates and consequently lowering food intake at a second meal because of longer stomach retention of the breakfast meal (Akhavan et al., 2014; Panahi et al., 2014).

Gastric emptying refers to the release of stomach chyme contents into the small intestine for further digestion and absorption and occurs through antral and pylorus contractions and gastric tone (Hasler, 2009). The rate of emptying depends on meal volume, composition, solid/liquid ratio, and post-ingestive and post-absorptive nutrients that may influence gastric hormone production of CCK, GLP-1 and PYY in enteroendocrine cells (Calbet and Holst, 2004; Aziz and Anderson, 2007; Akhavan et al., 2009).

Native bovine milk proteins are comprised of 80% casein and 20% whey. Casein proteins form insoluble micelle structures (30 – 300 nm) due to the large amounts of proline residues and their interaction with calcium, which disrupt the formation of  $\alpha$ -helical and  $\beta$ -sheets in the secondary structure (Dagleish and Corredig, 2012; Phadungath, 2005). Whey proteins form soluble globular tertiary structures due to the higher concentration of cysteine, compared to casein, as more cysteine AAs forms disulfide bridges. During gastric digestion, peptic hydrolysis of casein proteins and a pH which is close to casein's isoelectric point (pH 4.6), destabilises the micelle structure, leading to rearrangement into an aggregated network (Lentle and Janssen, 2011). The aggregated three-dimensional coagulate separates from the remaining soluble digesta and tends to resist emptying from the stomach, prolonging retropulsion, i.e. the process in which muscular movement helps to break larger lumps of food into smaller pieces by pushing food that has entered the duodenum backwards into the stomach for further mixing of chyme with gastric

juices. Whey protein, on the other hand, is soluble at pH 4.6 such that it remains dispersed and empties from the stomach earlier than casein. Based on the observed rises in plasma AAs following whey or casein ingestion, whey protein has been termed a “rapidly” digested protein that passes through the stomach quickly whereas casein is notably “slowly” digested (Boirie et al., 1997).

In the small intestine, milk proteins continue to be hydrolyzed, releasing peptides and free AAs that reflect their protein origin, since milk proteins contain distinct AA profiles and concentrations. Specifically, casein contains higher amounts of methionine, phenylalanine, proline and histidine, whereas whey proteins contain higher concentrations of lysine, threonine, tryptophan and BCAA (leucine, isoleucine and valine) (Fox and McSweeney, 1998). These differences in AA, in particular BCAA, may be the primary reason whey proteins are considered to be insulinotropic compared to casein (Dangin et al., 2001). It has been suggested that BCAA impacts on metabolic process such as stabilizing postprandial levels of BG and insulin, which has the potential impact to lower food intake (Layman, 2003). Furthermore, an insulin-independent mechanism is also proposed in which the consumption of whey protein improves glucose management by delaying gastric emptying (Akhavan et al., 2014).

Some studies have compared the AA composition between whey and casein and post-absorptive effects on delayed gastric emptying. Bowen et al. (2006) compared 55 g of whey or casein protein to 56 g of lactose or glucose in 100 mL of water in overweight males. They noted lowered plasma paracetamol AUC (reflecting lower rate of gastric emptying) and higher CCK with protein treatments compared to both carbohydrates, and differences in BCAA concentrations between whey and casein treatments. In another study, Veldhorst et al. (2009) compared the effects of high (25%) or normal (10%) energy intake from protein between casein, soy or whey protein at breakfast in healthy male and female participants (11 m/ 14 f). Increased

levels of AA accompanied by satiety hormonal (GLP-1 and ghrelin) response were observed. In that study, delayed gastric emptying was suggested as the mechanism for increased satiety, although food intake was not reduced. Furthermore, Sun et al. (2016) reported insulin-independent mechanisms involved in glycemic management and compared plasma AAs after consumption of soy or cow's milk (322 mL) in healthy males. However, it remains unclear if delayed gastric emptying is a response to the release of BCAA. Thus, we aim to investigate the alternative management of BG (from Chapter 2) through insulin-independent mechanisms, by whey protein and the release of BCAA. The purpose of this study in healthy adults is to test the effects of 3-fold increased protein concentration (from 3.1 wt% to 9.3 wt%) and modified 40% casein to 60% (i.e. higher BCAA) whey protein ratio. It is hypothesized that consumption of the higher (9.3 wt%) protein concentration and modified (40:60) ratio treatments will lead to increases in postprandial plasma free AAs, particularly BCAA, slower gastric emptying and decreased food intake at a subsequent meal.

### **3.3. MATERIALS AND METHODS**

#### *3.3.1. Treatment composition and preparation*

Breakfast drinks (250 mL) based on skim milk (Neilson Dairy – Saputo Dairy Products Canada, G.P., St.-Laurent, QC, Canada) or control (water with whey permeate) were formed as described in Chapter 2. Treatments were combined with 58 g of oat-based cereal (Honey Nut Cheerios, General Mills, Mississauga, ON, Canada) to form the breakfast meal totalling approximately 76.7 g available carbohydrate, from cereal (42 g) and lactose (34.7 g) (Chapter 2 Table 2.1 and 2.2). Protein powders and Honey Nut Cheerios were sent to Agricultural Experiment Station Chemical Laboratories (University of Missouri-Columbia, Columbia, MO, USA) for complete AA profiling (AOAC Official Method 982.30 E (a,b,c), chap. 45.3.05.2006).

**Table 2.1** Treatment composition (250 mL)

Treatment <sup>1</sup> (250 mL)	Water (mL)	Skim Milk Liquid (mL)	Permeate Powder (g)	Skim Milk Powder (g)	Whey Protein Concentrate Powder (g)
Control	250	-	47.3	-	-
3.1% MP (80:20) <sup>2</sup>	-	250	31.9	-	-
3.1% MP (40:60) <sup>3</sup>	125	125	31.6	-	12.5
9.3% MP (80:20) <sup>4</sup>	-	250	-	46.8	-
9.3% MP (40:60) <sup>5</sup>	-	250	-	11.8	37.7

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey; normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

**Table 2.2.** Combined composition of milk treatment (250 mL) with breakfast cereal (58 g)

Treatment <sup>1</sup>	Calories (kcal)	Available Carbohydrates (g)	Casein Protein (g)	Whey Protein (g)	Total Protein (g)
Control	403.8	76.7	0	0.3	4.6
3.1% MP (80:20)	433.1	76.7	6.2	1.6	12.4
3.1% MP (40:60)	434.3	76.7	3.1	4.8	12.4
9.3% MP (80:20)	485.6	76.7	18.6	4.7	27.9
9.3% MP (40:60)	499.1	76.7	9.3	14.1	27.9

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey; normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

AAs were grouped into EAA (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), NEAA (alanine, arginine, asparagine, ornithine, serine, cysteine, glutamine, glycine, proline, tyrosine), BCAA, and TAA (the sum of essential and non-essential) and were calculated based on the treatment composition (250 mL) with cereal (58 g) (Table 3.1). AA concentrations in the test meals are shown in Table 3.1. Accordingly, higher amounts of AAs (mg) were present in the high (9.3 wt%) concentration treatments compared to low (3.1 wt%) concentration treatments. More leucine, isoleucine, lysine, threonine, tryptophan, alanine, aspartic acid, and cysteine (mg) were found in the 40:60 treatments, and higher amounts of valine, histidine, methionine, phenylalanine, arginine, serine, glutamic acid, and proline (mg) were found in the 80:20 treatments (Table 3.1). AA amounts determined for the high (9.3 wt%)

treatment with breakfast cereal were as expected as that of the low (3.1 wt%) protein concentration meal, and the observed differences between the modified and normal protein ratios were as predicted based on formulation.

**Table 3. 1** Combined AA composition of milk treatments (250 mL) and breakfast cereal (58 g)

(g)	Treatment <sup>1</sup>				
	Control	3.1% MP (80:20)	3.1% MP (40:60)	9.3% MP (80:20)	9.3% MP (40:60)
<b>BCAA<sup>6</sup></b>	<b>0.78</b>	<b>2.43</b>	<b>2.50</b>	<b>5.74</b>	<b>5.96</b>
<i>Leucine</i>	0.35	1.10	1.14	2.60	2.72
<i>Isoleucine</i>	0.18	0.59	0.63	1.40	1.54
<i>Valine</i>	0.25	0.74	0.73	1.74	1.70
<b>Total EAA<sup>7</sup></b>	<b>1.60</b>	<b>5.05</b>	<b>5.18</b>	<b>11.95</b>	<b>12.40</b>
<i>Histidine</i>	0.10	0.31	0.28	0.73	0.64
<i>Isoleucine</i>	0.18	0.59	0.63	1.40	1.54
<i>Leucine</i>	0.35	1.10	1.14	2.60	2.72
<i>Lysine</i>	0.19	0.81	0.86	2.05	2.21
<i>Methionine</i>	0.07	0.24	0.23	0.60	0.54
<i>Phenylalanine</i>	0.24	0.61	0.55	1.35	1.19
<i>Threonine</i>	0.16	0.47	0.57	1.08	1.39
<i>Tryptophan</i>	0.06	0.18	0.20	0.41	0.47
<i>Valine</i>	0.25	0.74	0.73	1.74	1.70
<b>Total NEAA<sup>8</sup></b>	<b>2.70</b>	<b>6.97</b>	<b>6.86</b>	<b>15.51</b>	<b>15.22</b>
<i>Alanine</i>	0.22	0.46	0.52	0.94	1.15
<i>Arginine</i>	0.27	0.53	0.49	1.04	0.95
<i>Aspartic Acid</i>	0.36	0.93	1.05	2.06	2.43
<i>Serine</i>	0.19	0.54	0.53	1.23	1.21
<i>Cysteine</i>	0.11	0.16	0.22	0.26	0.43
<i>Glutamic Acid</i>	0.95	2.57	2.43	5.80	5.41
<i>Glycine</i>	0.22	0.36	0.36	0.64	0.66
<i>Proline</i>	0.26	0.97	0.85	2.39	2.02
<i>Tyrosine</i>	0.12	0.46	0.40	1.14	0.96
<b>TAA<sup>9</sup></b>	<b>4.30</b>	<b>12.02</b>	<b>12.04</b>	<b>27.46</b>	<b>27.62</b>

<sup>1</sup> Control (water with whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>6</sup> Branched-chained amino acids (leucine, isoleucine, valine)

<sup>7</sup> Total essential amino acids

<sup>8</sup> Total non-essential amino acids

<sup>9</sup> Total amino acids are the sum of essential and non-essential amino acids

### 3.3.2. Study design and protocol

Healthy young men and women (6 males and 6 females) adult participants were recruited from the University of Guelph and surrounding community. All participants met the following inclusion criteria: aged 18-30 y, body mass index 20-24.9 kg/m<sup>2</sup>, regular milk consumers (>2 servings/week), typical breakfast consumers (before 9:00am 5 days/week) and fasting BG 3.5-5.5 mmol/L. Study design and exclusion criteria were as described in Chapter 2. Blood samples were taken by an indwelling venous catheter to allow sufficient blood volume for paracetamol (acetaminophen), free AAs, insulin and gastric hormones (Appendix L). Sampling occurred at 30 min intervals (i.e. 0, 30, 60, 120, 140, 170, 200 min). At 120 min, participants, in isolation, were served an *ad libitum* pizza lunch and instructed to eat until comfortably full, as described in Chapter 2. Two female participants' consumed <150 kcal, which is not representative of a lunch caloric consumption 2 hrs after breakfast (Akhavan et al., 2010) and their data was thus excluded. Venous blood sampling continued for 1 hr after the pizza lunch (140, 170 and 200 min).

**Table 3. 2** Baseline characteristics for participants collected during in-person screening

	All Participants (n=12)		Male Participants (n=6)		Female Participants (n=6)	
	Mean	SD	Mean	SD	Mean	SD
Age (y)	22.8	3.0	23.2	2.9	22.5	3.3
Height (m)	1.7	0.1	1.8	0.1	1.7	0.1
Weight (kg)	70.0	11.2	78.1	7.7	62.0	7.7
BMI (kg/m <sup>2</sup> )	23.2	2.7	24.1	3.1	22.3	2.1
Systolic/ Diastolic Blood Pressure (mmHg)	124.5 / 71.3	13.8 / 10.0	14.9 / 72.1	14.9 / 9.6	116.8 / 70.6	7.1 / 11.2
Fasting BG (mmol/L)	5.0	0.4	5.1	0.3	5.0	0.4

### *3.3.3. Blood paracetamol and AAs analyses*

Blood for paracetamol analysis was collected in 8.5 mL BD P800 tubes (BD Diagnostics, Franklin Lakes, NJ, USA) containing spray-dried K<sub>2</sub>EDTA anticoagulant and proprietary additives to prevent their immediate proteolytic activity. The tubes were centrifuged at 2600 RCF for 20 min at 4°C. Plasma was aliquoted into Eppendorf tubes and stored at -80 °C for future analysis. Blood for free AAs was collected in 3.5 mL BD K<sub>2</sub>EDTA<sub>2</sub> tubes (Becton Dickinson, Franklin Lakes, NJ). The tubes were centrifuged at 2400 rpm for 10 min at 4°C. Collected plasma samples were aliquoted in Eppendorf tubes and stored at -80°C prior to analysis. Aliquotted samples from BD P800 tubes were sent to the Department of Nutritional Sciences, University of Toronto, for free paracetamol and hormone analyses. Free paracetamol was determined with commercially-available enzymatic assay from Cambridge Life Sciences (Ely, Cambridge, UK).

Another set of frozen aliquoted plasma samples was sent, on dry ice, to the Department of Food Sciences, Université Laval for AA analyses (alanine, glycine, valine, leucine, isoleucine, threonine, serine, ornithine, proline, asparagine, methionine, glutamine, phenylalanine, glutamic acid, lysine, histidine, tyrosine, tryptophan, cysteine) by EZ:faast kit (Phenomenex, Torrence, CA, USA) in GC-FID (GC-2010 Shimadzu) performed by a technician as per the instrument manual. Briefly, 100 µL of plasma was mixed with 100 µL of Norvaline and this solution was passed through sorbent tips. A washing solution (200 µL) was then pipetted to clean the sample. AAs were derivatized with 50 µL of propyl chloroformate octane and 100 µL of 1N HCl was added. Finally, the organic layer was transferred to a GC vial with insert. A 2 µL sample was injected to the ZB-AAA 10 m x 0.25 mm id GC column with a split ratio of 1:10 (v/v). The column flow was constant at 1.7 mL/min of hydrogen. Nitrogen (30 mL/min), hydrogen (40 mL/min) and air (400 mL/min) were used as auxiliary gases for flame ionisation detector set at

320°C. The oven temperature was initially set at 110°C, then increased to 320°C at 32°C/min. Repeatability was assessed for each AA from 3 samples injected 3 times for calculating mean relative standard of deviation (RSD). AA concentrations were compared to an internal standard (Norvaline) solution (200 nM each).

### *3.3.4. Data and statistical analyses*

Paracetamol and free AA (BCAA, EAA, NEAA, TAA) values were checked for normality using SAS PROC Univariate test. Free AAs were normally distributed, but paracetamol data was log transformed to become normally distributed before analyses. All milk treatments were firstly compared to the whey permeate control in the statistical analysis.

Pre-lunch meal AAs and paracetamol concentrations were compared based on incremental area under the curve (iAUC), which was calculated using GraphPad Prism version 5 using the trapezoidal method (GraphPad Software. San Diego, CA, USA). Pre-lunch paracetamol and free AAs iAUCs, energy intake, and paracetamol peak concentration (Cmax) data were analyzed using repeated measures PROC MIXED two-way (treatment and gender) ANOVA, followed by Tukey-Kramer test to compare treatments to the control by SAS version 9.3 (SAS Institutes, Cary, NC, USA). Results were pooled for males and females, unless a significant gender effect or gender and treatment interaction was found, in which case a one-way ANOVA (treatment) for each gender followed by Tukey's *post hoc* testing was performed to identify differences between treatments within each gender. Also, to complement iAUC data, a one-way ANOVA at each time point was utilized to compare absolute values of paracetamol and AA concentration followed by Tukey's *post hoc* test to compare treatments to the control.

Post-lunch paracetamol and free AA absolute mean concentrations were investigated using repeated measures PROC MIXED three-way (treatment, time and gender) ANOVA, followed by Tukey-Kramer testing to compare treatments to the control. When a significant

treatment and time interaction was observed, a one-way ANOVA (treatment) was utilized at each time point, followed by Tukey's *post hoc* test. Results were pooled for males and females, unless a significant gender effect or gender and treatment interaction was found, in which case a two-way ANOVA (treatment and time) for each gender followed by Tukey's *post hoc* testing was performed to identify differences between treatments within each gender.

Paracetamol time to peak concentration (Tmax) was investigated using non-parametric Kruskal-Wallis analysis (treatment x gender). Also, orthogonal contrast statements were used to group pairs of treatments (not including the whey permeate control) according to 1) protein concentration and 2) protein ratio as previously for Cmax as described in Chapter 2. Paracetamol Tmax values were also compared using pair-wise Wilcoxon non-parametric testing in the same treatment groups as above.

Linear regression and Pearson's correlation coefficient were used to explore pre and post-lunch change from baseline associations between paracetamol (mmol/L) and AAs group in pooled participants. Correlations were analyzed with the 4 treatments and the control together. Significance was set at P<0.05. Data are presented as mean  $\pm$  SEM.

### **3.4. RESULTS**

#### *3.4.1. Food intake*

At the lunch meal, male participants consumed  $1,036.7 \pm 47.7$  kcal of the cheese pizza while female participants consumed much less, i.e. only  $550.3 \pm 24.2$  kcal. There were no differences in food intake between milk treatment protein concentration or ratio ( $P>0.05$ ) for pooled, male or female participants. These subset results are similar to those for the overall participant group ( $n=32$ ), as presented in Chapter 2, although, in that case, there was an effect of gender ( $P<0.01$ ), not observed for the subset participants.

### *3.4.2. AAs pre-lunch (iAUC) and post-lunch (absolute values)*

The effects of treatments on pre-lunch AAs iAUC and post-lunch absolute values are shown for TAA, BCAA, EAA, and NEA in Table 3.3. Pre-lunch TAA, BCAA, EAA and NEAA were all affected by protein concentration ( $P<0.01$ ). The ratio affected BCAA, and EAA ( $P<0.05$ ) but not TAA and NEAA iAUC ( $P=0.05$  and  $P=0.18$ , respectively). Post-lunch AA mean concentrations were not affected by ratio, but protein concentration affected TAA, BCAA, NEAA, and EAA ( $P<0.01$ ). These summarized effects are detailed, as follows.

#### **TAA**

Pre-lunch TAA iAUC differed by treatment ( $P<0.01$ ), but was not affected by gender ( $P=0.68$ ) or treatment  $\times$  gender interactions ( $P=0.18$ ). Thus, all participant data were pooled. All milk treatments except for 3.1 wt% 80:20 treatment increased TAA iAUC compared to the control. At 30 and 120 min specifically, 9.3 wt% 40:60 treatment had the highest increase of TAA concentration compared to the control, i.e. by  $37.4 \pm 11.8 \mu\text{M}$  ( $P<0.01$ ) and  $651.7 \pm 211 \mu\text{M}$  ( $P<0.01$ ), respectively (Figure 3.1). Furthermore, high (9.3 wt%) increased TAA iAUC compared to low (3.1 wt%) protein concentration by  $71,477 \pm 15,692 \mu\text{M}\cdot\text{min}$  ( $P<0.01$ ) and modified (40:60) increased TAA iAUC compared to normal (80:20) by  $31,301 \pm 15,239 \mu\text{M}\cdot\text{min}$  ( $P<0.05$ ) (Table 3.3).

Post-lunch, TAA concentration differed by treatment ( $P<0.01$ ), treatment  $\times$  time ( $P=0.03$ ), but not time ( $p=0.05$ ) or gender ( $P=0.50$ ) and there was no treatment  $\times$  gender interaction ( $P=0.34$ ). Thus, all participant data were pooled. All milk treatments increased TAA concentration compared to the control (Tukey Kramer  $P<0.05$ ). At 140 and 170 min specifically, high (9.3 wt%) increased post-lunch TAA compared to low (3.1 wt%) protein concentration treatment by  $860.0 \pm 222.3 \mu\text{M}$  ( $P<0.01$ ) and  $742.4 \pm 186.3 \mu\text{M}$  ( $P<0.01$ ), respectively. At 200 min, 9.3 wt% (80:20) treatment had the highest TAA concentration compared to the control by  $428.0 \mu\text{M}$ .

$\pm 133.1 \mu\text{M}$  (Tukey-Kramer,  $P=0.02$ ) (Figure 3.1). Overall, post-lunch high (9.3 wt%) protein increased TAA compared to low (3.1 wt%) protein concentrations ( $644.8 \pm 112 \mu\text{M}$ ,  $P<0.01$ ), but there was no difference between protein ratios ( $P=0.90$ ) (Table 3.3).

**Table 3.3** Mean ( $\pm$ SEM) AAs iAUC during pre-lunch (0 – 120 min) and absolute concentrations during post-lunch (140 – 200 min) periods

	Treatments <sup>1</sup>					P-value <sup>2</sup>	Concentration P-value <sup>3</sup>	Ratio P-value <sup>4</sup>			
	Control	3.1 wt% (80:20)	3.1 wt% (40:60)	9.3 wt% (80:20)	9.3 wt% (40:60)						
<b>Pre-lunch iAUC (0 – 120 min, <math>\mu\text{M}\cdot\text{min}</math>)</b>											
All (n=12)											
TAA	$5,222 \pm 2979^a$	$24,997 \pm 4786^{ab}$	$40,629 \pm 12339^{bc}$	$60,717 \pm 10474^{cd}$	$76,385 \pm 7433^d$	<0.01	<0.01	0.05			
BCAA	$393 \pm 268^a$	$3,935 \pm 909^{ab}$	$8,531 \pm 3352^{bc}$	$12,720 \pm 2247^{cd}$	$17,752 \pm 2669^d$	<0.01	<0.01	0.02			
EAA	$1,032 \pm 630^a$	$8,701 \pm 1704^a$	$17,408 \pm 6986^{ab}$	$29,826 \pm 4820^{bc}$	$40,160 \pm 4697^c$	<0.01	<0.01	0.03			
NEAA	$6,154 \pm 2609^a$	$19,005 \pm 3459^{ab}$	$25,008 \pm 6182^{bc}$	$31,015 \pm 5987^{bc}$	$36,136 \pm 3292^c$	<0.01	<0.01	0.18			
<b>Post-meal (140 – 200 min, <math>\mu\text{M}</math>)</b>											
BCAA	$381 \pm 17^a$	$454 \pm 16^b$	$457 \pm 16^b$	$525 \pm 14^c$	$557 \pm 17^c$	<0.01	<0.01	0.12			
EAA	$913 \pm 33^a$	$1,035 \pm 27^b$	$1,067 \pm 27^b$	$1,235 \pm 22^c$	$1,283 \pm 28^c$	<0.01	<0.01	0.09			
NEAA	$1,717 \pm 54^a$	$1,834 \pm 48^{ac}$	$1,857 \pm 45^{abc}$	$1,997 \pm 46^{bc}$	$1,906 \pm 55^c$	<0.01	<0.01	0.34			
TAA	$2,681 \pm 81^a$	$2,923 \pm 68^b$	$2,979 \pm 68^b$	$3,295 \pm 62^c$	$3,252 \pm 79^c$	<0.01	<0.01	0.90			
<b>Males (n=6)</b>											
EAA	$1,009 \pm 50^a$	$1,071 \pm 35^a$	$1,055 \pm 24^a$	$1,238 \pm 25^b$	$1,300 \pm 33^b$	<0.01	<0.01	0.40			
<b>Females (n=6)</b>											
EAA	$817 \pm 29^a$	$999 \pm 41^b$	$1,080 \pm 48^{bc}$	$1,232 \pm 36^{cd}$	$1,265 \pm 45^d$	<0.01	<0.01	0.14			

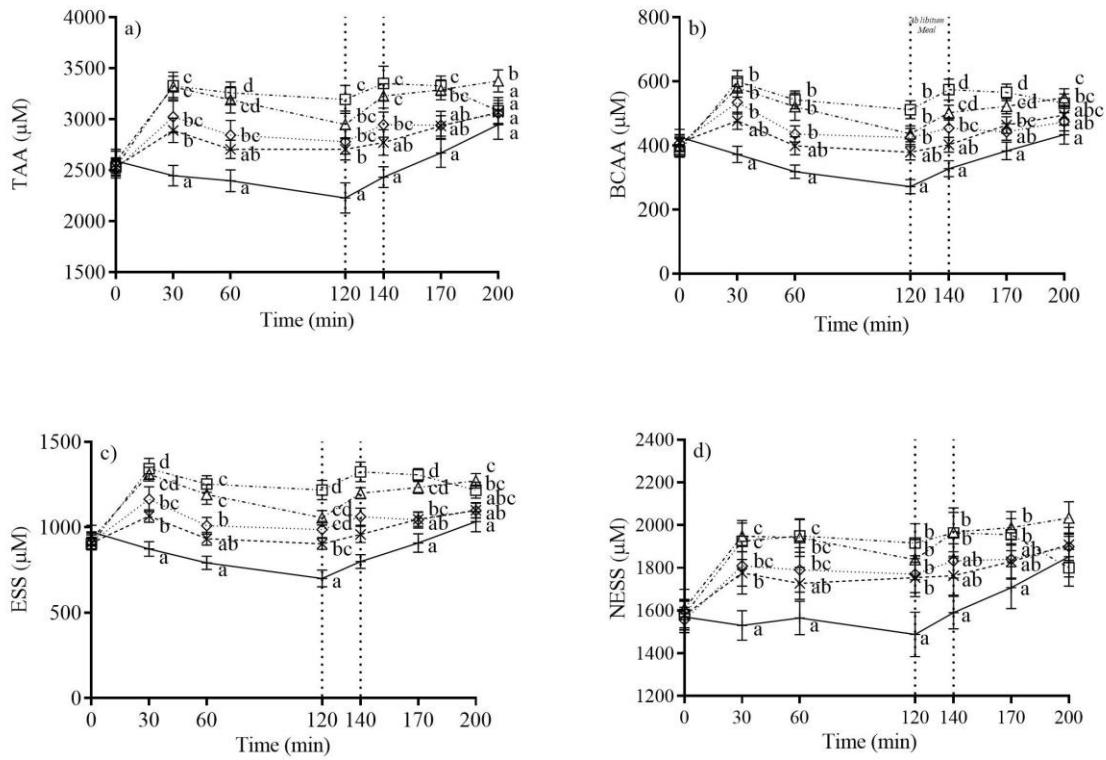
<sup>a-d</sup>Means within a row with different superscripts differ according to Tukey-Kramer ( $P < 0.05$ ).

<sup>1</sup> Control (water and whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey; normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>2</sup> ANOVA P-value for treatment effect

<sup>3</sup> Contrast between high and low protein concentration (mean 9.3 wt% (80:20) + 9.3 wt% (40:60) to mean 3.1 wt% (80:20) + 3.1 wt% (40:60))

<sup>4</sup> Contrast between modified and normal ratio (mean 9.3 wt% (40:60) + 3.1 wt% (40:60) to mean 9.3 wt% (80:20) + 9.3 wt% (80:20))



**Figure 3. 1** Mean ( $\pm$  SEM) a) TAA, b) BCAA, c) EAA, and d) NEAA for cumulative duration 0 – 200 min for all participants for control (+), 3.1% milk protein (MP) (80 casein:20 whey protein) (X), 3.1% MP (40 casein:60 whey protein) ( $\diamond$ ), 9.3% MP (80 casein:20 whey protein) ( $\triangle$ ), and 9.3% MP (40 casein:60 whey protein) ( $\square$ ). <sup>a-d</sup> Different letters at each measured time are different between treatment according to adjusted Tukey-Kramer ( $P < 0.05$ ).

### BCAA

Pre-lunch BCAA iAUC differed by treatment ( $P < 0.01$ ), but not gender ( $P = 0.75$ ) and no treatment  $\times$  gender interaction ( $P = 0.34$ ) was observed (Figure 3.1). Thus, all participant data were pooled. High protein treatments, regardless of protein ratio (9.3 wt% 80:20 and 9.3 wt% 40:60), increased BCAA iAUC compared to the control (Tukey-Kramer,  $P < 0.01$ ). High (9.3 wt%) increased BCAA compared to low (3.1 wt%) protein concentration by  $18,006 \pm 4,078.7 \mu\text{M} \cdot \text{min}$  ( $P < 0.01$ ) and modified (40:60) increased BCAA compared to normal (80:20) protein ratio by  $9,627.6 \pm 4,078.6 \mu\text{M} \cdot \text{min}$  ( $P = 0.02$ ) (Table 3.3).

In post-lunch, BCAA mean concentration differed by treatment ( $P<0.01$ ) and time ( $P<0.01$ ), but not gender ( $P=0.07$ ), and treatment  $\times$  time ( $P=0.01$ ), but no treatment  $\times$  gender ( $P=0.07$ ) interactions were observed. At 140 min specifically, high (9.3 wt%) increased BCAA compared to low (3.1 wt%) protein concentration by  $220.0 \pm 41.9 \mu\text{M}$  ( $P<0.01$ ) and modified (40:60) increased BCAA compared to normal (80:20) protein ratio by  $124.2 \pm 41.9 \mu\text{M}$  ( $P<0.01$ ). Furthermore at 170 and 200 min, high protein (9.3 wt%) continued to increase BCAA compared to low (3.1 wt%) protein concentration by  $182.0 \pm 39.3 \mu\text{M}$  ( $P<0.01$ ) and  $111.4 \pm 36.0 \mu\text{M}$  ( $P<0.01$ ), respectively (Figure 3.1). Also, normal (80:20) slightly increased BCAA compared to modified (40:60) ratio for low 3.1 wt% by  $20.9 \pm 25.8 \mu\text{M}$  ( $P=0.93$ ) and high 9.3 wt% protein concentration by  $17.5 \pm 25.8 \mu\text{M}$  ( $P=0.96$ ) at 200 min. Overall, there was no difference between protein ratio ( $P=0.12$ ), but high (9.3 wt%) increased BCAA concentration relative to low (3.1 wt%) protein concentration by  $171.1 \pm 22.4 \mu\text{M}$  ( $P<0.01$ ) (Table 3.3).

### **EAA**

Pre-lunch EAA iAUC differed by treatment ( $P<0.01$ ), but no significant gender effect ( $P=0.59$ ) or treatment  $\times$  gender interaction ( $P=0.26$ ) were observed. Thus, all participant data were pooled. High protein treatments (9.3 wt% 80:20 and 9.3 wt% 40:60) increased EAA iAUC relative to the control (Tukey-Kramer  $P<0.01$  and  $P<0.01$ , respectively). At 60 min specifically, high (9.3 wt%) increased EAA iAUC compared to low (3.1 wt%) protein concentration by  $82.2 \pm 31.2 \mu\text{M}$  ( $P=0.01$ ). Also at 120 min, high (9.3 wt%) increased EAA iAUC compared to low (3.1 wt%) protein concentration by  $384.8 \pm 89.1 \mu\text{M}$  ( $P<0.01$ ) and modified (40:60) increased EAA iAUC compared to normal (80:20) ratio by  $245.5 \pm 89.1 \mu\text{M}$  ( $P<0.01$ ) (Figure 3.1). Overall, high (9.3 wt%) increased EAA iAUC compared to low (3.1 wt%) protein concentration

by  $43,877 \pm 8,343 \mu\text{M}\cdot\text{min}$  ( $P<0.01$ ) and modified (40:60) increased EAA iAUC compared to the normal (80:20) ratio by  $19,042 \pm 8,343 \mu\text{M}\cdot\text{min}$  ( $P=0.02$ ) (Table 3.3).

Post-lunch, EAA mean concentration differed by treatment ( $P<0.01$ ) and time ( $P=0.03$ ), and there were treatment  $\times$  time ( $P=0.01$ ) and treatment  $\times$  gender ( $P=0.01$ ) interactions, but no significant gender effect ( $P=0.25$ ) (Table 3.3). At 140 min, high (9.3 wt%) increased EAA compared to low (3.1 wt%) protein concentration by  $219.9 \pm 41.9 \mu\text{M}$  ( $P<0.01$ ) and modified (40:60) increased EAA compared to normal (80:20) ratio by  $124.2 \pm 41.9 \mu\text{M}$  ( $P<0.01$ ). However, by 200 min, normal (80:20) slightly increased EAA compared to modified (40:60) by  $54.1 \pm 55 \mu\text{M}$  ( $P=0.86$ ) for high (9.3 wt%) protein concentration (Figure 3.1). Overall, high (9.3 wt%) increased EAA compared to low (3.1 wt%) protein concentration by  $415.2 \pm 46.7 \mu\text{M}$  ( $P<0.01$ ), but there was no difference between ratio ( $P=0.09$ ) in all participants (Table 3.3). Furthermore, EAA mean concentration differed by treatment ( $P<0.01$ ), effect of time ( $P=0.02$ ), but not treatment  $\times$  time interactions ( $P=0.09$ ) in male participants. High protein treatments (9.3 wt% 80:20 and 40:60) increased EAA compared to low protein treatments and the control (Tukey-Kramer,  $P<0.05$ ). Overall, high (9.3 wt%) increased EAA compared to the low (3.1 wt%) protein concentration by  $412.1 \pm 55.2 \mu\text{M}$  ( $P<0.01$ ), but there was no difference between protein ratio ( $P=0.40$ ) in male participants (Table 3.3). Also, EAA mean concentration differed by treatment ( $P<0.01$ ), but there was no time effect ( $P=0.52$ ) or treatment  $\times$  time interactions ( $P=0.26$ ), in female participants. All milk treatments increased EAA mean concentration compared to the control ( $P<0.01$ ). Overall, high (9.3 wt%) increased EAA compared to low (3.1 wt%) protein concentration by  $418.3 \pm 75.4 \mu\text{M}$  ( $P<0.01$ ), but there was no difference between protein ratio ( $P=0.14$ ) in female participants (Table 3.3).

## **NEAA**

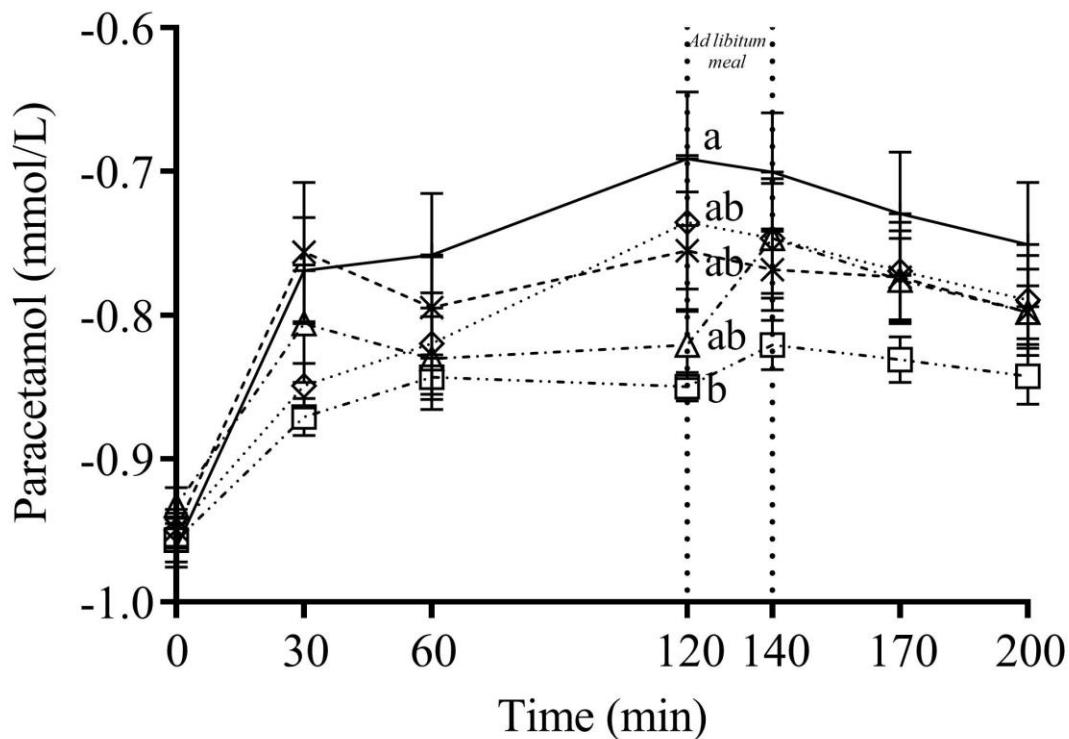
Pre-lunch NEAA iAUC differed by treatment effect ( $P<0.01$ ), but no significant gender effect ( $P=0.75$ ) or treatment  $\times$  gender interaction ( $P=0.22$ ) were observed (Table 3.3). Thus, all participant data were pooled. All milk treatments except for low protein concentration normal ratio (3.1 wt% 80:20) treatment increased NEAA iAUC relative to the control (Tukey-Kramer  $P<0.05$ ). At 30 and 60 min specifically, high (9.3 wt%) increased NEAA mean concentration compared to low (3.1 wt%) protein concentration by  $286.2 \pm 116.4 \mu\text{M}$  ( $P=0.02$ ) and  $373.0 \pm 111.2 \mu\text{M}$  ( $P<0.01$ ) (Figure 3.1). Overall, high (9.3 wt%) increased NEAA iAUC compared to low (3.1 wt%) protein concentration by  $23,138 \pm 8,191 \mu\text{M}\cdot\text{min}$  ( $P<0.01$ ), but there was no difference between ratios ( $P=0.18$ ) (Table 3.3).

In post-lunch, NEAA concentration showed a treatment effect ( $P<0.01$ ), but not time ( $P=0.17$ ) or gender ( $P=0.71$ ) effects, nor treatment  $\times$  time ( $P=0.09$ ) or treatment  $\times$  gender interactions ( $P=0.16$ ) (Figure 3.1). Thus, all participant data were pooled. High milk treatments (9.3 wt% 80:20 and 40:60) increased NEAA compared to low protein treatments and the control ( $P<0.05$ ). Overall, high (9.3 wt%) increased NEAA compared to low (3.1 wt%) protein concentration by  $212.2 \pm 69.5 \mu\text{M}$  ( $P<0.01$ ), but there was no difference between protein ratio ( $P=0.34$ ) (Table 3.3).

### *3.4.3. Paracetamol pre-lunch (iAUC) and post-lunch (mean concentration)*

Pre-lunch paracetamol iAUC differed by treatment ( $P<0.01$ ), but no significant gender effect ( $P=0.99$ ) or treatment  $\times$  gender interaction ( $P=0.17$ ) was observed (Table 3.4). Thus, participant data were pooled. Only the high protein concentration treatments (9.3 wt% 80:20 and 9.3 wt% 40:60) lowered paracetamol concentration relative to the control (Tukey-Kramer  $P=0.02$  and  $P=0.02$ , respectively). At 30 min specifically, modified (40:60) lowered paracetamol

### 3.4.3. Paracetamol pre-lunch (iAUC) and post-lunch (mean concentration)



**Figure 3. 2** Mean ( $\pm$  SEM) paracetamol (acetaminophen) concentration for all subset participants from 0 - 200 min after the consumption of breakfast meal for control (+), 3.1% milk protein (MP) (80 casein:20 whey protein) (X), 3.1% MP (40 casein:60 whey protein) ( $\diamond$ ), 9.3% MP (80 casein:20 whey protein) ( $\triangle$ ), and 9.3% MP (40 casein:60 whey protein) ( $\square$ ). <sup>a-d</sup> Different letters at each measured time are different between treatment according to adjusted Tukey-Kramer ( $P < 0.05$ ).

concentration compared to normal (80:20) protein ratio by  $-0.2 \pm 0.1$  mmol/L ( $P=0.01$ ). Also at 120 min, high (9.3 wt%) lowered paracetamol concentration compared to (3.1 wt%) protein concentration by  $-0.2 \pm 0.1$  mmol/L ( $P=0.02$ ) (Figure 3.2). Overall, high (9.3 wt%) lowered paracetamol iAUC when contrasted to low (3.1 wt%) protein concentration by  $-11.3 \pm 4.9$  mmol/L·min ( $P=0.03$ ), but there was no difference between protein ratios ( $P=0.41$ ).

Post-lunch paracetamol concentration was affected by treatment ( $P<0.01$ ), but not time ( $P=0.12$ ) or gender ( $P=0.89$ ) and there were no treatment  $\times$  gender ( $P=0.52$ ) or treatment  $\times$  time

(P=1.0) interactions observed (Table 3.4). Thus, participant data were pooled. Overall, 9.3 wt% (40:60) treatment lowered post-lunch paracetamol concentration compared to the control by an average of  $-0.10 \pm 0.02$  mmol/L (Tukey-Kramer, P<0.01). There were no differences based on protein concentration (P=0.09) or ratio (P=0.17).

**Table 3. 4** Mean ( $\pm$ SEM) paracetamol iAUC during pre-lunch (0 – 120 min) and absolute concentration for post-lunch (120 – 200 min) durations

	Treatments <sup>1</sup>				P-value <sup>2</sup>	Concentration P-value <sup>3</sup>	Ratio P-value <sup>4</sup>	
	Control	3.1 wt% (80:20)	3.1 wt% (40:60)	9.3 wt% (80:20)	9.3 wt% (40:60)			
Pre-lunch iAUC (0 – 120 min, mmol/L·min)								
All (n=12)	$23.1 \pm 3.3^a$	$18.6 \pm 4.2^{ab}$	$15.4 \pm 2.9^{ab}$	$11.8 \pm 2.4^b$	$10.9 \pm 1.5^b$	<0.01	0.03	0.41
Post-lunch absolute (140 – 200 min, mmol/L)								
All (n=12)	$-0.7 \pm 0.0^a$	$-0.8 \pm 0.0^{ab}$	$-0.8 \pm 0.0^{ab}$	$-0.8 \pm 0.0^{ab}$	$-0.8 \pm 0.0^b$	<0.01	0.09	0.17

<sup>a-d</sup> Means within a row with different superscripts differ according to Tukey-Kramer (P < 0.05).

<sup>1</sup> Control (water and whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>2</sup> ANOVA P-value for treatment effect

<sup>3</sup> Contrast between high and low protein concentration (mean 9.3 wt% (80:20) + 9.3 wt% (40:60) to mean 3.1 wt% (80:20) + 3.1 wt% (40:60))

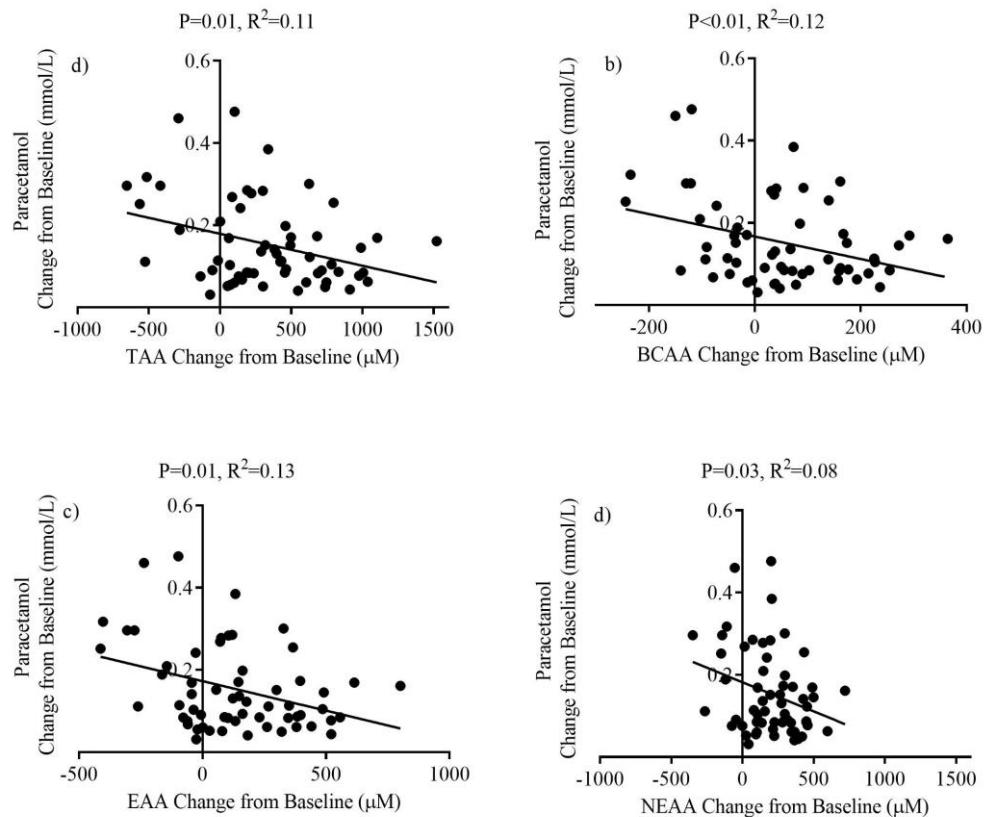
<sup>4</sup> Contrast between modified and normal ratio (mean 9.3 wt% (40:60) + 3.1 wt% (40:60) to mean 9.3 wt% (80:20) + 9.3 wt% (80:20))

### Paracetamol Cmax and Tmax

Pre-lunch paracetamol Cmax differed by treatment (P=0.02), but no significant gender effect (P=0.30) nor a treatment  $\times$  gender interaction (P=0.58) were observed. Thus, participant data were pooled. High protein concentration and modified ratio (9.3wt% 40:60) treatment significantly lowered paracetamol Cmax compared to the control (Tukey-Kramer, P=0.03). Overall, high (9.3 wt%) protein lowered paracetamol Cmax compared to low (3.1 wt%) by  $-0.21 \pm 0.07$  mmol/L (P=0.01), but there was no difference between protein ratios (P>0.05). However, pre-lunch Tmax was not affected by treatment (P=0.71). In post-lunch paracetamol Cmax was not affected by treatment (P=0.75), gender (P=0.84), or treatment  $\times$  gender interaction (P=0.95).

There was no difference between protein concentration and ratio ( $P=0.50$  and  $P=0.81$ , respectively). Also, post-lunch paracetamol Tmax was not affected by treatments ( $P=0.78$ ).

### 3.4.4. Paracetamol $\times$ AAs correlation



**Figure 3.3** Mean ( $\pm$  SEM) paracetamol with AAs change from baseline for all participants ( $n=12$ ) a) TAA, b) BCAA, c) EAA, and d) NEAA from 0-120 min after the consumption of breakfast meal.

Overall, pre-lunch paracetamol was negatively correlated with BCAA ( $P=0.01$ ,  $R^2=0.1$ ), EAA ( $P=0.01$ ,  $R^2=0.12$ ), NEAA ( $P=0.03$ ,  $R^2=0.08$ ) and TAA ( $P=0.01$ ,  $0.11$ ) for pooled participants (Figure 3.3). Post-lunch, correlations were not observed between paracetamol and BCAA, EAA, NEAA, or TAA in pooled participants ( $P>0.05$ ).

### **3.5. DISCUSSION**

This study was undertaken to explore plasma AAs, gastric emptying, and food intake after consumption of milk products with enhanced protein concentration (9.3 wt%) or enhanced whey ratio, when compared with commercial milk (3.1 wt% concentration and 80:20 casein ratio). It is nested within a larger study (n=32) that included postprandial BG and participant ratings of satiety and which concluded that high (9.3 wt%) protein concentration significantly lowered pre-lunch BG iAUC and lowered post-lunch appetite tAUC, but there was no difference in second meal food intake (Chapter 2, Kung et al., 2018). In this Chapter, pre-lunch high (9.3 wt%) protein was associated with higher plasma levels of TAA, BCAA, EAA, NEAA and lower paracetamol iAUC. The high protein concentration modified ratio (9.3 wt% 40:60) treatment had the lowest paracetamol Cmax compared to control (water with whey permeate). Furthermore, there was a modest effect of modified (40:60) protein ratio on increased BCAA and EAA and lowered paracetamol iAUC, compared to normal protein (80:20). As such, these results suggest a correlation between the early appearance of AAs, particularly those from whey, and slow gastric emptying – a mechanism that may contribute to the observed BG attenuations. There was no treatment effect on food intake. Post-lunch high (9.3 wt%) protein concentration increased TAA, BCAA, EAA, and NEAA iAUC. There was only modest increase by modified (40:60) ratio on BCAA at 140 min and EAA at 120 min, and by normal (80:20) ratio on TAA, BCAA and EAA at 200 min. These results support the hypothesis that postprandial plasma AAs reflect the consumption of dairy protein intake, i.e. concentration and ratio. The hypothesis that differences in protein consumption would alter gastric emptying was generally supported based on examination of the pre- and post-lunch duration, but there was no association with food intake at a subsequent meal. This result deviates from the current understanding that increasing consumption of protein or AAs decreases food intake in a subsequent meal. Plasma AAs are

considered reasonable predictors of relative changes in uptake of AAs in the brain and regulate food intake. Certain areas of the brain are responsive to the concentration and composition of plasma AAs, such as specific areas of the hypothalamus and anterior piriform cortex. Also, plasma AAs act by signalling neurochemical systems (Tome et al., 2009; Anderson and Li, 1987). However, despite previous observations (Aziz and Anderson, 2007), no relationship between plasma BCAA and subsequent food intake were observed in this study. In particular, it did not validate the aminostatic theory proposed by Mellinkoff et al. (1956) which suggests a relationship between increasing plasma AAs and food intake. Nor were there any evidence that increased plasma BCAA may led food intake suppression as suggested by Layman (2003), and it is also consistent with the finding from Bowen et al. (2006) which did not support a role for BCAA in satiety regulation. The discrepancy may also be related to large breakfast volume consumption by all participants although differing in body weight, and short meal interval between the breakfast and lunch meal as discussed in Chapter 2.

This study did show that the consumption of differing proportions and amounts of dairy proteins led to differences in AA plasma appearance. Specifically, from 0 – 120 min whey protein releases a “fast” increase of plasma AAs compared to casein, which demonstrates a “slow” AAs release . Bowen et al. (2006) also observed a greater rise of BCAA for whey compared to casein protein after 30 min, but no differences in TAA. Our results did show a fast, but short, transient increase in plasma AAs that peaked between 40 min to 2hr after ingestion of the treatment and also some indications of increased plasma AAs from casein at a later duration (Anderson et al., 2011; Boirie et al., 1997; Lambers et al., 2013; Frühbeck, 1998; Hall et al., 2003). Our results showed a significant difference between the increase in BCAA and EAA iAUC by modified (40:60) when contrasted to normal (80:20) ratio over 2 hr.

Interestingly, since there was no treatment effect on food intake, the post-lunch duration may provide a novel insight into the appearance of plasma AAs after subsequent meal consumption. The appearance of plasma AAs after consumption of treatment and beyond the subsequent meal has not been previously commented on. At post-meal, there was still evidence that the high (9.3 wt%) protein breakfast meal continued to be associated with higher plasma AAs compared to the low (3.1 wt%) protein concentration treatments. Also, the appearance of AAs associated with the proteins consumed at breakfast showed that modified (40:60) ratio modestly increased BCAA and EAA beyond the lunch meal at 140 min. Also, there was some modest evidence of “slow” protein digestion, as the normal (80:20) increased TAA and slightly increased BCAA and EAA compared to modified (40:60) protein ratio in high (9.3 wt%) protein treatments at 200 min. This latter observation was consistent with the prediction that casein AAs absorption occurs between 3 – 4 hr after consumption (Anderson et al., 2011). However, since the study ended after the 200 min time point, we only observed the beginning of the appearance of AA from casein.

Pre-lunch paracetamol concentration seems to reflect treatment protein concentration and ratio, as there was a significant difference between mean high and low protein concentration ( $-11.3 \pm 4.9$  mmol/L·min,  $P=0.03$ ). Also, there was a difference between protein ratios in terms of plasma paracetamol concentration at 30 min. This may be related to whey protein emptying earlier from the stomach compared to casein aggregation, and the release of AA to stimulate gastric hormones (Boirie et al., 1997; Dangin et al., 2002; Hall et al., 2003). The gastric hormone results (analyzed at the University of Toronto) seem to validate the insulin-independent mechanism for glycemic management by delayed gastric emptying (Appendix L). High protein (9.3 wt%) treatments increased GLP-1 and CCK responses compared to normal (3.1 wt%)

protein concentration during the pre-lunch duration. The effect of modified ratio was observed on the attenuation of subset BG and increased GLP-1, but not CCK and paracetamol. Ingestion of the high protein concentration modified ratio (9.3 wt% 40:60) treatment resulted in the highest GLP-1 concentration compared to other milk treatments. Thus, glycemic management seems to be in response to gastric hormones and is insulin-independent, as there were no differences between insulin and C-peptide (proinsulin) concentrations among treatments. Also, correlations during the pre-lunch period seem to provide further validation by relating hormonal with glycemic responses. BG was inversely correlated with GLP-1 ( $R=-0.32$ ,  $P<0.05$ ), and paracetamol concentration was inversely correlated with CCK ( $R=-0.30$ ,  $P<0.05$ ) and GLP-1 for the cumulative duration (0-200 min, R value was not reported) (Appendix L). The hormonal and plasma AA results from the subset participants follows previous studies that related “fast” protein digestion and AA absorption with changes in gastric hormones leading to delays in gastric emptying (Boirie et al., 1997; Hall et al., 2003). Particularly in Akhavan et al. (2014), plasma paracetamol concentrations were lower post-consumption of 10 and 20 g of whey protein compared to 10 g of glucose and water (control) in healthy men. PYY and GLP-1 were only higher than the water control after 20 g of whey protein (Akhavan et al., 2014). Similarly, this study observed lower paracetamol iAUC compared to control after a comparable amount of total protein (27.9 g) from the high protein treatments (9.3 wt% 40:60 and 9.3 wt% 80:20) were ingested. Thus, subset insulin and gastric hormone results as well as observations from Akhavan et al. (2014), Luhovyy et al. (2007) and Tome et al. (2009) suggest that the presence of gastric hormones (particularly GLP-1 and CCK but not limited to) are a possible mechanism for BG attenuations.

Pre-lunch paracetamol concentration did not mirror BG concentration trends reported in Chapter 2. This may be explained by the fact that the design of the paracetamol absorption test is best suited to reflect liquid-phase emptying (Willems et al., 2001). Strong correlation of liquid-phase gastric emptying between the paracetamol test and the “gold standard”, scintigraphy assessment, was observed. However, the treatments in this study required co-ingestion of both liquid (beverage) and solid particles (protein aggregates and cheerios). The co-ingested solid particles may interfere and modulate with the absorption of paracetamol and influence gastric emptying. Firstly, co-ingestion of both phases may affect the distribution of paracetamol between the solid or liquid components. Paracetamol may only reflect the liquid phase if not properly integrated into the solid phase of the meal and thus cannot be used to draw inferences about emptying of solids (Johansson et al., 2003). Secondly, co-ingestion of both phases may alter the emptying pattern of the liquid phase into a two-step process: initially, paracetamol will reflect the liquid component entering the duodenum at a rapid rate, and subsequently at a slower rate that is equivalent to the emptying of the solid component (due to the remaining liquid integrating with the solid components) (Sanaka et al., 2002). Paracetamol concentration showed an effect by ratio at 30 min, which may be an indication of early gastric emptying due to difference between casein and whey protein whereas an overall pre-lunch (0-120 min) BG was affected by ratio (Chapter 2). Also, the paracetamol Cmax (the extent of emptying (Sanaka et al., 2002)) results suggested that whey protein reduced the release of chyme, but there was no treatment differences between Tmax. Post-lunch, paracetamol concentration remained low for the 9.3 wt% (40:60) treatment, potentially due to the lasting effects of whey protein. Post-lunch treatment effects on plasma paracetamol after consumption of a standardized second meal were not previously observed (Akhavan et al., 2014).

Exploring correlations gave greater insights into the effect of AAs on gastric emptying and potential BG management as discussed in Chapter 2. Our study revealed pre-lunch increases in plasma BCAA, EAA and Total AA were inversely correlated with paracetamol concentrations in pooled participants. This result is aligned with previous literature. However, the current understanding of this relationship with BG management is conflicting. Two studies from Sun et al. (2016 and 2017) suggested that, relative to soy, cow's milk when co-ingested with white bread may attenuate glycemic response due to the higher release of BCAA leading to a greater GLP-1 response, which may be in turn be responsible for the lower glycemia. GLP-1 was positively correlated with BCAA release at 30 min (Sun et al., 2016). Also, slower gastric emptying (measured using real-time ultrasonography) was observed at 45 and 90 min compared to the control meal (water and bread) in a later study (Sun et al., 2017). However, Hall et al. (2003) did not observe any relationship between slower gastric emptying, higher BCAA profile and BG (no difference between BG was observed), when whey was compared to casein. Similarly, Veldhorst et al. (2009) did not observe any BG difference between treatments, although higher BCAA iAUC were accompanied by higher GLP-1 concentrations after consumption of 25% of whey protein compared to casein. In addition, whey protein elicited higher plasma GLP-1 and leucine iAUC compared to the control and casein, respectively, in Gunnerud et al. (2012). Leucine and valine were positively correlated with GLP-1, and inversely correlated with BG response (Gunnerud et al. 2012). In this study, post-lunch paracetamol results mostly reflect plasma AAs in that 9.3 wt% (40:60) treatment increased BCAA and EAA and lowered paracetamol concentration compared to the control. However, the lack of post-lunch correlation may be explained by contradictions observed at 200 min, as the normal (80:20) ratio slightly increased plasma AAs, but 9.3 wt% (40:60) was associated with the lowest paracetamol

concentration. Thus, the pre-lunch AAs in association with slower gastric emptying via gastric hormone feedback may provide some evidence of BG management.

### **3.6. CONCLUSION**

High (9.3 wt%) protein concentration breakfast milk increased postprandial TAA, BCAA, EAA, and NEAA iAUC and reduced paracetamol compared to the low (3.1 wt%) protein equivalent. Also, modified (40:60) protein ratio increased BCAA and EAA iAUC compared to normal (80:20) protein ratio. Similarly, modified (40:60) protein ratio modestly lowered paracetamol concentration at 30 min, pointing to slowing in gastric emptying. This suggests that pre-lunch attenuations in BG (Chapter 2) were, at least in part, attributable to slower gastric emptying elicited by whey protein, as suggested by similar trends in AA kinetics and gastric hormone results (Appendix L). Furthermore, the lack of difference between lunch meal food intake can allow for novel observations in post-lunch duration. Specifically, post-lunch, the high (9.3 wt%) protein concentration continued to be associated with increased TAA, BCAA, EAA, and NEAA iAUC. Also, the 9.3 wt% (40:60) treatment lowered paracetamol concentration compared to the control. However, there was no effect of protein ratio on any AA grouping, nor a relationship between paracetamol and AA concentrations in the post-lunch duration. Thus, this paper has relevance for understanding the roles of dairy protein when consumed with a high carbohydrate meal for diet-related food intake and glycemic management through impacting gastric emptying.

### **3.7. ACKNOWLEDGEMENT**

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### **3.8. BRIDGE TO CHAPTER 4**

Human clinical trials provide the best evidence regarding dietary intervention strategies, but are costly to conduct, time-consuming and present potential risk to participants. *In vitro* digestion studies may replace or complement human clinical trials, as they can provide explanations to specific physiochemical research questions without the challenges associated with human participants. However, physiological relevance must be established. The human work described in Chapters 2 and 3 provides data that may be used to assess the accuracy of the *in vitro* digestion results and to explore relationship between the two types of studies via *in vitro*-*in vivo* correlation analyses. Moreover, the combination of *in vitro* and *in vivo* research can provide a more complete picture of human digestion, including offering mechanistic insights about carbohydrate and protein digestion impacting postprandial metabolism. This is investigated in the following chapter.

**CHAPTER 4: CORRELATING *IN VITRO* DIGESTION VISCOSITIES AND  
BIOACCESSIBLE NUTRIENTS OF MILKS CONTAINING ENHANCED PROTEIN  
CONCENTRATION AND NORMAL OR MODIFIED CASEIN: WHEY PROTEIN  
RATIO WITH BREAKFAST CEREAL TO *IN VIVO* HUMAN GLYCEMIC AND  
SATIETY STUDY**

*In Preparation*

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#### **4.1. ABSTRACT**

This work investigated the hydrolyses of breakfast cereal and two milk treatments containing high total protein concentrations (9.3 wt%) and either normal protein ratio (80 casein:20 whey) or modified protein ratio (40 casein: 60 whey) compared to a dairy protein-free control during *in vitro* digestion. Previous human clinical findings indicated that milk protein ratio may modestly delay the postprandial appearance of BG. However, the mechanism behind this attenuation has not been fully elucidated. It is possible that increased viscosity may delay gastric emptying and inhibit amylolysis. Therefore, milk beverages and a dairy protein-free control (water and permeate) were digested in a rheometer with pH-stat system to determine proteolytic profiles. Sampling occurred at the end of each digestion phase: oral (O, 2 min), gastric (G, 62 min) and duodenal (D, 92 min). Gastrointestinal viscosity, breakfast disintegration, protein solubility, buffering capacity, protein hydrolysis (SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and the bioaccessibility of TAA, D-glucose and reducing sugars were determined. The high protein normal ratio (9.3 wt% 80:20) treatment demonstrated higher gastric and duodenal viscosity, more intact casein at the end of oral digestion, and higher buffering capacity between pH 5.5 - 4.8 (G) and pH 5 – 7.0 (D) ( $P<0.05$ ). With the 9.3 wt%

(40:60) treatment, more  $\beta$ -lg and  $\alpha$ -lactalbumin ( $\alpha$ -la) were present at the end of oral digestion, and higher gastric buffering capacity was found between pH 4.8 - 3.5 (G) and pH 3.6 - 4.8 (D)(P<0.05). At the end of the duodenal digestion, there were no remaining intact proteins or peptides detected for all treatments and the control, but higher free TAA (mg/g protein of undigested breakfast) was indicated for the 9.3 wt% (80:20) sample. Also, both protein-containing treatments had lower reducing sugars (mg/g available carbohydrates of undigested breakfast) compared to the control at the end of duodenal phase. Overall, there were no treatment differences for breakfast disintegration, protein solubility and D-glucose. There were similar trends observed between the *in vitro* and *in vivo* results. Specifically, there was higher gastric viscosity (Pa.s) and slower gastric emptying (paracetamol, mmol/L) because of casein aggregation. Also, both high protein treatments compared to the control attenuated release of *in vitro* and plasma TAA (mg and  $\mu$ M/g protein of undigested breakfast) at the end of gastric phase and lowered release of reducing sugar (mg/g of available carbohydrates) and BG (mmol/L\*g of available carbohydrates) at the end of duodenal phase. However, increased viscosity, elicited by higher proportions of casein, did not seem to have a direct influence on the inhibition of *in vitro* starch hydrolysis and appearance of *in vivo* BG.

#### **4.2. INTRODUCTION**

T2D is a chronic metabolic disease that occurs when the body cannot effectively utilize insulin to metabolize BG (World Health Organization, 2016). Thus, dietary strategies for the prevention or management of T2D are under investigation. In particular, dairy proteins have demonstrated BG lowering effects when consumed with a high carbohydrate (McGregor and Poppitt, 2013; Anderson et al., 2011). Bovine milk proteins concentration is typically 3.1 wt% and consists of 80% casein proteins and 20% whey proteins. Caseins are associated with micelle

structure, whereas whey proteins are globular (Fox and McSweeney, 1998). Casein and whey have been characterized as “slow” and “fast” proteins, respectively, due to their different digestion kinetics and rate of release of AAs (Boirie et al., 1997; Hall et al., 2003). Casein micelles form clotted aggregates in the acidic environment of the stomach and are retained for longer in this compartment, until they are further broken down (Lentle and Janssen, 2011), whereas whey protein remains soluble in the stomach and more rapidly empties and is hydrolyzed to release AAs in the small intestine.

Co-ingestion of starchy foods with other food products potentially elicits different rate and extent of digestion that would ultimately influence BG response (Wolever and Jenkins, 1986). Our previous human study showed that enhanced protein concentration (9.3 wt%) from commercial (3.1 wt%) and modified (40:60) casein to whey protein ratio compared to normal (80:20) had the lowest attenuation of BG when co-ingested with breakfast cereal (Chapter 2). There was some evidence that one of the mechanisms contributing to the attenuated BG was delayed gastric emptying leading to nutrient-stimulated hormonal feedback with AA absorption (Chapter 3 and Appendix L). These results are in accordance with previous studies showing that rapidly digested whey protein initiates an earlier delay in gastric emptying. It has been proposed that the mechanism for delay gastric is by stimulated gastric hormones feedback, such as GLP-1 and CCK (Appendix L; Panahi et al., 2014; Akhavan et al., 2014). However, other mechanisms behind attenuation of BG by co-ingestion of dairy proteins with starch have not been fully investigated. Of interest are possible effects of increased viscosity through the interaction of cereal soluble fibre and milk protein on the inhibition of enzyme activity, and the subsequent rate and extent of starch digestion. Previous research indicates that increased viscosity by dietary fibres can influence BG, but did not validate delays in amylolysis (Repin et al., 2017). Still, the

role of gastrointestinal content viscosity on BG in a mixed-meal remains unclear. Thus, there is a need to explore the effect of viscosity on lowering BG for carbohydrate-rich meals, particularly in the presence of dairy and dairy proteins. Postprandial BG is the result of digestible carbohydrate absorption. Starch is a complex carbohydrate composed of two glucose polymers, amylose and amylopectin. Amylose is hydrolysed by  $\alpha$ -amylase enzyme to produce reducing sugars such as maltose, maltotriose and branched  $\alpha$ -limit dextrans (Dona et al., 2010). Amyloglucosidase acts on hydrolysing  $\alpha$ -(1->6) linkages, which  $\alpha$ -amylase is unable to attack. Further hydrolysis of maltose by brush border enzymes allows starch digestion to proceed to completion, final conversion to glucose monomers (Dona et al., 2010). The presence of increased digesta viscosity may inhibit the effect of enzymes by acting as a barrier for access or transport of nutrients (Pflugfelder and Rooney, 1986).

*In vitro* digestion is a tool that mimics human digestion and aims to elucidate results that complement or expand on those available from *in vivo* research. Thus, the purpose of this paper is to use an *in vitro* digestion system to simulate digestion of breakfast cereal and milk with modified (40:60) casein to whey protein ratio to extend and validate the previous *in vivo* results, to provide mechanistic insights. Treatments from the *in vitro* viscosity, free TAAs and sugar results will also be correlated with *in vivo* gastric emptying (indirectly measured by paracetamol) (mmol/L), plasma TAA ( $\mu$ M) and BG concentrations (mmol/L). It is hypothesized that the digestion of milk with modified (40:60) casein to whey proteins ratio will have a different proteolytic profile (breakfast disintegration, protein solubility, buffering capacity, protein hydrolysis (SDS-PAGE) and free TAA released and decrease gastric viscosity, which would consequently increase accessibility of digestive enzymes and the bioaccessibility of nutrients (free TAA, D-glucose and reducing sugar) compared to a normal (80:20) protein ratio and the

control (dairy protein-free). *In vitro* viscosity, TAA, D-glucose and reducing sugar results will be related with *in vivo* gastric emptying (paracetamol), plasma TAA and BG concentrations.

### 4.3. MATERIALS AND METHODS

#### 4.3.1. Treatment composition and preparation

**Table 4. 1** Combined composition of milk treatment (18 mL) with breakfast cereal (3.5 g) sample in the digestion system

	Treatment (18 mL) <sup>1</sup>		
	Control	9.3 wt% (80:20)	9.3 wt% (40:60)
Carbohydrates (g)			
Lactose	2.1	2.1	2.1
Cereal <sup>2</sup>	2.5	2.5	2.5
<b>Total available carbohydrates<sup>3</sup></b>	<b>4.6</b>	<b>4.6</b>	<b>4.6</b>
Proteins (g)			
Whey	0.02	0.33	1.01
Casein	-	1.34	0.71
Cereal	0.28	0.28	0.28
<b>Total proteins (including cereal)<sup>4</sup></b>	<b>0.30</b>	<b>1.95</b>	<b>2.00</b>
<b>Total amino acids (TAA, g)<sup>5</sup></b>	<b>0.26</b>	<b>1.93</b>	<b>1.94</b>

<sup>1</sup> Control (water and whey permeate); commercial skim milk (MP) 3.1 wt% 80 casein : 20 whey); normal MP 3.1 wt% (40 casein : 60 whey protein); high MP 9.3 wt% (80 casein : 20 whey protein); high MP 9.3 wt% (40 casein : 60 whey protein)

<sup>2</sup> Combination of starch and sugar

<sup>3</sup> Combined lactose and cereal available carbohydrates

<sup>4</sup> Combined total protein from whey, casein and cereal.

<sup>5</sup> Combined total amino acids from treatments and cereal. AOAC Method 2006. Section 982.3 E (a,b,c), chp. 45.3.05

Breakfast drinks ( $18.0 \pm 0.01$  mL) based on skim milk (Quebon – Agropur Cooperative, Saint-Hubert, QC, Canada) or dairy protein-free control (water with whey permeate) were formulated and cold mixed together using whey permeate (DariSweet200, #215503), skim milk powder (Low Temp, #202001), and whey protein concentrate (Prodel 35, #33703) (Parmalat Canada, London, ON). Both breakfast drinks contained three-fold (9.3 wt%) protein concentration and either modify 40:60 casein:whey protein ratio or (normal) 80:20 while holding the lactose ( $2.10 \pm 0.01$  g) contents constant (Chapter 2, Table 2.1). Treatments were combined

with  $3.5 \pm 0.01$  g of oat-based cereal (Honey Nut Cheerios, General Mills, Mississauga, Canada), which were milled by hand with a kitchen rolling pin and sifted through sieve stack with apertures of 4.00, 2.00 and 1.00 mm (Canadian Standard Sieve Series, WS Tyler, Saint Catharines, ON, Canada) to produce particles with the mean size of 1.5 mm (Table 4.1) (Bleis et al., 2013). The final mixture contained 60% of 1 mm and 40% of 2 mm particles.

#### *4.3.2. In vitro digestion*

##### *Chemicals*

Acids and bases (2N HCl, 2N NaOH and 0.2N NaOH were purchased from Fisher Chemicals (Markham, ON, Canada). 0.25N HCl solution was prepared from dilution of 2N HCl. Enzymes ( $\alpha$ -amylase (A3176), pepsin (P7000), lipase (L3126), bile (B8631), and pancreatin (P7545), and a trypsin-chymotrypsin inhibitor (Rioux and Turgeon, 2012) were purchased from Sigma Aldrich (Oakville, ON, Canada). The activities of the enzymes used (units per g of sample) were described by Rioux and Turgeon (2012). In addition, porcine pancreatic  $\alpha$ -amylase had an activity of  $\geq 5$  U/mg as defined by the manufacturer, one unit was defined by the manufacturer as the amount of enzyme required to liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C. Fungal  $\alpha$ -amyloglucosidase (*Aspergillus niger*) was obtained from Megazyme® (E-AMGDF, Megazyme International Ireland Ltd., Bray, Ireland) and had an activity of 3,260 U/mL, as defined by the manufacturer. One unit was defined by the manufacturer as the amount of enzyme required to release one micromole of glucose from soluble starch per minute (10 mg/mL starch; pH 4.5; 40°C).

##### *In vitro digestion model*

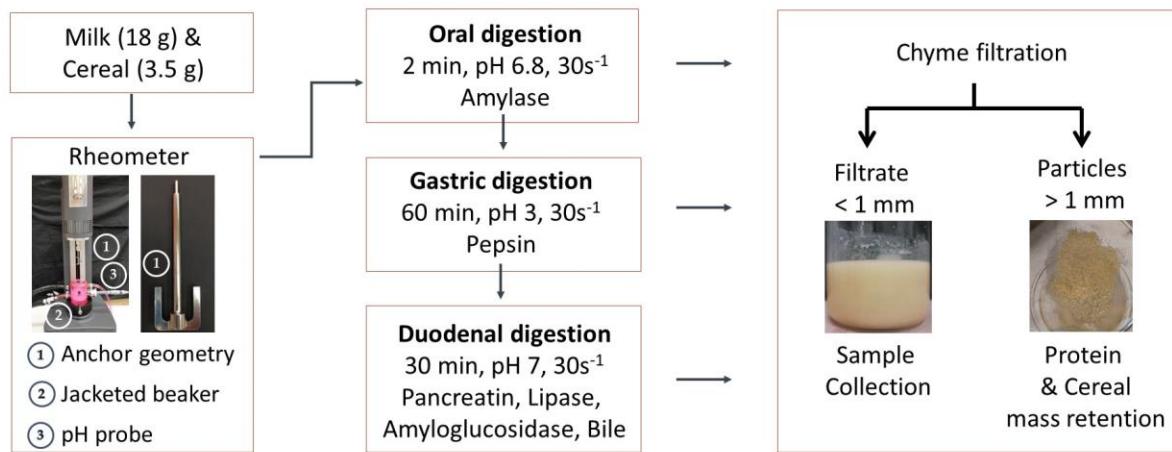
The digestion process involves three steps simulating digestive processes in the mouth, stomach and small intestine. Details about the model design and composition of the simulated juices were presented previously (Rinaldi et al., 2014; Rioux and Turgeon, 2012) (Figure 4.1). Briefly, a pH-stat titrator (Mettler Toledo Excellence T9 and Labx software, Columbus, Ohio,

USA) was programmed to reach and maintain the targeted sample digestion pH of  $3.0 \pm 0.05$  using 2N and 0.25N HCl for the gastric phase and  $7.0 \pm 0.05$  using 2N and 0.20N NaOH for the duodenal phase (Mat et al., 2016, 2018). The *in vitro* digestion took place in a rheometer (AR-G2, TA Instruments New Castle, DE USA) equipped with a jacketed beaker (52 mm internal diameter, 74 mm internal height, Adams & Chittenden, Berkeley, CA, USA) and a mixing geometry (anchor, 38 mm height and 44 mm diameter, TA Instruments, New Castle, DE, USA, Figure 4.1) as presented previously in Fang et al. (2016). The rotating anchor geometry in the jacketed beaker ensured no meal sedimentation by creating a fluid flow that provides a continuous force to mix and disintegrate the cereal and milk particles. A constant shear rate was set to  $30 \text{ s}^{-1}$  for each of the oral, gastric and duodenal stages. Enzymes were preheated in a hot water bath set to  $37^\circ\text{C}$  prior to adding to the digesta (Isotemp 2100, Fisher Scientific, Ottawa, ON, Canada). Milk treatment samples ( $18.0 \pm 0.1 \text{ g}$ ) and cereal ( $3.5 \pm 0.1 \text{ g}$ ) were added into the jacketed beaker to represent the initial combination of the breakfast for 1 min. 12 mL of saliva was then added to initiate oral digestion that lasted 2 min. Gastric digestion started with the addition of 18 mL gastric juice and lasted 1 hr. In this study, 431.6 mg pepsin solubilized in 6 mL gastric solution was added after the pH had been stabilized to 3 with 2N HCl since a preliminary experiment with milk showed that proteolysis was too fast (Rinaldi et al., 2014). To facilitate fluid flow, the agitation of an anchor (gap 1000  $\mu\text{m}$ ,  $30 \text{ s}^{-1}$ ) was used and the temperature during the whole digestion was maintained at  $37 \pm 1^\circ\text{C}$  (Thermocube 300, Solid State Cooling Systems Inc., Pleasant Valley, New York, USA). Initial duodenal pH was adjusted to 7 by the addition of 2N NaOH. Then 28.5 mL of duodenal juice, 12.0 mL of bile solution, 2.0 mL of lipase and 0.5 mL of amyloglucosidase was added to start the duodenal digestion, which lasted for 30 min.

The digestion procedure was carried out 3 times and stopped to obtain the digesta samples at the end of oral (2 min), gastric (62 min) and duodenal (92 min) phases, respectively. For the oral and gastric digestion trials, the quantities of the breakfast and the digestive juices used were doubled, because the anchor-type geometry needs to be fully immersed to measure the viscosity, and data was recorded using TRIOS V.4.3.1. 39215 software package (TA Instrumentals, New Castle, DE, USA). At the end of digestion, the digesta was neutralized ( $\text{pH } 7.00 \pm 0.05$ ) with NaOH/HCl and immediately passed through a metallic mesh sieve filter ( $\sim 1$  mm) that sat on top of a beaker (250 mL) to mimic the human gastric emptying process in which only food particles  $< 1\text{-}2$  mm can pass through the stomach into the small intestines for further digestion and absorption (Dressman, 1986; Thomas, 2006). The retained material remained on the filter for 3 min at room temperature and a spatula was used to gently stir and fold the sample to drain the liquid into the bottom beaker. Then the filtration system (retained material on the filter and liquid) was inserted into an incubator set at  $37^\circ\text{C}$  for 5 mins. Afterwards, the solid retentate particles retained in the mesh sieve and the liquid filtrate were immediately weighed to analyze for breakfast disintegration (defined as small fragments and food dissolution into the gastric juices) and protein solubility, as below.

For sugar analysis (D-glucose and reducing sugar), 1 mL of the digesta was added to 4 mL of absolute ethanol to stop enzymatic activity. The sample was then centrifuged for 5000 g, 10 min at  $24^\circ\text{C}$  and resulting supernatants were aliquoted before cooling in ice for 10 min and then frozen at  $-80^\circ\text{C}$  for later analysis. Considering that the duodenal enzymes are still active after pH neutralization, a trypsin-chymotrypsin inhibitor solution (24  $\mu\text{g/mL}$  supernatant) was added in the duodenal supernatant samples once the supernatants were aliquoted. To differentiate the role of enzyme activity and other factors (water adsorption and acidic hydrolysis) occurring

during digestion on treatment and the control, a water digestion (without any treatment nor cereal) was carried out under similar conditions. The mechanical disintegration (solid content), protein solubility, degree of hydrolysis, free AAs, reducing sugars and D-glucose were determined at the end of each digestion phase, as described below.



**Figure 4. 1** *In vitro* digestion model components, experimental parameters and filtration schematics

#### 4.3.3. Breakfast disintegration and protein solubility

Breakfast disintegration (solid content, %) represented the dispersal of the milk and cereal components into the water (digesta) phase. It was determined by drying the remnants (filtered solid particles and the liquid filtrate) at 100°C in an air-forced oven for 16 hr (AOAC, section 2.5.3.1, 2008) and calculating the percentage of solid retentate of the digesta at the end of each phase relative to the total solids of the initial breakfast meal (Eq. 1):

$$(Eq. 1) \text{ Breakfast Disintegration (\%)} = \frac{(W_0 \times S_0) - (W_p \times S_p)}{W_0} \times 100$$

where  $W_0$  is the weight of the initial breakfast before digestion, g.

$W_p$  is the weight of the solid retentate after filtration at end of digestion phase, g.

$S_0$  is the dry matter content in the initial breakfast, g /100 g.

$S_p$  is the dry matter content in the retentate at the end of digestion, g/100 g.

The dry matter content in the retentate was analyzed in duplicate (AOAC, 2008).

After drying, the protein solubility in the filtrate (liquid after filtration) was measured by Dumas combustion (Leco, Saint-Joseph, MI, USA)(IOS, 2002) to determine the proportion of soluble proteins (AOAC section 2.5.3.3, 2008). The amount of protein in the filtrate was subtracted from the amount of initial breakfast protein before digestion. The protein solubility (%) was calculated as presented in Fang et al. (2016)(Eq. 2):

$$(Eq. 2) \text{ Protein Solubility (\%)} = \frac{(W_p \times P_p)}{(W_0 \times P_0)} \times 100$$

where  $W_0$  is the initial breakfast weight (g) before digestion, g.

$W_p$  is the filtrate weight at the end of digestion phase, g.

$P_0$  is the protein content (g/100g) in the initial breakfast

$P_p$  is the protein content (g/100g) in the filtrate at time, t.

#### 4.3.4. Buffering Capacity

The buffering capacity expresses the resistance of the dairy proteins to a pH change during gastric (lowering pH) and duodenal (raising pH) digestion. Data for calculating buffering capacity was modified based on Mat et al. (2018). Recorded time, volume and pH values from 2 to 62 min for gastric digestion and 62 to 92 mins for duodenal digestion were smoothed with a moving average on R 3.4.4 statistical program (R Core Team, 2018) over 55 points. Then volume and pH values were interpolated using the first order derivative and calculated using the Savitsky-Golay algorithm with a linear fit (Savitzky and Golay, 1964). These smoothed data were input into Eq. 3 for each 3 repetitions before calculating means and standard deviations (Salaün et al., 2005):

$$(Eq. 3) \text{ Buffering Capacity (pH)} = \frac{\text{(Volume of acid added)} \times \text{(Normality of the acid)}}{\text{(Volume of the sample)} \times \text{(pH change produced)}}$$

#### *4.3.5. Protein hydrolysis by SDS-PAGE*

Digested samples and milk treatments were solubilized in 1.5 mL SDS before SDS-PAGE analysis, on a pre-set gel (4-20% wt/vol polyacrylamide precast gels, Bio-Rad Laboratories, Hercules, Ca, USA). Protein standards of  $\alpha$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin were purchased at Sigma Aldrich. Equal parts of proteins were combined for the final concentration of the standard of 8  $\mu$ g/L, then 10  $\mu$ g of diluted sample and protein standard were loaded per well. The gels were stained with Coomassie blue (Bio-Rad) overnight at room temperature and scanned into Bio-Rad ChemiDoc<sup>TM</sup> MP Imaging System and then quantified in Image Lab<sup>TM</sup> Software (Bio-Rad Laboratories). Protein bands for caseins,  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin and peptides (<10 kDa) were identified and the volumes were quantified relative to the protein standard marker.

#### *4.3.6. TAA*

Free TAA were analyzed by modified Cd-Ninhydrin Method C (Doi et al., 1981; Jamdar et al., 2017) with leucine standard purchased from Sigma Aldrich. Cd-ninhydrin reagent was created by mixing 0.8 g of ninhydrin dissolved in a mixture of 80 mL of absolute ethanol and 10 mL of acetic acid, followed by the addition of 1 g of CdCl<sub>2</sub> dissolved in 1 mL of distilled water. For colour development, 320  $\mu$ L of ninhydrin reagent was combined with 80  $\mu$ L of sample extract. The reaction was tightly covered and heated for 5 min in a boiling water bath and then cooled in an ice bath for 5 min before absorbance was measured at 505 nm using a UV-Visible spectrophotometer (Evolution 60S, Thermo-Scientific, UK). The amount of free TAA in the digested samples (filtrate) was expressed as mg/g protein measured in undigested breakfast.

#### *4.3.7. D-glucose and reducing sugars*

D-glucose was assayed by the glucose oxidase-peroxidase GOPOD assay (Megazyme International Ireland Ltd.) as per the manufacturer's instructions. Briefly, 0.1 mL supernatant was aliquoted from the 1 mL digesta sample combined with 4 mL absolute ethanol that was

centrifuged, as previously described. This 0.1 mL was added to 3.0 mL of the GOPOD reagent and then incubated at 47°C for 20 min. Absorbance was read at 510 nm in the UV-Visible spectrophotometer (Evolution 60S) against a water blank. The amount of D-glucose was expressed as mg/g available carbohydrate in the undigested breakfast meal.

Reducing sugars are products of starch hydrolysis, released by  $\alpha$ -amylase and  $\alpha$ -amyloglucosidase enzymes. They have a free aldehyde group or a free ketone group and can act as a reducing agent and include, for example glucose, maltose, maltotriose, maltotetraose and  $\alpha$ -dextrans. In this study, dinitrosalicylic acid (DNS), a colorimetric method described by Miller (1959), was used to determine the concentration of reducing sugars based on the quantity of free carbonyl groups from starch digestion. 1 mL of supernatant sample was combined with 1 mL of DNS reagent (1% (w/v) dinitrosalicylic acid, 0.2 % (w/v) phenol, 1% (w/v) sodium hydroxide and 0.05% (w/v) sodium sulfate). Samples were covered with aluminum foil and placed into a hot water bath at 100°C for 15 min. Afterwards, 0.20 mL of Rochelle salt (40% (w/v) potassium sodium tartrate solution) was added to each sample before placing the tubes into an ice water bath for 10 min. The absorbance of the samples was read using a UV-Visible spectrophotometer (Evolution 60S) at 575 nm. A series of  $\alpha$ -dextrin standards were also analyzed to obtain a standard curve (Butterworth et al., 2012, Zhang et al., 2015) and the amount of reducing sugars was expressed as mg/g available carbohydrates in the undigested breakfast meal.

#### *4.3.8. Data and statistical analyses*

The milk treatments, including the control, were each tested in 3 digestion replicates, in random order. TAA, D-glucose and reducing sugar analyses were carried out in duplicate. One replicate of 9.3 wt% (80:20) sample at duodenal (92 min) was spoiled and therefore excluded from the AAs analysis. The effect of meal treatment at each digestion stage (oral (O, 2 min), gastric (G, 62 min) and duodenal (D, 92 min)) and treatment  $\times$  time interactions were analyzed

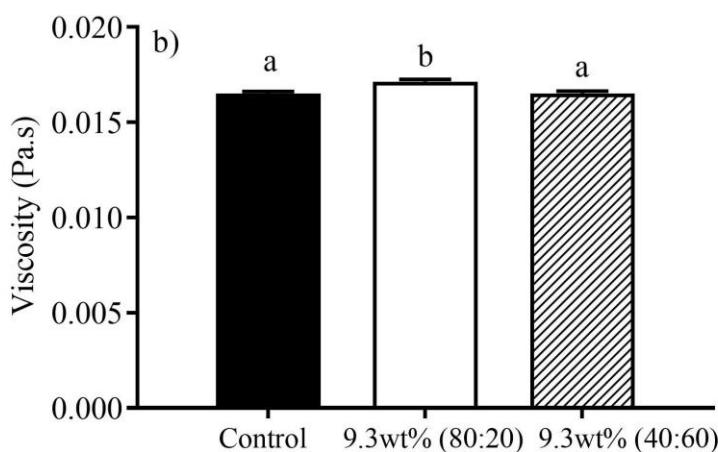
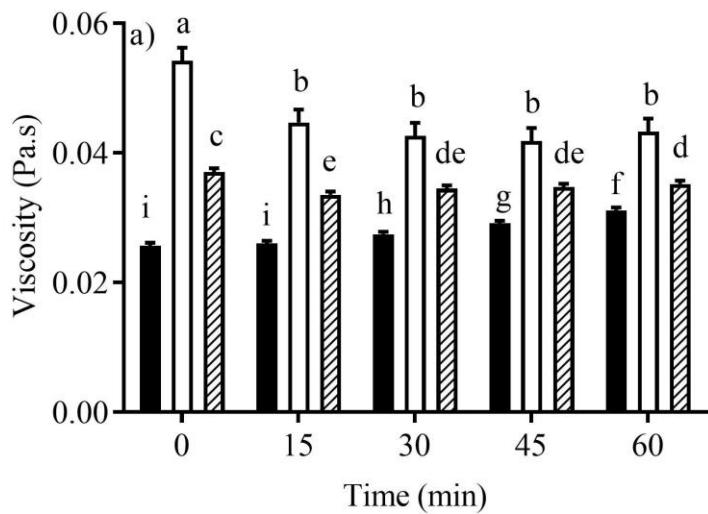
by PROC MIXED ANOVA with least square means in SAS (SAS version 9.4, SAS Institute, Cary, NC, USA) for viscosity, breakfast disintegration, protein solubility, buffering capacity, protein hydrolysis (SDS-PAGE), TAA, D-glucose and reducing sugar. Results are expressed as mean  $\pm$  SEM. Significance was set at P<0.05.

*In vitro* digestion data (i.e. 62 min for gastric and 92 min for duodenal) were compared with the *in vivo* data. In the human study, milk treatments (250 mL) and breakfast cereal (58 g) were consumed in a randomized, controlled, doubled-blind study with healthy young adults (n=12 6M/6F, 22.8  $\pm$  3.0 y, BMI 23.2  $\pm$  2.7 kg/m<sup>2</sup>) (Chapter 3), as described in Chapter 2 for BG, and in Chapter 3 for plasma AAs and gastric emptying. Only the high protein treatments (9.3 wt% 80:20 and 9.3 wt% 40:60) and the control from the *in vivo* experiment were used in the analysis. Paracetamol values were log transformed because the data was not normally distributed (Chapter 3). Comparisons were made with data from the subset group at 60 min for gastric digestion and at 90 or 120 min for duodenal digestion (Chapter 3). Within the *in vitro* and *in vivo* data sets, treatment effect was analyzed by PROC MIXED ANOVA with least square means at each digestion stage. Treatment trends between apparent viscosity (Pa.s) and gastric emptying (paracetamol, mmol/L), BG (mmol/L) with reducing sugar (mg/g of available carbohydrate) and plasma TAA ( $\mu$ M/g protein of undigested breakfast) with *in vitro* TAA (mg/g protein of undigested breakfast) were explored.

## 4.4. RESULTS

### 4.4.1. Viscosity

Gastric viscosity differed by treatment (P<0.01), time (P<0.01), and there was a treatment x time interaction (P<0.01). The highest mean viscosity was observed with the 9.3 wt% (80:20) treatment compared to 9.3 wt% (40:60) treatment and then the protein-free control (Figure 4.2a).



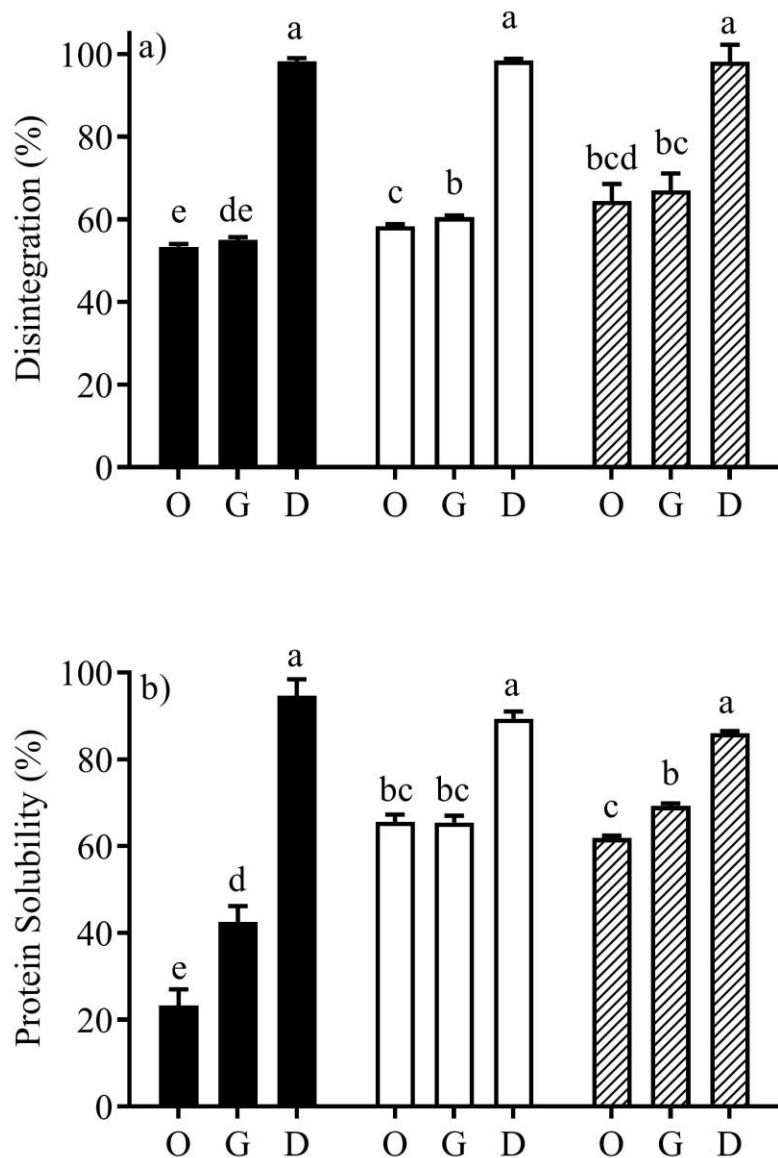
**Figure 4.2** Mean (± SEM) viscosity (Pa·s) for a) gastric (15 min interval, 0 - 60 min) and b) duodenal (5 min interval, 0 - 30 min) for the control (■), 9.3 wt% (80:20) (□), and 9.3 wt% (40:60) (▨). <sup>a-i</sup>Superscript letters differ according to LS Means multiple comparison testing for treatment × time interactions for all treatment bars, except for b) duodenal phase treatment effect ( $P < 0.05$ ).

Treatments and the control were all significantly different from each other ( $P < 0.01$ ).

Furthermore, duodenal viscosity differed by treatment ( $P < 0.01$ ) and time ( $P < 0.01$ ), but there was no treatment × time interaction ( $P = 0.25$ ). Again, higher mean duodenal viscosity was induced by 9.3 wt% (80:20) treatment compared to the 9.3 wt% (40:60) treatment and the protein-free

control ( $P<0.01$ ) (Figure 4.2b) (Supplemental figure of the viscosity curve can be found in Appendix J).

#### 4.4.2. Breakfast disintegration and protein solubility

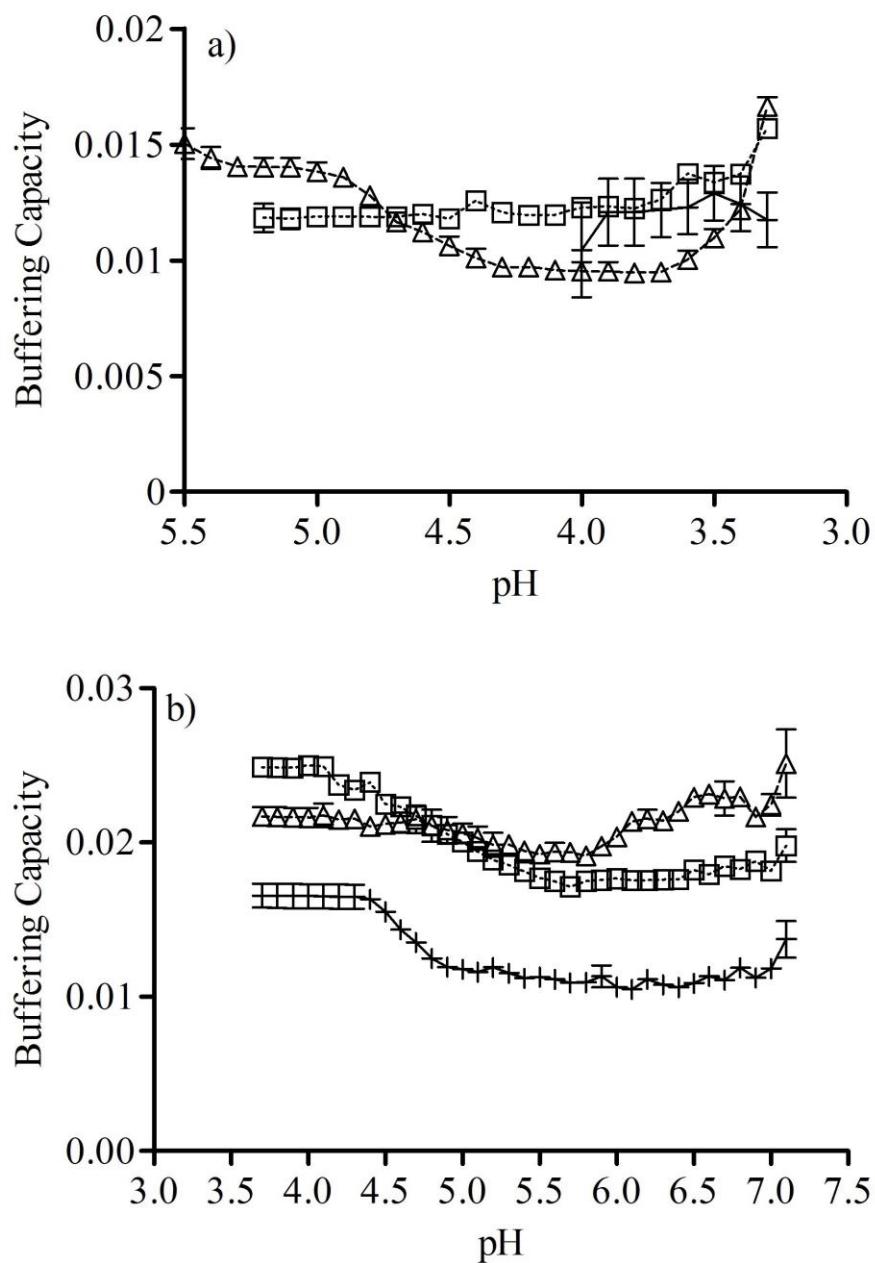


**Figure 4.3** Mean ( $\pm$  SEM) a) breakfast disintegration (%) and b) protein solubility (%) for the control (■), 9.3 wt% (80:20) (□), and 9.3 wt% (40:60) (▨) at the end of each of the oral (O), gastric (G) and duodenal (D) phases. <sup>a-e</sup>Superscript letters differ according to LS Means multiple comparison testing for treatment  $\times$  time interactions for all treatment bars ( $P < 0.05$ ).

Breakfast disintegration (%) significantly differed by treatment ( $P<0.01$ ), time ( $P<0.01$ ), and there was a treatment x time interaction ( $P<0.01$ ) (Figure 4.3a). Breakfast disintegration was not significantly different between the 80:20 and 40:60 treatments at each of the three digestion phases. Lower breakfast disintegration was observed with the protein-free control during the oral and gastric phase, and this may be related to the initial low protein of the treatment. Yet, by the end of the duodenal phase, breakfast disintegration was nearly complete in all cases and there were no differences amongst all treatments and the control. Protein solubility (%) significantly differed by treatment ( $P<0.01$ ), time ( $P<0.01$ ), and there was a treatment x time interaction ( $P<0.01$ )(Figure 4.3b). Similar trends as for the disintegration were observed, i.e. protein solubility did not significantly differ between 80:20 and 40:60 treatments at each digestion phase. Moreover, lower protein solubility was observed for the control during the oral and gastric phases, but no protein solubility differences were observed at the end of the duodenal phase.

#### *4.4.3. Buffering capacity*

During the initial decrease of pH for gastric digestion, buffering capacity for the control ranged between pH 3.3 and 4.0, whereas with the protein treatments buffering capacity pH ranged between 3.3 and ended after 5.0 (Figure 4.4a). Additional information on mean buffering capacity is shown in Appendix K. As expected, the control had the lowest buffering capacity during gastric digestion. Interestingly, from pH 5.5 to 4.6 buffering capacity was lower with the 80:20 compared to the 40:60 treatment. Thus, higher whey proportions seem to resist the change in pH better than casein during the addition of acid. However, by pH 4.7 the reverse was observed in that buffering capacity was higher for the 80:20 compared to the 40:60 treatment.

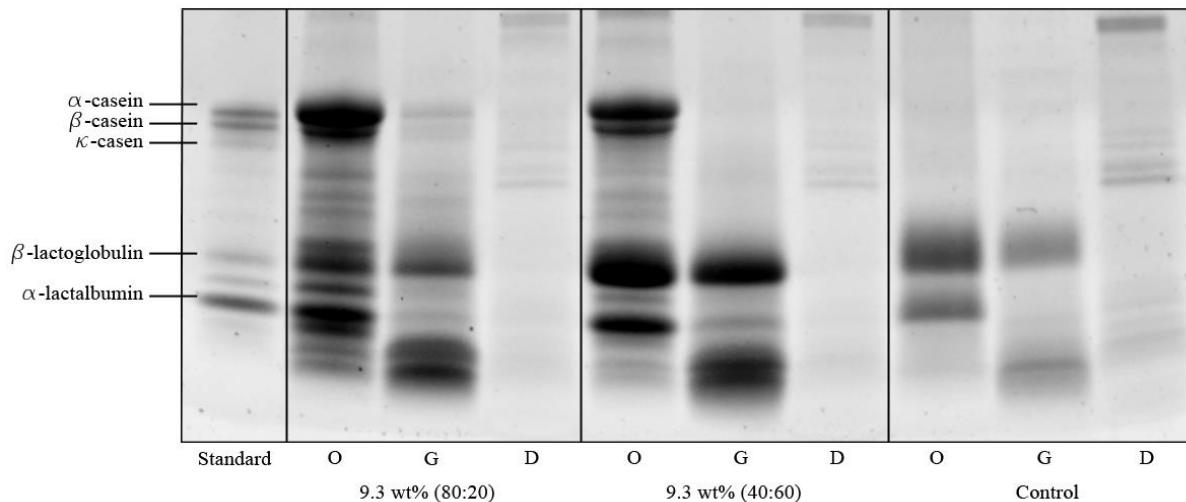


**Figure 4.4** Buffering capacity during a) gastric and b) duodenal digestion for the control (+), 9.3% MP (80 casein:20 whey protein) ( $\triangle$ ), and 9.3 MP (40 casein:60 whey protein) ( $\square$ ).

Duodenal buffering capacity differed by treatment ( $P<0.01$ ), pH ( $P<0.01$ ) and treatment  $\times$  pH interactions ( $P<0.01$ ) (Figure 4.4b). Comparable to gastric trends, the control had the lowest buffering capacity during duodenal digestion. In addition, from pH 3.5 to 4.7, buffering capacity

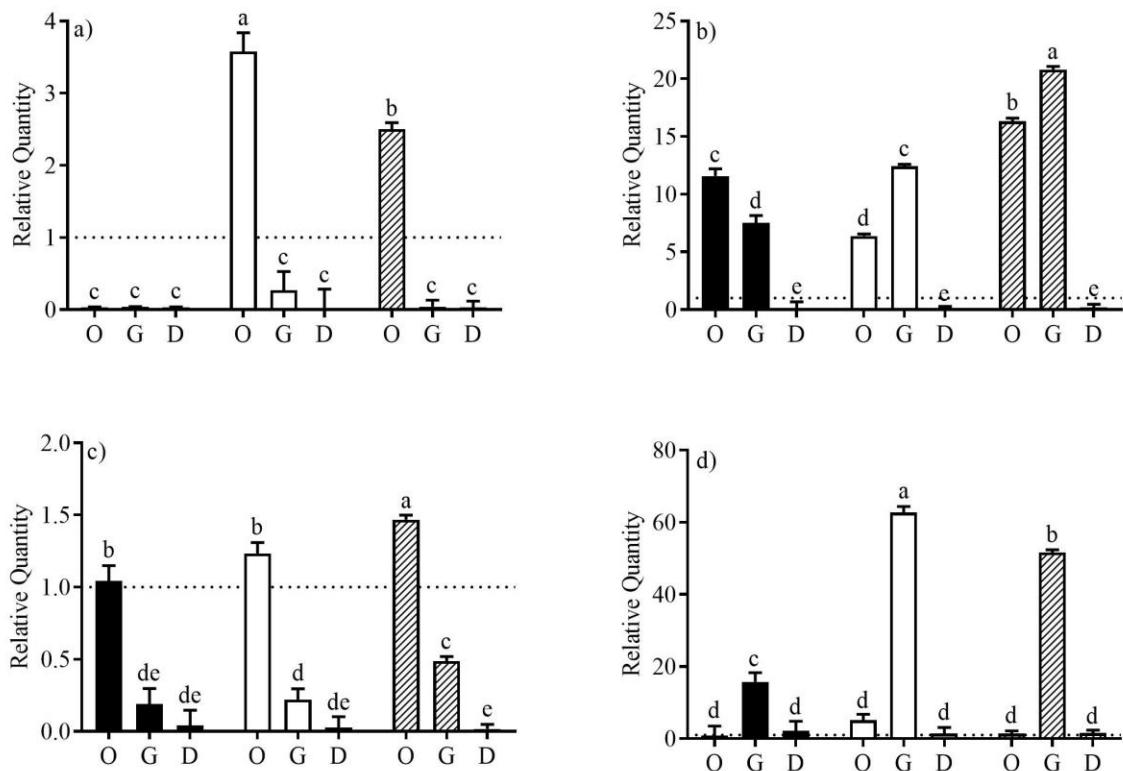
was lower in normal (80:20) protein ratio compared to modified (40:60) treatment and from pH 4.7 to 7.1, higher resistance to the change in pH for the modified (80:20) treatments was observed.

#### 4.4.4. Protein hydrolysis by SDS-PAGE



**Figure 4. 5** SDS-PAGE protein bands for 9.3 wt% (80:20), 9.3 wt% (40:60) and the control at the end of oral (O), gastric (G), duodenal (D) phase.

The relative volumes of protein and peptide in treatments and the control from the protein standard was estimated with SDS-PAGE (Figure 4.5). Caseins,  $\beta$ -lg and peptides volumes differed by treatment ( $P<0.05$ ), time ( $P<0.01$ ), and treatment  $\times$  time interactions ( $P<0.01$ ). Similarly,  $\alpha$ -la differed between treatment ( $P<0.01$ ), and effect of time ( $P<0.01$ ), but there were no treatment  $\times$  time interactions ( $P=0.06$ ) (Figure 4.6). At the end of the oral digestion (before proteolysis) protein profiles associated with the composition of the treatment were observed. The 9.3 wt% (80:20) treatment was associated with significantly more intact caseins than 9.3 wt% (40:60) treatment ( $P<0.05$ ) (Figure 4.6a). The reverse was observed as more intact  $\beta$ -lg and  $\alpha$ -la in the 9.3 wt% (40:60) compared to 9.3 wt% (80:20) ( $P<0.05$ ) (Figure 4.6b and c). Moreover, more  $\beta$ -lg was observed in the control compared to the 9.3 wt% (80:20) treatment, most

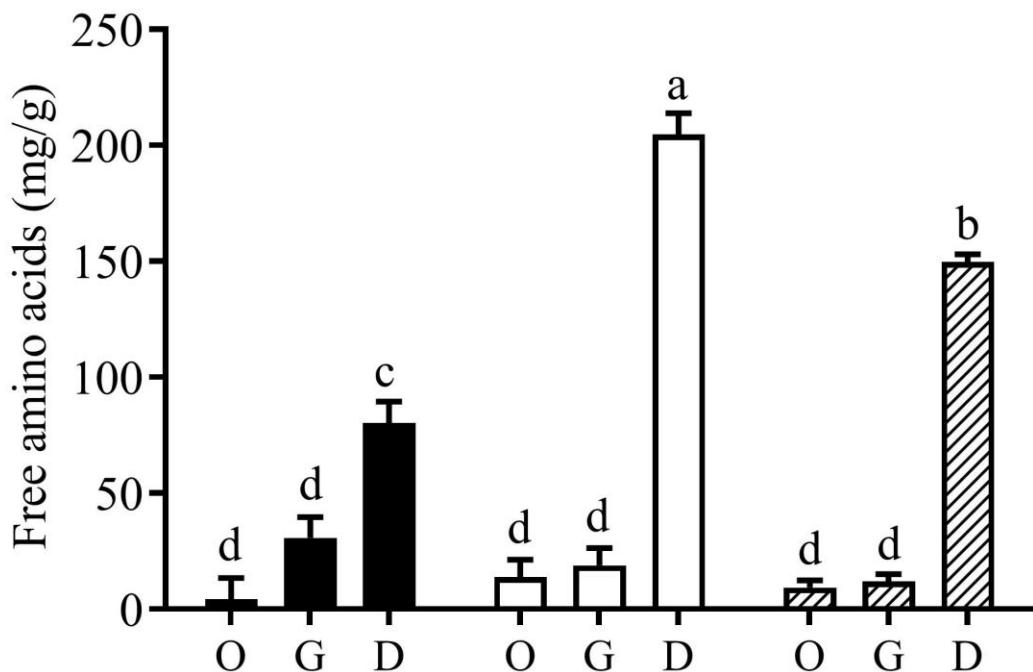


**Figure 4.6** Relative quantity of protein volume to the standard for a) casein, b)  $\beta$ -lg, c)  $\alpha$ -la and d) peptides for the control (■), 9.3 wt% (80:20) (□), and 9.3 wt% (40:60) (▨) at the end of each of the oral (O), gastric (G) and duodenal (D) phases. Superscript letters differed according to LS Means multiple comparison for treatment  $\times$  time interactions ( $P<0.05$ ).

likely due to permeate being sourced from cheese whey. At the end of the gastric phase, casein bands faded among treatments, but more  $\beta$ -lg and  $\alpha$ -la seems to remain intact between treatment. This was confirmed by higher presence of peptides by the 9.3 wt% (80:20) treatment (Figure 4.6d). At the end of the duodenal phase, overall there were no intact proteins and peptides detected for all treatments and the control. In summary, the results indicate a greater presence of intact casein in the 9.3 wt% (80:20) treatment, and  $\beta$ -lg and  $\alpha$ -la in 9.3 wt% (40:60) at the end of the oral phase. Then at the end of the gastric phase, higher presence of  $\beta$ -lg and  $\alpha$ -la were observed in the 9.3 wt% (40:60) treatment, as more peptides were observed in the 9.3 wt%

(80:20) treatment. Finally, at the end of the duodenal phase, there were no proteins or peptide differences between treatments.

#### 4.4.5. TAA



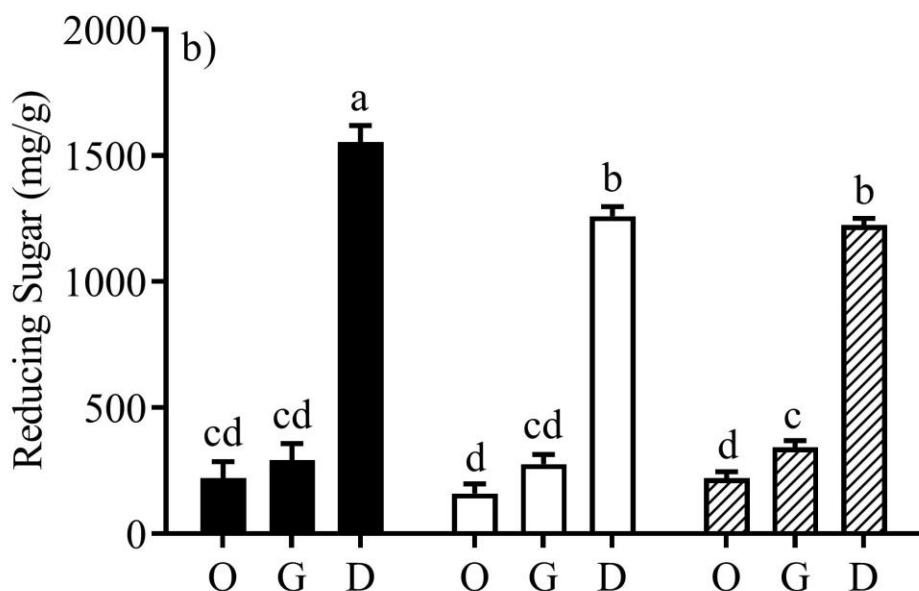
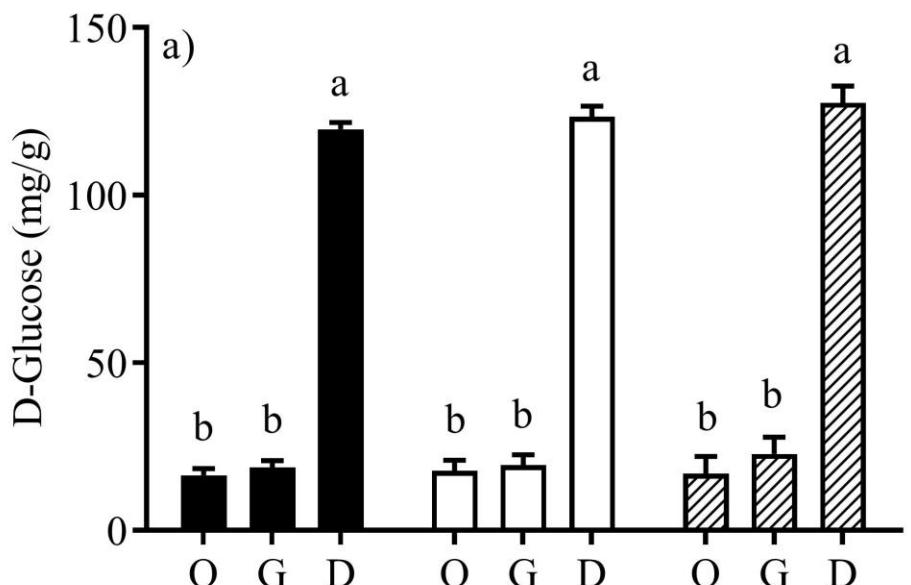
**Figure 4. 7** Mean ( $\pm$  SEM) free TAA (mg/g protein of undigested breakfast) for the control (■), 9.3 wt% (80:20) (□), and 9.3 wt% (40:60) (▨) at the end of each of the oral (O), gastric (G) and duodenal (D) phases. <sup>a-d</sup>Superscript letters differ according to LS Means multiple comparison for treatment  $\times$  time interactions for all treatment bars ( $P<0.05$ ).

TAA released differed by treatment ( $P=0.01$ ), time ( $P<0.01$ ), and treatment  $\times$  time interactions ( $P<0.01$ ) (Figure 4.7). No significant differences were observed between treatments and control during the oral and gastric phase ( $P>0.05$ ). After gastric digestion, minimal TAA were released by the 80:20 ( $18.9 \pm 7.5$  mg/g) and 40:60 treatments ( $11.9 \pm 3.2$  mg/g). For the control, overall less amount of TAA was observed at all digestion phases. This is probably related to the low protein content of the control and consequently less TAA present. At the end of the duodenal phase, 80:20 released the highest amounts of TAA ( $204.7 \pm 9.2$  mg/g) compared

to 40:60 treatment and the control ( $149.7 \pm 3.2$  and  $80.3 \pm 9.1$  mg/g, respectively), pointing to differences in relative digestibility of casein and whey, since both treatments contained 9.3 wt% protein.

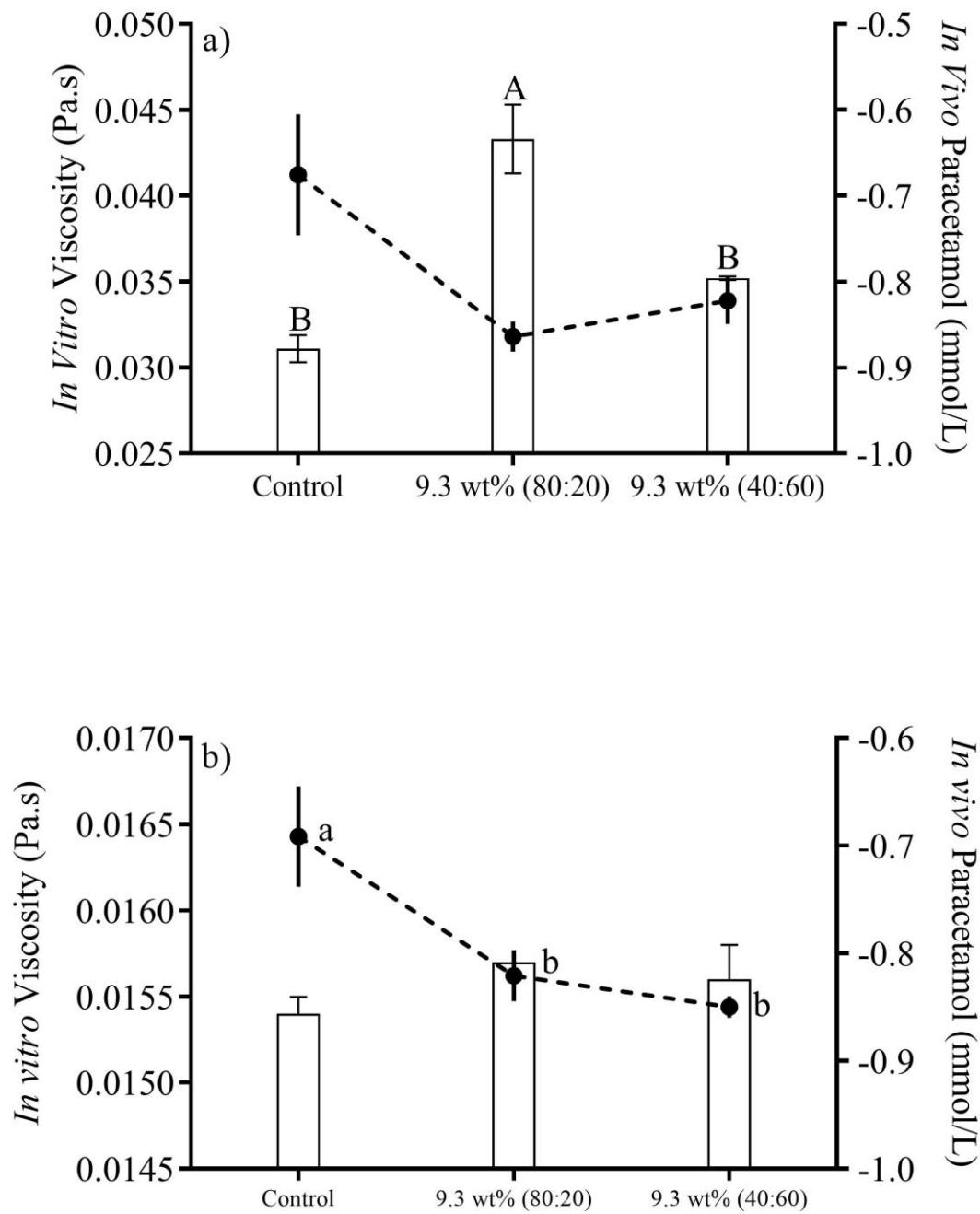
#### *4.4.6. D-glucose and reducing sugars*

Overall, D-glucose differed by time ( $P<0.01$ ) but showed no significant difference between treatments ( $P=0.39$ ), or treatment  $\times$  time interactions ( $P=0.89$ ) (Figure 4.8a). Similarly, reducing sugar analysis showed no treatment effect ( $P=0.06$ ), but there was an effect of time ( $P<0.01$ ) and treatment  $\times$  time interactions ( $P=0.03$ ) (Figure 4.8b). In the absence of  $\alpha$ -amylglucosidase,  $\alpha$ -amylase releases very little glucose and reducing sugar from cereals. Thus, similar amounts were detected at the end of the oral and gastric digestion. By the end of the duodenal digestion, there was no significant difference between treatments for D-glucose but lower amounts of reducing sugar was observed in the 80:20 and 40:60 protein treatments ( $1259.7 \pm 38.4$  and  $1223.9 \pm 26.3$  mg/g, respectively) compared to the control ( $1554.7 \pm 65.1$  mg/g). Results for D-glucose and reducing sugar are similar, except protein treatments seems to interfere with  $\alpha$ -amylglucosidase and the production of reducing sugars, but this was not observed in D-glucose release.



**Figure 4. 8** Mean ( $\pm$ SEM) a) D-glucose and b) reducing sugar (mg/g available carbohydrate of undigested breakfast) for the control (■), 9.3 wt% (80:20) (□) and 9.3 wt% (40:60) (▨) at the end of each of the oral (O), gastric (G) and duodenal (D) phases. <sup>a-d</sup>Superscript letters differ according to LS Means multiple comparison for treatment  $\times$  time interactions for all treatment bars ( $P<0.05$ ).

*4.4.7. Relationships between in vivo and in vitro endpoints*  
*Gastric emptying and digestate viscosity*

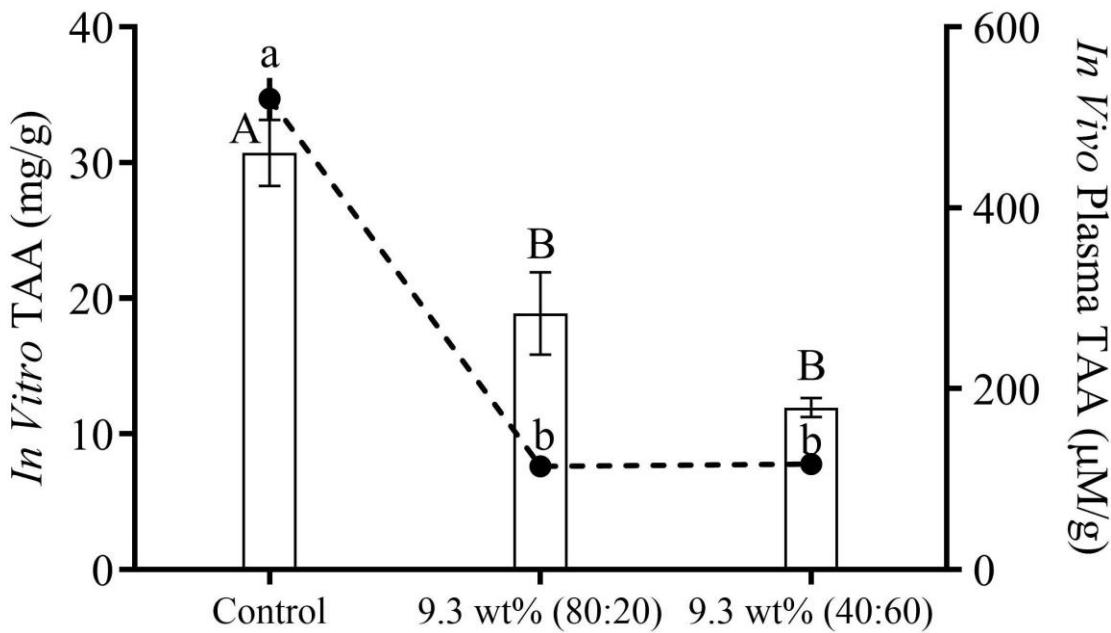


**Figure 4.9** Mean ( $\pm$ SEM) viscosity (Pa.s) ( $\square$ ) and paracetamol concentration (mmol/L) (-●-) for a) females (n=6) at the end of gastric digestion (60 min) and b) all participants (n=12) at the end of duodenal digestion (120 min) for the control, 9.3 wt% (80:20), and 9.3 wt% (40:60). Superscript letters ( $A-B$  within *in vitro* and  $a-b$  within *in vivo* treatments) differ according to LS Means multiple comparison for treatment effect ( $P<0.05$ ).

Treatment trends at the end of gastric digestion between *in vitro* apparent viscosity (Pa.s) at 62 min and *in vivo* paracetamol (mmol/L) at 60 min were observed in females (n=6), but not in males or all subset participants. Slower gastric emptying ( $-0.86 \pm 0.05$  mmol/L) and higher *in vitro* gastric viscosity ( $0.04 \pm 0.00$  Pa.s)(P<0.05) by 9.3 wt% (80:20) was observed (Figure 4.9a). At the end of duodenal digestion, both treatments (9.3 wt% 80:20 and 40:60) had higher apparent viscosities (Pa.s) (92 min) and lower plasma paracetamol concentrations (120 min) in all participants and both genders (Figure 4.9b). However, the 9.3 wt% (80:20) treatment with the highest viscosity was not associated with the lowest paracetamol concentrations. Overall, the results may suggest an association between slower gastric emptying and higher apparent viscosity, but with influence from gender differences.

#### *Plasma TAA and in vitro TAA*

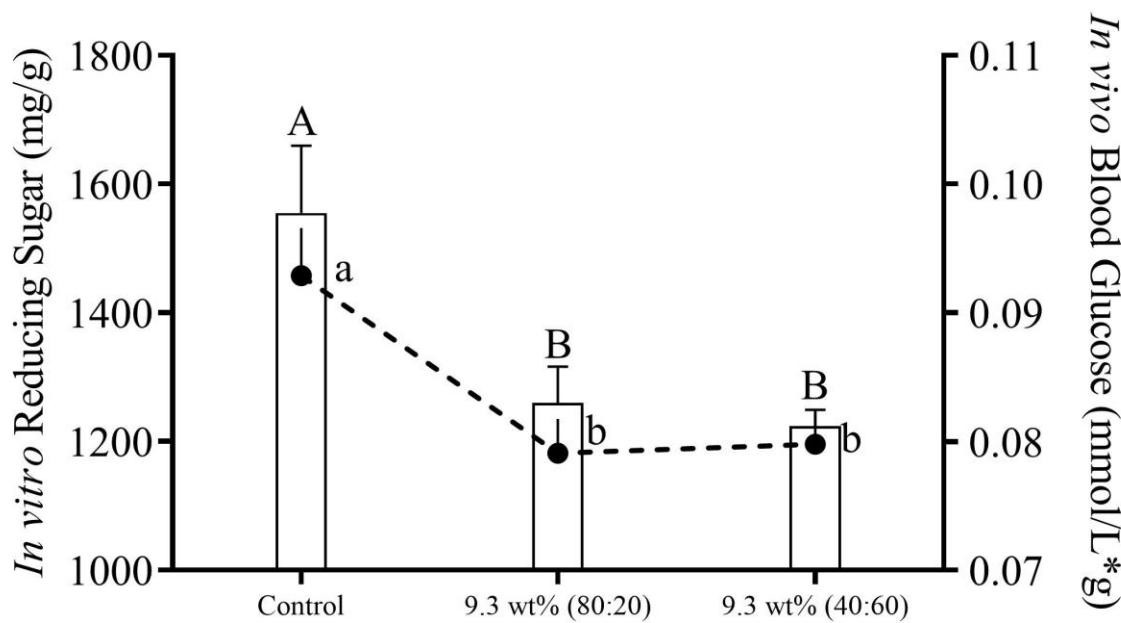
Comparable treatment trends between *in vitro* TAA (mg/g protein of undigested breakfast) (62 min) and *in vivo* plasma AAs ( $\mu$ M/g protein of undigested breakfast) (60 min) in all participants and both genders (data not presented) were observed at the end of gastric digestion (Figure 10). Therefore, the *in vitro* treatment trends reflect the *in vivo* plasma TAA. Both milk treatments had lower plasma and *in vitro* TAA compared to the control (P<0.05), but there was no difference between treatments (P>0.05). However, trends between *in vitro* (92 min) and plasma TAA (120 min) were not comparable at the end of duodenal digestion (data not presented). Plasma TAA treatment trends remain unchanged following gastric to duodenal digestion, but higher TAA was observed by the 9.3 wt% (80:20) treatment in *in vitro*.



**Figure 4. 10** Mean ( $\pm$ SEM) *in vitro* TAA (mg/g) (□) (62 min) and plasma TAA ( $\mu$ M/g) (●) for all participants (n=12) (60 min) at the end of gastric digestion for the control, 9.3 wt% (80:20), and 9.3 wt% (40:60). Superscript letters (<sup>A-B</sup> within *in vitro* and <sup>a-b</sup> within *in vivo* treatments) differ according to LS Means multiple comparison for treatment effect ( $P<0.05$ ).

#### BG and sugar release

There were no comparable treatment trends between BG and D-glucose or reducing sugar at the end of gastric digestion (62 min) (data not presented). However, similar treatment trends were observed for duodenal digestion in terms of BG (90 min) and reducing sugar concentrations (92 min) in male, female (data not reported) and all participants (Figure 4.11). Both milk treatments significantly attenuated BG (90 min) and *in vitro* reducing sugars (92 min) at the end of duodenal digestion compared to the control ( $P<0.05$ ). There were no comparable treatment trends between BG and D-glucose results at the end of duodenal phase.



**Figure 4. 11** Mean ( $\pm$ SEM) *in vitro* reducing sugar (mg/g) (□) (92 min) and BG (mmol/L\*g) (●) for all participants ( $n=12$ ) (90 min) at the end of duodenal digestion for the control, 9.3 wt% (80:20), and 9.3 wt% (40:60). Superscript letters (<sup>A-B</sup> *in vitro* and <sup>a-b</sup> *in vivo*) differ according to LS Means multiple comparison for treatment effect ( $P<0.05$ ).

#### 4.5. DISCUSSION

This study investigated the *in vitro* digestion of cereal and milk with modified casein-to-whey (40:60) ratio and the influence of varying protein on proteolytic profile and starch hydrolysis. It furthers research on stimulated digestion of proteins by including more complex meal such as combining both dairy proteins with carbohydrates (Lambers et al., 2013). The main hypothesis was mostly validated as there were different proteolytic and buffering capacity profiles between treatments richest in casein versus whey proteins and the control and some inhibition of amyloylsis by protein treatments. However, increased gastrointestinal viscosity content as the underlying mechanism for BG attenuation was not supported. At the end of the duodenal phase, no intact protein and peptides, nor differences between breakfast disintegration or protein solubility, were detected between treatments, although, more AAs were found in the

case of the 9.3 wt% (80:20) treatment. Thus, some proteolytic results were conflicting and highlight the challenges of working with a mixed-meal product.

The induction of higher gastric and duodenal viscosities by the 9.3 wt% (80:20) treatment may be due to the presence of clotted casein aggregates. Thus, simulated digestion of dairy proteins provided complementary evidence of “fast” and “slow” digestion kinetics of whey and casein as observed in human studies (Boirie et al., 1997). A modest increase of remaining casein, although not significant, was quantified in 9.3 wt% (80:20) ( $0.27 \pm 0.3$ ) compared to the 9.3 wt% (40:60) ( $0.04 \pm 0.1$ ) treatment at the end of the gastric digestion. Still, the presence of higher proportion of casein (80:20) resulted in a significantly higher gastric viscosity. Enhanced viscosity may be somewhat explained by synergistic interaction of the protein and undigested  $\beta$ -glucan from the oat-based cereal (Rinaldi, 2013; El Khoury et al., 2012). Similar enhanced viscosity observations from the protein- $\beta$ -glucan interaction were found in high dairy protein beverages (Vasquez-Orejarena et al., 2018), yogurts (Rinaldi et al., 2015) and sodium caseinate mixtures (Agbenorhevi et al., 2013). This synergy was described as a complex coacervation between network formation of aggregates or by electrostatic interaction between the aggregates and polysaccharide (Lambers et al., 2013; De Kruif and Tuinier, 2001; Agbenorhevi et al., 2013). However, as in other studies, the protein-fibre interactions would have been diluted and weakened due to the addition of simulated juices, leading to phase separation between protein and  $\beta$ -glucan and a lower impact of viscosity (Rinaldi, 2013; Corredig et al., 2011; Kontogiorgos et al., 2009).

These results did not indicate that increased viscosity influenced starch hydrolysis and release of sugars. Higher apparent gastric and duodenal viscosities with the 9.3 wt% (80:20) treatment were observed. However, reducing sugars were lowered by both protein treatments

compared to the control, and there was no D-glucose difference amongst the treatments at the end of duodenal digestion. This result was surprising since increased digesta viscosity has been suggested to delay amylolysis, as well as the subsequent breakdown of starch by limiting the contact between digestive enzymes with starch (Repin et al., 2017; Singh et al., 2010). In the current experiments, the viscosity measured was low (<1 Pa.s), which could explain the lack of impacts on starch hydrolysis. Nonetheless, there seems to be some inhibition of pancreatic  $\alpha$ -amylase and release of reducing sugar from the presence of peptides, independent of viscosity. This inhibition of pancreatic  $\alpha$ -amylase may be due to the presence of oat components such as phenols and antioxidants and/or the interactions of oats components with inhibitory bioactive peptides (Admassu et al., 2018; Hanhineva et al., 2010). Perhaps the attenuation of reducing sugar was not reflected in the amount of D-glucose because the reaction was early in the duodenal stage and lower fungal  $\alpha$ -amyloglucosidase activity relative to pancreatic  $\alpha$ -amylase activity (Warren et al., 2015). A surpassing amount of  $\alpha$ -amyloglucosidase was utilized over  $\alpha$ -amylase, but the activity was possibly affected by lower digestion temperature than the optimal range (60°C) and short digestion duration (30 min). Whereas,  $\alpha$ -amylase sourced from porcine pancreatin operates at a closer optimal temperature (40°C) to human digestion (37°C). Thus, our results may suggest some amylolysis inhibition by proteins, although probably not related to chyme viscosity, and further verification is necessary by utilizing supplemental  $\alpha$ -amyloglucosidase.

Despite the potential for *in vitro* digestion models to elucidate *in vivo* results, there are limitations to their use. Specific to this project, the mono-chamber does not facilitate the transport of chyme from the stomach to duodenal digestion. Thus, higher duodenal viscosity was maintained beyond the gastric phase due to the remnants of clotted casein aggregates together

with soluble  $\beta$ -glucan (Rinaldi, 2013). The absence of gastric emptying further complicates the release of nutrients in the duodenal stage. During human digestion, AAs kinetics suggest that clotted casein aggregates have remained in the stomach for further antral grinding until < 1-2 mm to enter the intestines, whereas soluble whey protein would enter the intestine earlier resulting in a faster appearance of AAs (Chapter 3, Figure 3.1). However, deprived of the gastric emptying mechanism, our result suggests resistance of whey protein to gastric hydrolysis. Whereas most of the casein proteins were hydrolyzed at the end of the gastric phase (Figure 4.5). Hence, this may be the reason for a higher release of TAA by the 80:20 treatment compared to 40:60 at the end of the duodenal phase. The resistance of whey protein to pepsin have been observed in other *in vitro* studies (M. R. Guo et al., 1995; Kim et al., 2007; Mat et al., 2018), as well as in animal model (Tari et al., 2018).

Furthermore, the heterogenous composition and complexities of the treatments, containing both milk and cereal, posed analytical challenges contributing to proteolytic results. The maximum amount of free TAA (approximately <200 mg/g protein of undigested breakfast) at the end of duodenal phase was lower than expected. Conversely, Rinaldi et al. (2015) observed approximately 200 – 300 (mg/g protein of undigested breakfast) of free TAA in yogurts with similar protein concentration and digestion parameters using gas chromatography. The reason for lower release of TAA may be due to the difficulties in analyses by the Cd-Ninhydrin method and low and unstable color yield. Inconsistent color yield via the ninhydrin method has been previously reported due to water content and presence of some carbohydrates (Kopfraum and Maier, 1970). This method has been validated for barley, corn, lima bean, soyabean, rice and wheat seeds (Friedman, 2004), but to our knowledge this method has not been validate for oat cereal. To compensate for color instability, the method was adapted to 1:4.25 sample-to-reagent

ratio, prevented possible evaporation by covering with a tight cap, and immediate sampling (Doi et al., 1981). In summary, based on the SDS-PAGE results towards the end of the duodenal digestion, no intact dairy proteins, peptides and enzymes were present amongst the treatments, signifying that all proteins have been hydrolyzed into smaller peptides and AAs as previously reported (Rinaldi et al., 2015; Rioux and Turgeon, 2012). Yet, the comparison of differing quantity of duodenal free TAA by the Cd-ninhydrin method to SDS-PAGE may be difficult as the source (hormones, proteins) of free TAA is unknown.

#### *In vitro-in vivo relationships*

*In vitro* methods have been deemed as an useful measure of the behaviour of food products, which can be examined under simulated digestion to elucidate mechanisms and predict physiological responses while negating the risk and the expensive of conducting a clinical trial. The 9.3 wt% (80:20) treatment increased apparent gastric viscosity and was associated with the lowest paracetamol concentrations in female participants at 60 mins (Figure 4.9a), suggesting some influence of casein aggregate-induced viscosity on gastric emptying (and early subjective appetite rating although not at the exact 60 min timepoint, (Chapter 2, Figure 2.3)). Interestingly, the presence of gastric viscosity was not associated with reductions in TAA, reducing sugars or D-glucose at the end of the *in vitro* gastric phase. This result seems to reflect the *in vivo* results, as paracetamol concentration (gastric emptying) did not mirror the lowered plasma TAA and BG by protein treatments at the end of 60 min. This may be explained by in-progress gastric digestion by a higher volume meal and minimal nutrient release into the small intestine. Also, the attenuation of TAA release both *in vivo* and *in vitro* for the protein treatments may indicate incomplete hydrolysis, as protein treatments should release more TAA than the control (Figure 4.10). At the end of duodenal digestion, there was no apparent viscosity difference amongst

treatments, but reduced paracetamol concentrations (gastric emptying) by protein treatments relative to the control were observed (Figure 4.9b). The reduction of paracetamol may indicate slower gastric emptying by unaccounted physiological factors such as gastric hormonal response. Likewise, plasma TAA and BG were attenuated by protein treatments compared to the control. Comparable results were observed as reducing sugars was attenuated by protein treatments, but this is only indicative of starch hydrolysis inhibition as we are unable to measure gastric emptying or hormonal response in the simulated digestion model.

The correlations between *in vitro*-*in vivo* data was challenging due the design of the *in vitro* models. Particularly in this study, nutrient sampling was limited to the end of each *in vitro* digestion phase to accurately measure the digesta viscosity. Thus, there were too few sampling points for the productions of digestion curves, area-under-the-curve, regression and correlations analyses. For the future, 5 timepoints at 15 min intervals could be utilized during the pre-lunch duration (0, 15, 30, 45 and 60 min) which would represent the gastric digestion stage and another 4 timepoints (70, 80, 90 and 100 min) at 10 min intervals would represent the duodenal digestion stage for nutrient sampling. Besides this, *in vitro*-*in vivo* time points for duodenal digestion were not synchronized as the duration was limited to 30 min to prevent complete hydrolysis and/or arriving at a plateau. Nonetheless, the *in vitro* conditions allowed for the studying specific enzymatic activities and nutrient bioaccessibility, and several *in vitro*-*in vivo* trends were observed.

#### **4.6. CONCLUSION**

This study demonstrated the challenges of analyzing a commonly consumed heterogenous food matrix – cereal and milk. The presence and digestion of different whey and casein proteins ratios were observed. There was no indication of influence of viscosity on starch

hydrolysis nor was *in vitro* viscosity associated with the attenuation of postprandial BG. Our results may suggest stronger influence of other physiological factors on BG such as gastric hormone. *In vitro* digestion of the breakfast meal with 9.3 wt% protein content and a normal (80:20) casein-to-whey ratio showed inverse trends between gastric viscosity and *in vivo* gastric emptying (paracetamol, mmol/L). At the end of duodenal digestion, nutrients were hydrolyzed to absorbable forms amongst all treatments and the control, but free TAAs could not be quantified due to instability of the Cd-ninhydrin method. There also seems to be some inhibition of starch hydrolysis due to the presence of protein, but not in association with chyme viscosity. Thus, attenuation of BG seen in the presence of milk protein in the clinical trial could not be explained by starch hydrolysis inhibition by increased viscosity, as studied by the *in vitro* method, and may be due to the influence of other physiological factors.

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## CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

### 5.1 PREAMBLE

The purpose of this work was to address gaps in the literature regarding the ingestion of dairy proteins with a carbohydrate rich breakfast meal on postprandial glycemia and appetite. The precise mechanisms by which whey proteins alter these processes are not well understood, but there is evidence that it is related to the ability of whey to influence gastric emptying earlier than casein protein. Understanding the digestion profile of dairy proteins and the mechanisms involved in the attenuation of appetite and postprandial glycemic responses will help to formulate foods that reduce these responses more effectively. Specifically, this work focused on the use of a novel dairy beverage with enhanced protein concentration (wt%) and modified protein ratio from commercially available milk products. Also, the testing of co-ingestion of the dairy proteins with high carbohydrate cereal as a breakfast meal on BG, appetite and subsequent lunch intake. This assessment was carried out as per three specific objectives: to determine the effects of the novel beverages on glycemia and appetite in healthy individuals; to determine the appearance of plasma AAs and relationship with gastric emptying (paracetamol concentration); and to explore the relationship between *in vitro* and *in vivo* digestions in an attempt to elucidate physiochemical *in vitro* measurements that can help to validate or accompany *in vivo* results. Thus, this thesis provides multilevel support for the attenuation of BG and modest attenuation of appetite by higher whey protein proportions and could potentially aid glycemic and satiating responses in healthy individuals.

### 5.2 SUMMARY AND DISCUSSION OF MAJOR FINDINGS

Study 1 (Chapter 2) tested the co-ingestion of enhanced (9.3 wt%) milk protein concentration (from 3.1 wt%) and reduced (40:60 casein-to-whey) protein ratio (from normal 80:20) with a high carbohydrate breakfast cereal on postprandial BG, satiety and subsequent *ab*

*libitum* lunch intake. Pre-lunch BG change from baseline and iAUC were significantly reduced after consumption of all milk treatments relative to the control. High protein (9.3 wt%) contrasted to normal protein (3.1 wt%) treatment lowered BG change from baseline and iAUC. Modified (40:60) protein ratio lowered BG change from baseline only, but not iAUC. Also, normal (80:20) protein ratio lowered pre-lunch appetite change from baseline, but not tAUC. However, there was no treatment effect on subsequent lunch energy intake. Post-lunch, high protein (9.3 wt%) treatments contrasted to normal protein (3.1 wt%) treatments lowered appetite change from baseline and tAUC. Therefore, a decrease in BG was seen following consumption of enhanced (9.3 wt%) protein concentration and a modest reduction following modified (40:60) ratio prior to the lunch meal, accompanied by a decrease in appetite following enhance (9.3 wt%) protein concentration after the lunch meal. This shows that consumption of higher (9.3 wt) protein concentration is more efficient at lowering BG than a beverage with altered whey: casein protein ratio which increases the proportion of whey.

Study 2 (Chapter 3) looked more closely at the appearance of plasma AAs in a subset group. The appearance of plasma AAs groups (TAA, BCAA, EAA, NEAA) may induce slower gastric emptying (determined via an indirect measure; paracetamol absorption) and offer a potential explanation for the BG attenuation observed in Study 1. Pre-lunch, high (9.3 wt%) protein milk increased all AA groups (TAA, BCAA, EAA and NEAA) when contrasted to normal (3.1 wt%) protein concentration. Also, reduced casein to whey ratio (40:60) milks increased BCAA and EAA iAUC compared to normal (80:20). Blood paracetamol iAUC was lower after ingestion of the high (9.3 wt%) protein milks, but there was only an effect of modified ratio at 30 min. Further support for slower gastric emptying via gastric hormone feedback was shown through increased GLP-1 and CCK responses after consumption of high

protein concentration. The effect of ratio was only observed on GLP-1 response but not CCK and paracetamol concentration. Yet, there were no insulin and C-peptide differences observed between treatments, confirming insulin-independent mechanism on BG management. All pre-lunch AA groups were inversely correlated with paracetamol change from baseline concentration, suggesting proportional post-absorptive association between the release of plasma AAs and slower gastric emptying. However, food intake was not affected by treatment as coincided with Study 1. Post-lunch, the high (9.3 wt%) protein milks increased TAA, BCAA, EAA and NEAA iAUC when contrasted to those with low (3.1 wt%) protein concentration. Normal (80:20) ratio increased TAA compared to modified (40:60) protein at 200 min indicating the release of AAs by casein, but paracetamol concentration did not reflect the appearance of AAs. Thus, the inverse correlation between pre-lunch plasma AAs and paracetamol concentration further supports the role of AAs in reduced gastric emptying and supporting BG management.

Study 3 (Chapter 4) complemented the clinical trials by analyzing the high protein milks (9.3 wt% 80:20 and 9.3 wt% 40:60) via *in vitro* digestion. Furthering investigation of the attenuation of postprandial BG, possible mechanisms such as gastric viscosity and amylolysis inhibition were explored. Higher gastric and duodenal viscosities with the high protein normal ratio (9.3 wt% 80:20) treatment were observed. At the end of the duodenal digestion, both protein treatments lowered reducing sugars compared to the protein-free control, suggesting some amylolysis inhibition by the presence of protein. Several observations were made by comparing the *in vitro* and *in vivo* studies, particularly in terms of higher gastric viscosity and attenuated gastric emptying in the presence of clotted casein aggregation, and the fact that both protein treatments attenuated *in vitro* and plasma TAA at the end of gastric digestion and lowered

the release of reducing sugar and BG at the end of duodenal phase. Therefore, increased viscosity at the low levels studied, did not seem to have a direct influence on the inhibition of starch hydrolysis and the appearance of *in vivo* BG.

### **5.3 STRENGTHS AND LIMITATIONS**

This study provided a deeper understanding on the effects of protein when co-ingested with a carbohydrate-rich meal. Studying co-ingestion of multiple macronutrients is challenging and research that includes more than one macronutrient is limited due to the confounding effects of how one macronutrient influences the other. A large human study (n=32) was employed including an equal number of healthy participants from both genders, which resulted in study differences due to hormonal factors, body composition and weight and food consumption. Also, multiple blood sampling sites and timepoints were obtained from both fingerpricks and intravenous sites to provide sufficient results for analyses. A full understanding of glycemic management, hormonal responses and mechanisms were presented through a large collaborative effort and novel insights about nutrient digestibility and gastrointestinal factors were offered based on the inclusion of the *in vitro* digestion methods. Moreover, the comparisons of plasma AAs and gastric emptying extend this focus. In addition, the study of our treatment composition was multileveled from the effects of intact dairy protein concentrations and proportions of casein and whey to AAs.

The study treatments were designed to test milks containing higher protein concentrations and modified ratio, enhanced in whey, with a high-carbohydrate source, as a typical North American breakfast meal. A commercially available oat-based cereal was chosen because it was a commonly consumed product. Although this meal represents a realistic scenario, the cereal contained a substantial amount of soluble fibre and this may have affected certain outcome measures such as viscosity, precluding the ability to differentiate effects specific to the protein

differences. Thus, it is important to test other sources of carbohydrates to solidify the effect based solely on dairy protein, and to eliminate any affect due to carbohydrate source. Also, the dairy beverage was formulated with WPC, which contained a high amount of lactose (34.7 g) which was shown to cause some slight difference in overall gastrointestinal function in our participants 24 h after the study visit [22 (13-40)] to that at the time of pre-screening [10 (4-17)]. Although, pre-screening criteria included regular dairy consumers (at least 2 servings) and excluded individuals with gastrointestinal intolerances, this may or may not have influenced appetite and energy intake. Reformulating the dairy beverage with WPI would minimize the amount of lactose. Regarding the study design, plates of pizzas were cut into quarter slice but the amount of pizza was not blinded to participants and thus participants could quantify the number of slices consumed. This may have cognitive consequences to restricted or unbounded food intake.

Food digestion is a complex biological process involving several variables that occur together. Thus, the mimicking of food digestion by *in vitro* is a challenging endeavour to accurately emanate postprandial responses. *In vitro* digestion models cannot simulate all physiological mechanisms, including hormonal feedback, immune system and gastrointestinal contractions that influence transit. Also, enzyme activities were not added based on feeding state, age and physical constitutions as it would be *in vivo*. Nor were study treatment volumes based on participant body weight or energetic/nutritional needs and body weight differs, particularly between male and female participants which may help to explain why different trends in subsequent food intake were observed. In this study, the motility utilized as the mechanical forces was an anchor stirrer and the gastrointestinal containment was a mono chamber. The limitation of this static system eliminates compartments in the stomach and thus there were no

stacking of the food bolus nor separation between solid and liquid phases. A solid food bolus would have resulted in a slower digestion due to the inability of the digestive juices to access the interior of the bolus. Moreover, there was no continual removal of nutrients out of the stomach, which allowed continual access of enzymes. Sampling only occurred at the end of the digestion time and the *in vitro-in vivo* time point for duodenal digestion did not match. This limited our ability to correlate between *in vitro* and *in vivo* studies as there were insufficient data points to produce a linear regression or correlation coefficient.

#### **5.4 FUTURE DIRECTIONS**

There are many opportunities to expand and build upon the current field of research. Specific to this study design, the volume served was large and may have affected subsequent food intake due to the short meal interval. Thus, it is recommended to either decrease the volume of the breakfast meal, individually calculate food intake based on BMI or lengthen the interval between breakfast meal and lunch meal. The latter recommendation would extend the overall study period which may help to characterize the release of AAs from casein protein. Overall, a long-term study can assess if consumption of higher protein breakfast will benefit BG response, insulin sensitivity and weight loss.

Further physiochemical and data analyses could be conducted to provide understanding of relationships and additional validation and accuracy. Correlations between plasma AA concentrations and gastric hormones could be explored. Measuring gastric emptying rate using MRI and scintigraphy radioactive markers would provide a more accurate characterization of phase separation and biorelevant results. Additional *in vitro* bioaccessibility work are required to complete the understanding of protein solubility such as calculating the degree of hydrolysis and quantifying D-glucose and free AAs. Particularly, the attenuation of reducing sugar production and D-glucose liberation in the presence of peptides needs further exploration and better

understanding of  $\alpha$ -amylase inhibition by dairy derived peptides but could provide another strategy to lessen the release of glucose. Thus, the challenges of studying a mixed-meal matrix are complicated because there is no standard method and each food product may require different methodological modifications for accurate analyses.

Regarding product development, varying protein proportions should be assessed in other dairy matrices. A further endeavour can also be undertaken to create a superior breakfast meal that influences pre- and post-absorptive satiety signals. This may be achieved through a product enriched with whey protein and novel soluble fibres that would interact with whey proteins to create a strong gelled or viscous network. A strong viscous chyme would act on post-ingestive satiety signals and as the digestion process releases AAs, gastric emptying would be delayed via gastric hormone feedback in the post-absorptive phase.

## 5.5 CONCLUSION

In summary, whey proteins were shown in this study to have a beneficial effect on postprandial BG response by slowing gastric emptying following consumption of a carbohydrate rich breakfast meal. The effect of whey protein on appetite and energy intake remains unclear. *In vitro* digestion experiment provided further insights to the human study by relating higher gastrointestinal viscosity with slower gastric emptying and characterization of casein and whey protein by enzymatic hydrolysis and some amyloylsis inhibition during digestion. Several trends were found between *in vitro* and *in vivo* measurements suggesting that *in vitro* digestion can be a good resource for complement and supporting human work. Therefore, consumption of higher protein concentration, and to a modest extent higher whey protein proportions, may offer a feasible dietary strategy for prevention and management of glycemic response.

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## APPENDICES

### Appendix A: University of Guelph Human Research Ethics Board certificate



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

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APPROVAL PERIOD:	07-Jul-2014
EXPIRY DATE:	06-Jul-2018
REB:	Research Ethics Board – Natural, Physical and Engineering Sciences
REB#:	REB16-12-702
TYPE OF REVIEW:	Full Board
PRINCIPAL INVESTIGATOR:	AMANDA WRIGHT
DEPARTMENT:	Human Health and Nutritional Science
SPONSOR(S):	Dairy Farmers of Canada~
TITLE OF PROJECT:	The effect of milk proteins on satiety, food intake and metabolic control (glycemia) in early adulthood 14JN004

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 a Annual Renewal to the REB upon completion of the project. If the research is a multi-year project, an Annual Renewal must be submitted annually prior to the expiry date. Failure to submit an Annual Renewal will lead to your research being suspended and potentially terminated.

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

## Appendix B: Participant recruitment poster

The poster features the University of Guelph logo on the left, which includes the text "UNIVERSITY of GUELPH" and "CHANGING LIVES IMPROVING LIFE". To the right is a photograph of milk being poured into a glass, creating a splash. Below the image is a large, bold title: "YOUR PARTICIPATION IS NEEDED FOR A MILK STUDY!"

Healthy, non-smoking men and women between the ages of 18 – 30 years are needed to participate in a human nutrition study at the University of Guelph. The purpose of this study is to determine the effects of milk products on food intake, satiety, metabolic response (glycemic).

**Participants will be required to visit the University of Guelph for:**

- One hour screening visit to determine the eligibility
- 30 minute Orientation session
- Five weekly 3.5 hours morning study visits where you will consume milk products, provide blood samples and answer questionnaires periodically.

This study has been reviewed and has received clearance through the University of Guelph Human Research Ethics Board (REB#14JN004) and will be conducted at the Human Nutraceutical Research Unit in the Department of Human Health and Nutritional Sciences.

### FINANCIAL COMPENSATION PROVIDED

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

Human Nutraceutical Research Unit  
University of Guelph  
88 McGilvray Street  
Guelph ON N1G 2W5

Bonnie & Alex  
Milk Study Coordinator  
519-824-4120 x56314  
[Milk@uoguelph.ca](mailto:Milk@uoguelph.ca)

**Appendix C:** Screening 1 questionnaire

## **PHONE SCREENING QUESTIONNAIRE**

**The Milk Study – The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)**

1. How did you hear about the Milk Study? <input type="checkbox"/> Poster <input type="checkbox"/> Friend <input type="checkbox"/> Department Email <input type="checkbox"/> Other: _____			
2. How old are you?			
3. What is your height?			
4. What is your weight?			
$BMI = \frac{\text{weight (kg)}}{\text{height (m)}^2}$ Space for BMI Calculation (Range 18.5 < BMI < 29.9)			
5. Are you pregnant or currently breast-feeding? (female callers only)		YES	NO
6. Do you smoke?		YES	NO
7. Do you consume alcohol? If YES, how many drinks per sitting? (<4)		YES	NO
8. Have you ever been diagnosed with diabetes or told you are pre-diabetic?		YES	NO
9. Do you suffer from hypoglycemia (i.e. low blood sugar levels?)		YES	NO
10. Are you currently taking any medications? This includes over-the-counter or prescription medications, vitamins and dietary supplements (especially acetaminophen (Tylenol) or protein supplements). If YES, what are they?		YES	NO
11. Have you ever taken acetaminophen? This is the primary ingredient in the brand Tylenol. If YES, did you have any reactions to the drug?		YES	NO
12. Do you have any other major diseases or medical conditions (i.e. Liver or renal disease, cardiovascular disease – including hypertension, celiac disease, lactose intolerance)? If YES, please describe:		YES	NO
13. Do you have any gastrointestinal disorders (Celiac disease, gluten intolerant, lactose intolerance, irritable bowel syndrome, etc.)? If YES, please specify:		YES	NO
14. Do you have any food allergies? If YES, please describe:		YES	NO
15. Do you have any anaphylactic or life-threatening allergies? If YES, please specify:		YES	NO
16. Are you currently trying to lose or gain weight? If YES, please specify (i.e. weight loss or weight gain of more than 5 kg during the previous 2 months):		YES	NO
17. Elite athletes are defined as anyone currently competing as a varsity player (individual or team), a professional player or a national or international level player. Training athletes are participating in intense practice and exercise for individual or team events.		YES	NO

Based on this explanation, do you consider yourself an elite or training athlete? If YES, please provide an example of your exercise regime:								
18. Do you exclude cow's milk products from your diet? YES NO								
19. In one week, how many times do you drink cow's milk or consume milk products? 0      1      2      3      4      5      6      7      8      9+								
20. This study involves consuming cow's milk products. Do you have any concerns about this? If YES, please expand:								
21. This study involves consuming acetaminophen, which is the main ingredient in Tylenol. Do you have any concerns about this? If YES, please explain:								
22. Do you regularly consume breakfast? YES NO								
23. Do you regularly skip meals? YES NO								
24. This study involves consuming a sweetened breakfast cereal, Honey Nut Cheerios. Do you have any concerns about this? If YES, please explain:								
25. This study involves consuming cheese pizza. Do you like pizza? Do you have any other concerns about this? If YES, please expand:								
26. Are you comfortable providing blood samples by fingerprick? YES NO								
27. There is an optional Part 2 of this study where eligible participants will be providing blood samples by catheter. Would you be comfortable providing blood samples by catheter? YES NO								
28. The study visits require participants to fast for 12 hours overnight. Water is permitted until 1 hour before each study visit. Would you be comfortable with this? YES NO								
29. Are you able to come to the HNRU at the University of Guelph on two occasions for a 30-60 minute in-person screening and orientation visit? YES NO								
30. If eligible to participate in the study, you will be asked to visit the University of Guelph to attend five 3.5 hour study visits (approx. 8am – noon), over a 2 month period. Would your schedule accommodate these visits? YES NO								

*This concludes the questionnaire. Do you have any questions?*

**UNSURE**

*Thank you for your time. We will be in touch within a few days to let you know if you are eligible to continue in the screening process. Have a nice day.*

**INELIGIBLE**

*Based on the questionnaire, you have not met the requirements for eligibility. We hope you will consider the HNRU for future studies. Please check the HNRU website for our current studies. Thank you for your time and interest. Have a nice day.*

- *Fill out the Phone Screening on the Tracking Log and Trial Activity Checklist.*

## **ELIGIBLE**

*Based on the questionnaire, you have met the initial eligibility requirements. Are you still interested in moving further with the study? If NO: Thank you for your time.*

**If YES:** *We would like to set up an in-person screening visit which will involve filling out a more detailed questionnaire, fingerpick sample and learning more about the study. We ask you to fast for 12 hours prior to your arrival at the HNRU so that we can take a fasting fingerprick blood sample. We also want you to complete a food record of a typical day and bring it to the screening. We will email you instructions on how to complete the food record. A \$5 Tim Horton's gift card will be given to you as compensation for your in-person screening. Thank you again for your time, we will contact you with specific details about booking this visit and send a reminder closer to the date. Included in the email will be a map and directions for finding the HNRU, in case you do not know where we are located. We will also be sending out a sheet for you to record your typical food intake. We appreciate the time you are investing in our study. Do you have any more questions?*

- *Set up an in-person screening visit.*
- *Enter the scheduled date and time of the screening visit into the Google calendar.*
- *Fill out the Phone Screening on the Tracking Log and Trial Activity Checklist.*
- *Arrange to send the potential participant a map to the HNRU and provide parking information.*

In-person screening visit scheduled for:

Date:	Time:	Coordinator Signature:

## **Appendix D:** Screening 2 consent form

### **IN-PERSON SCREENING VISIT INFORMED CONSENT**

#### **The Milk Study**

#### **The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)**

You are being asked to participate in a screening visit for a research study conducted by MSc. students (Bonnie) Hau Ming Kung and Alexander Lazier; Professor Douglas Goff Ph.D. from the Department of Food Science, Associate Professor Amanda Wright Ph.D. and HNRU Manager Amy Tucker Ph.D. from the Department of Human Health & Nutritional Sciences, at the University of Guelph. Results of this study will contribute towards a MSc. thesis for (Bonnie)Hau Ming Kung. This research is sponsored by the Dairy Farmers of Ontario.

If you have any questions or concerns about the research, please feel free to contact the study personnel at any time:

**1. Study Coordinator:** (Bonnie) Hau Ming Kung, B.Sc.H. (MSc graduate student) at 519-824-4120 x56314, [kungh@uoguelph.ca](mailto:kungh@uoguelph.ca)

**2. Study Coordinator:** Alexander Lazier, B.Sc.H. (MSc graduate student) at 519-824-4120 x56314, [laziera@uoguelph.ca](mailto:laziera@uoguelph.ca)

**3. Study Principal Investigator:** Professor Amanda Wright, Ph.D. at 519-824-4120 x54697, [ajwright@uoguelph.ca](mailto:ajwright@uoguelph.ca)

**4. Study Faculty Co-Investigator:** Professor Douglas Goff, Ph.D. at 519-824-4120 x53878, [dgoff@uoguelph.ca](mailto:dgoff@uoguelph.ca)

**5. Study Faculty Co-Investigator:** Amy Tucker, Ph.D. (HNRU Manager) at 519-824-4120 x53749, [aborland@uoguelph.ca](mailto:aborland@uoguelph.ca)

#### **PURPOSE OF THE SCREENING VISIT**

The purpose of the screening visit is to determine your eligibility to participate in the study. The study will examine the effects of novel milk products incorporating modified protein ratio and amount, and how they affect satiety, food intake, and metabolic control (glycemic response) in about 33 healthy men and women. Therefore, participants must have normal fasting glucose. Body measurements will also be taken to ensure you are qualified to participate in the trial.

#### **PROCEDURES**

This screening visit will occur at the Human Nutraceutical Research Unit (HNRU) in room 144 of the Food Science-Guelph Food Technology Centre Building, 88 McGilvray Street at the University of Guelph. At this screening visit you will complete two questionnaires and have your

height, weight, waist circumference and blood pressure taken by a study coordinator. If you have any questions, please feel free to ask one of the study coordinators.

If you volunteer to participate in this screening visit, we would ask you to do the following things:

**PRIOR TO THE SCREENING VISIT:** It is critical that you fast for 12-14 hours prior to your screening visit and that you arrive for the visit fasted. During the fast, you **should** drink water, but **should not** consume any other drinks or any foods. We also ask that you avoid drinking alcohol, participating in strenuous exercise or taking acetaminophen products for 24 hours before your screening visit.

**DURING THE SCREENING VISIT:** The following describes the activities associated with the screening visit:

When you arrive at the HNRU, one of the study coordinators will welcome you at the main entrance. The study coordinator will then go through a health questionnaire, similar to the one you completed over the phone, but with more detail. You will also complete a Gastrointestinal Questionnaire and a Three Factors Eating Questionnaire, which asks questions about your eating habits. The study coordinator will measure your height, weight, waist circumference and blood pressure in private. The study coordinator will also take a fasting blood sample to determine your fasting blood glucose concentration. You will receive a \$5 gift card from Tim Horton's as compensation for the screening visit.

**AFTER THE SCREENING VISIT:** Following your screening visit, the study coordinator will then discuss the results of your questionnaire with the study's principal investigator to determine your eligibility. The process of getting your results back could take up to 5 days. Therefore, the study coordinator will contact you within one week of your screening visit. If you meet the eligibility requirements, you will be invited to participate in the study. The study coordinator will answer any questions you might have, arrange for the study orientation visit during which you will learn all about the participant responsibilities and benefits, try a sample of a study product and review the Study Informed Consent Document. Please note that the results from your screening visit should not be used for diagnostic purposes. If you are concerned about your results you should seek the advice of a physician.

#### **POTENTIAL RISKS AND DISCOMFORTS**

There are minimal risks associated with participation in this screening visit. The following summarizes the potential risks:

- During the visit, the study coordinator will ask if he/she can take your body measurements, including height, weight, waist circumference and blood pressure. You may experience discomfort during this process but steps will be taken to minimize this. These measurements will be taken in a closed off room with only the study coordinator

present to ensure your privacy and comfort.

- During the visit, a trained graduate student coordinator will draw blood from your fingertip on one occasion. There is a chance that this process could cause you some slight discomfort as a lancet is used to prick your finger and as with any needle there may be some minimal bruising afterwards. These risks and potential discomforts from the blood draws will be managed by having a trained student perform the sampling. In addition, consuming plenty of water the night before and the morning of your visit can facilitate blood sampling.

### **POTENTIAL BENEFITS TO PARTICIPANTS AND/OR TO SOCIETY**

You will benefit from participating in this screening process by gaining the experience as a study participant, through the compensation you will receive and also because of the summary you will receive about glycemic response.

Your potential involvement in the study will lead to results that will provide valuable insights into milk protein products, satiety and metabolic control (glycemic response). This information may lead to the development of dietary strategies that could improve human health, particularly as it relates to foods, which decrease the risk or aid in the management of type II diabetes.

### **CONFIDENTIALITY**

Every effort will be made to ensure confidentiality of any identifying information that is obtained in connection with this study. All participants will be assigned a number, and a study code will be used. Your name will never be used in communicating results of the study. Records will be kept on an encrypted computer and/or in a locked file cabinet in a locked office. All data will be kept indefinitely. In following these guidelines, participants' confidentiality will be maintained to the best of our ability. The investigator's institution will permit trial-related monitoring, audits, REB review, and regulatory inspection(s), providing direct access to source data/documents as required. Results from the study may be published, but will be presented as group data.

### **PARTICIPATION AND WITHDRAWAL**

You can choose whether to participate in this screening visit. Even if you meet the requirements for taking part in this study, your participation is not obligatory and you may choose not to participate. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. You may also refuse to answer any questions you don't want to answer and still remain in the study. 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 investigator may withdraw you from this research if circumstances arise that warrant doing so.

**RIGHTS OF RESEARCH PARTICIPANTS**

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board. If you have questions regarding your rights as a research participant, contact:

Director, Research Ethics  
Telephone: (519) 824-4120, ext. 56606  
E-mail: sauld@uoguelph.ca

**SIGNATURE OF RESEARCH PARTICIPANT/LEGAL REPRESENTATIVE**

I have read the information provided for the screening visit for the study "*The Milk Study –The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)*" as described herein. My questions have been answered to my satisfaction, and I agree to participate in this screening visit. I have been given a copy of this form.

Name of Participant (please print): \_\_\_\_\_

Signature of Participant: \_\_\_\_\_ Date: \_\_\_\_\_

**SIGNATURE OF WITNESS**

Name of Witness (please print): \_\_\_\_\_

Signature of Witness: \_\_\_\_\_ Date: \_\_\_\_\_

## Appendix E: Screening 2 questionnaire

### IN-PERSON SCREENING QUESTIONNAIRE

#### The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)

Thank you very much for your interest in this study. The purpose of this questionnaire is to gather more information about you and to ensure your safety as a participant in this study. Please feel free to NOT answer any questions that you are uncomfortable with answering. Please feel free to ask the study coordinator any questions you might have.

**All information provided in this questionnaire will be kept strictly confidential.**

1. How are you feeling today (e.g. nauseous, sick, etc)?																	
2. How would you describe your general health? POOR                    GOOD                    VERY GOOD                    EXCELLENT																	
3. Do you have gastrointestinal disorders (Celiac disease, lactose intolerance, irritable bowel syndrome, etc.)? YES / NO If Yes, please specify:																	
4. Do you have any other medical conditions (heart disease, diabetes, hypoglycaemia, gum disease, etc.)? YES / NO If Yes, please specify:																	
5. Do you smoke? YES / NO a) If NO, have you ever smoked? YES / NO b) If YES, how long since you quit smoking?																	
6. Do you use any medication and/or over-the-counter drugs (e.g. Tylenol, Advil, Claritin, Sudafed, etc?) YES / NO a) If YES, please complete the following table:																	
<table border="1"><thead><tr><th>PRODUCT NAME</th><th>REASON FOR USE</th><th>MEDICINAL INGREDIENTS</th><th>DOSE</th><th>HOW OFTEN</th><th>HOW LONG</th></tr></thead><tbody><tr><td> </td><td> </td><td> </td><td> </td><td> </td><td> </td></tr></tbody></table>						PRODUCT NAME	REASON FOR USE	MEDICINAL INGREDIENTS	DOSE	HOW OFTEN	HOW LONG						
PRODUCT NAME	REASON FOR USE	MEDICINAL INGREDIENTS	DOSE	HOW OFTEN	HOW LONG												


7. Do you use any dietary supplements (i.e. vitamins, protein powders, probiotics?)

**YES / NO**

a) If YES, please complete the following table:

PRODUCT NAME	REASON FOR USE	ACTIVE INGREDIENTS	DOSE	HOW OFTEN	HOW LONG

8. Have you ever taken acetaminophen before? This is the primary ingredient in the brand Tylenol.

**YES / NO**

a) If YES, did you have any reaction to the drug:

9. This study requires that participants continue their usual medication and dietary supplement routine (i.e. dosage and frequency) for the 2-month duration of the study. **Would you be comfortable with this?**

**YES / NO**

**10.** Have you ever donated blood through the Canadian Blood Services? **YES / NO**

a) If YES, when was the last time you donated blood?

b) Would you be comfortable NOT donating blood for the duration of the study?

11. Do you have ANY allergies (i.e. food, medications, ragweed or pollen)?

**YES / NO**

a) If YES, please list:

b) Please describe your type of allergic reactions: (i.e. anaphylaxis, difficulty breathing, swelling, etc.)

12. If FEMALE, are you pregnant or planning to become pregnant?				
YES / NO				
13. How would you describe your fitness/activity level?				
<b>VERY LOW</b>	<b>LOW</b>	<b>MODERATE</b>	<b>HIGH</b>	<b>VERY HIGH</b>
14. Do you participate in any of the following types of exercise:				
a) Weight training?	YES	NO	If YES, how often? _____	
b) Running/jogging?	YES	NO	If YES, how often? _____	
c) Aerobics?	YES	NO	If YES, how often? _____	
d) Team sports?	YES	NO	If YES, how often? _____	
e) Other? (please list)	YES	NO	How often? _____	
_____				
How often? _____				
_____				
How often? _____				
_____				
15. This study requires that participants DO NOT participate in vigorous exercise for 24 hours prior to the HNRU study visits (a total of 5 occasions). <b>Would you be comfortable with this?</b>				
YES / NO				
16. Do you have unusual sleeping patterns (shift work etc.)?				
YES / NO				
17. How many hours of sleep do you usually get per night? <b>0 – 3, 4 – 6, 7 – 9, 10+</b>				
18. Do you eat breakfast?				
YES / NO				
19. On average, what time do you usually eat breakfast?				
20. How many days/week do you eat breakfast before 9 am? <b>0 – 1, 2 – 3, 4 – 5, 6 – 7</b>				
21. Approximately how many alcoholic drinks do you consume per week? (1 drink = 12 oz beer, 5 oz wine, or 1.5 oz hard liquor)				

22. This study requires that participants DO NOT consume any alcoholic beverages for 24 hours prior to the HNRU study visits (a total of 5 occasions). <b>Would you be comfortable with this?</b>	
YES / NO	
23. Do you consume caffeinated beverages (coffee, tea, pop, energy drinks etc.)?	
YES / NO	
a) If YES, approximately how many caffeinated drinks do you consume per day?	
b) If YES, please list the types of beverages you consume:	
24. Are you vegan, vegetarian or do you exclude dairy products?	
YES / NO	
25. Do you regularly drink cow's milk?	
YES / NO	
a) If YES, how often per week?	
b) Please provide specific examples of milk products (1%, 2%, skim milk or homo milk) that you consume and how often:	
26. Have you ever had a reaction to any milk or dairy products?	
YES / NO	
a) If YES, please describe:	
27. Do you like cheese pizza?	
YES / NO	
28. In this study, participants will be asked to remain at the HNRU, University of Guelph for approximately 3.5 hours in duration and to consume a provided cheese pizza lunch at the HNRU. <b>Would you be comfortable with this?</b>	
YES / NO	
29. This study requires 6 additional visits to the HNRU at the University of Guelph as follows:	
- One - 30-60 minute study orientation	
- Five - 3.5 hour study visits	
a) Can your schedule accommodate these visits?	
YES / NO	
b) Would you prefer study visits on Monday, Wednesday or Fridays?	
YES / NO	
30. Are you currently participating in any other research studies?	
YES / NO	
a) If YES, please describe:	

\*\*Provide the TFEQ to participants for them to complete. Once TFEQ is completed, then lead to sampling bay for height and weight measurements, record values on fasting blood glucose.

The study coordinator will measure your body weight, height, waist circumference and blood pressure.	HEIGHT (m)	
	WEIGHT (kg)	

	<i>Space for BMI Calculation</i>	
	WAIST CIRCUMFERENCE (cm)	
	BLOOD PRESSURE (mm/Hg)	
The study coordinator will perform a fingerprick blood sample.  TIME: _____ HEMOCUE #: _____	FASTING GLUCOSE (mmol/L)	

Continue with the following questions.

31. This study requires that participants maintain their body weight for the 2-month duration of the study. <b>Would you be comfortable with this?</b>	<b>YES / NO</b>
32. Has your body weight changed in the past: a) 6 months? YES    NO    If YES, how much? _____ b) 1 year?    YES    NO    If YES, how much? _____	
33. Are you currently on a diet or weight loss program?  a) If YES, please describe:  _____	<b>YES / NO</b>

\_\_\_\_\_

HNRU Coordinator Signature

*NB: Complete the In-person Screening CRF, Trial Activity Checklist and discuss results with the research team.*

## **Appendix F: Study consent form**

### **The Milk Study**

#### **The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)**

### **CONSENT DOCUMENT**

You are asked to participate in a "Milk Study" for a research study conducted by MSc. students (Bonnie) Hau Ming Kung and Alexander Lazier, Associate Professor Amanda Wright from the Department of Human Health & Nutritional Sciences, Professor Douglas Goff from the Department of Food Science, and Dr. Amy Tucker from the HNRU at the University of Guelph. Results of this study will contribute towards a MSc. thesis for (Bonnie) Hau Ming Kung. This research is sponsored by the Dairy Farmers of Canada.

If you have any questions or concerns about the research, please feel free to contact the study personnel at any time:

**1. Study Coordinator:**

(Bonnie) Hau Ming Kung, BSc.H (MSc. graduate student) at 519-824-4120 x56314,  
kungh@uoguelph.ca

Alexander Lazier, B.Sc.H (MSc. graduate student) at 519-824-4120 x56314,  
laziera@uoguelph.ca

**2. Study Principal Investigator:**

Associate Professor Amanda Wright, PhD. at 519-824-4120 x54697,  
ajwright@uoguelph.ca

**3. Study Co-Investigator:**

Professor Douglas Goff, PhD. at 519-824-4120 x53878, [dgoff@uoguelph.ca](mailto:dgoff@uoguelph.ca)  
HNRU Manager Amy Tucker, PhD. at 519-824-4120 x53749, [aborland@uoguelph.ca](mailto:aborland@uoguelph.ca)

#### **PURPOSE OF THE STUDY**

The purpose of this study is to develop and test modified milk protein products to determine the effects of amino acids on food intake, satiety, insulin secretion and glucose control by insulin-independent action in healthy adults.

#### **PROCEDURES**

If you volunteer to participate in this study, we would ask you to do the following things: This study involves 5 visits to the Human Nutraceutical Research Unit (HNRU) of the Food Science-Guelph Food Technology Centre Building, 88 McGilvray Street at the University of Guelph. This is the same location as the screening visit. The following describes in detail what will happen during each of the 5 treatments:

**PRIOR TO EACH VISIT:** For 24 hours prior to each study visit you will need to avoid over-the-counter medications, alcohol and strenuous activity. Prior to each study visit you will also need

to observe a 12 hour overnight fast where you consume no food or drink, except for water until 1 hour prior to the study visit. Before starting each fast, you will need to consume a standard dinner meal which will be created based on your 24 hour food record. You will have some choice in the meal option. You should be fasted when you arrive to the Human Nutraceutical Research Unit for your scheduled study visit.

**STUDY VISITS:** At each study visit the study coordinator will ask you questions about your sleep habits, wellness and stress from the past 24 hours. The study coordinator will then take one fasting blood sample. You will then consume 250ml of the milk product with Honey Nut Cheerios within 5 minutes at a constant pace. You will then fill out the palatability questionnaire about the taste and mouth feel of the milk product. Fingerprick blood samples will be obtained at the 15, 30, 45, 60, 90, 120 minute intervals, following the start of ingestion of the milk product and Honey Nut Cheerios. A pizza buffet lunch and 500ml of water will be served at 120 minutes, at this time food and caloric intake will be measured. You will be given 20 minutes to eat the pizza meal until you are comfortably full and you will be asked to fill out a Palatability Questionnaire at the end of your pizza meal. Fingerprick blood samples will resume at 140, 155, 170, 185, and 200 minutes from the start of the study. This will total 12 fingerprick blood samples per study session. During the 3 hours of blood sampling, you will be asked to fill out a Satiety Questionnaire every 5 minutes prior to a fingerprick blood sample. You will be asked to remain seated with minimal activity. There will be magazines and movies available to watch, but you are also invited to bring your work to do or books to read. At the end of each fingerprick, a bandage or dressing will be applied. After that, you will be provided with a light snack, which you will need to consume in the presence of the study coordinator before you may leave (for example juice box and granola bar). You will be asked to fill out a Gastrointestinal Questionnaire 24 hours after each study visit, to be returned at your next visit.

**IF YOU ARE PART OF THE INTRAVENOUS SUBSET:** Participants in the subset will simultaneously have a qualified and department-approved phlebotomist obtain 8-12ml of venous blood samples at the fasting, 30, 60, 120 (prior to the pizza meal) 140, 170 and 200 minute intervals. This is a total of 7 time slots per study session. Participants are asked to not donate blood for the duration of the study

## **STUDY TEST PRODUCTS**

### **Breakfast Product**

On each of the 5 study visits, you will be asked to consume approximately 250ml of a milk product with 58g of Honey Nut Cheerios (General Mills; Mississauga, Canada). The milk ingredients include water, skim milk, various amount of casein and whey isolate, and permeate (lactose and milk salts). 1500mg of acetaminophen is added into all milk treatments. All of the products will be prepared in the Pilot Plant or Sensory kitchen (Food Science, Room 146A), following good manufacturing practices (GMPS), including complete cleaning and sanitation of equipment before and after use and personnel hygiene requirements for safe and sanitary production of food product. All ingredients will be purchased from food ingredient suppliers or the local grocery store.

**Honey Nut Cheerios Ingredients:** Whole grain oat, golden sugar and/or sugar, oat bran, corn starch, honey, salt, golden syrup, calcium carbonate, high monounsaturated canola oil, trisodium phosphate, monoglycerides, tocopherols, wheat starch, natural almond flavour  
**Contains:** Almond, Wheat and Oat Ingredients

Nutritional Information Per ¼ cup (29g)	Honey Nut Cheerios
Protein (g)	2
Total Fat (g)	1.5
Carbohydrates (g)	23
Calories	110

### Lunch Product

We will be serving McCain Deep 'N Delicious 5" Three Cheese Pizza, McCain Foods Ltd. Florenceville, NB. The pizza will be delivered to the University of Guelph by McCain Ltd., stored frozen in a food safe walk-in freezer (-30C) and baked in the Sensory Kitchen (Food Science, Room 146A) according to package directions under sanitary and hygienic requirements.

### **Pizza Ingredients:**

**Crust:** Enriched wheat flour, water, yeast, sugar, soya oil, salt, soy lecithin

**Cheeses:** Mozzarella, cheddar, parmesan (milk ingredients, modified milk ingredients, bacterial culture, salt, calcium chloride, microbial enzymes, lipase, natural colour), cornstarch

**Sauce:** Water, tomato paste, sugar, salt, cornstarch, spices (mustard), dehydrated garlic, wheat flour, natural flavour

Nutritional Information Per 1 Pizza (81g)	McCain Deep 'N Delicious Three Cheese Pizza
Protein (g)	10
Total Fat (g)	5
Carbohydrates (g)	23
Calories	180

### **STUDY SAMPLE LABORATORY ANALYSIS**

Fingerprick blood samples from each time point will be analyzed for glucose in order to determine the glycemic response to each test meal.

Intravenous blood samples (collected from the **subset group**) from each time point will be sent to the University of Toronto and Laval University for further analysis. The University of Toronto will analyze the collected blood samples for satiety hormones: C-peptide, amylin, ghrelin, glucagon-like peptide 1 (GLP-1), gastric inhibitory polypeptide (GIP), cholecystokinin (CCK) and peptide tyrosine-tyrosine (PYY); free acetaminophen and insulin. Laval University will analyze the blood for amino acid analysis.

## **STUDY RESULTS PUBLICATION**

Results from this study may be published, but will always be presented as group data with no ability to link data back to individuals. Your decision to be a participant in this study is voluntary and you are free to withdraw from the study at any time.

## **POTENTIAL RISKS AND DISCOMFORTS**

There are minimal risks associated with participation in this study. The following summarizes the potential risks:

At each of the 5 study visits a graduate student will obtain fingerprick blood samples. You may experience discomfort and soreness at your finger tips as a result of 12 fingerpricks in 3.5 hours. A phlebotomist will obtain intravenous blood samples from the subset group. There is a chance that this process could cause you some slight discomfort. These risks and potential discomforts from the blood draws will be managed by having qualified and experienced personnel obtaining your blood samples. In addition, consuming plenty of water the night before and the morning of the study visit can facilitate the flow of blood.

## **POTENTIAL BENEFITS TO PARTICIPANTS AND/OR SOCIETY**

You will benefit from participating in this study by gaining the experience as a study participant, through the financial compensation you will receive and because of the written summary you will receive about the overall research results as well as your individual results. Your involvement in the study will lead to results that will provide valuable insights into milk proteins, food intake, satiety and glycemic response. This information may lead to the development of dietary strategies that could improve human health, particularly as it relates to foods which decrease the risk or aid in the management of type II diabetes.

## **COMPENSATION**

You will be financially compensated for your time and effort at an amount of \$30 upon completion of each study visit for a total of \$150 (5 visits x \$30 per visit). The subset group will be financially compensated at an amount of \$45 upon completion of each study visit for a total of \$225 (5 visits x \$45 per visit). Following completion of the study analysis, a summary of the research results (both group data and your individual data) will be mailed to you. We request for you to disclose your mailing address so that we can mail a cheque and the results to you at the end of the study period.

If you withdraw from the study before completion, your compensation will be prorated for your involvement. For example, if you complete 3 study visits, you will receive \$90 (subset amount is \$135).

## **PARTICIPATION AND WITHDRAWAL**

You can choose whether to be in this study or not. If you volunteer to be in this study you may withdraw at any time without consequences of any kind. You may exercise the option of removing your data from the study. You may also refuse to answer any questions you don't want to answer and still remain in the study. 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 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. All participants will be assigned a number, and a study code will be used. Your name will never be used in communicating results of the study. Records will be kept on an encrypted computer and/or in a locked file cabinet in a locked office. All data will be kept indefinitely. In following these guidelines, your confidentiality will be maintained to the best of our ability. The investigator's institution will permit trial-related monitoring, audits, REB review, and regulatory inspection(s), providing direct access to source data/documents as required. Results from the study may be published, but will be presented as group data.

### **RIGHTS OF RESEARCH PARTICIPANTS**

You may withdraw your consent at any time and discontinue participation without penalty. You are not waiving any legal claims, rights or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board. If you have questions regarding your rights as a research participant, contact:

Director, Research Ethics  
Telephone: (519) 824-4120, ext. 56606  
E-mail: sauld@uoguelph.ca

### **SIGNATURE OF RESEARCH PARTICIPANT/LEGAL REPRESENTATIVE**

I have read the information provided for the "The Milk Study – The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)" 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.

Name of Participant: \_\_\_\_\_  
(please print)

Signature of Participant: \_\_\_\_\_ Date: \_\_\_\_\_

#### ***I want to participate in the subset group:***

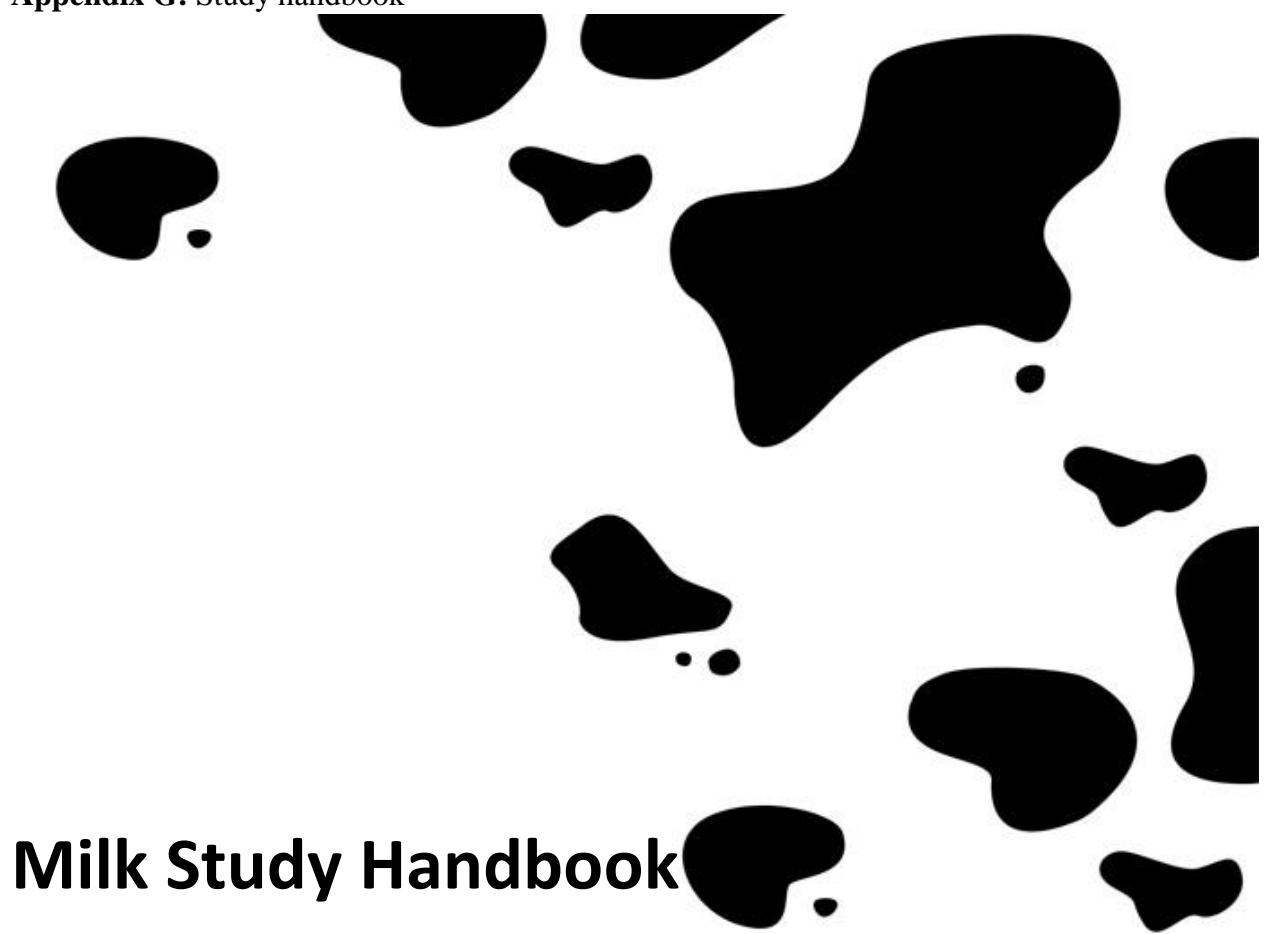
Signature of Participant: \_\_\_\_\_ Date: \_\_\_\_\_

### **SIGNATURE OF WITNESS**

Name of Witness: \_\_\_\_\_

Signature of Witness: \_\_\_\_\_ Date: \_\_\_\_\_

**Appendix G: Study handbook**



Participant I.D. \_\_\_\_\_

Contact Information:

M.Sc. Student Coordinators: (Bonnie) Hau Ming Kung & Alexander Lazier

E-mail: [kungh@uoguelph.ca](mailto:kungh@uoguelph.ca)

Telephone: 519-824-4120 ext. 56314

## **Dear Study Participant,**

Welcome to the Milk Study – The Effect of Milk Proteins on Food Intake, Satiety and Metabolic Control (Glycemia)! This handbook has been created with the purpose of guiding you through all of the study details.

This study is conducted on behalf of the Human Nutraceutical Research Unit (HNRU) within the Department of Human Health & Nutritional Sciences at the University of Guelph. The HNRU supports experiential learning and research opportunities with human-based clinical trials in the area of foods and natural health products. We welcome you to visit the HNRU website: [www.uoguelph.ca/hnru](http://www.uoguelph.ca/hnru)

The Milk Study is conducted under the supervision of Professors Amanda Wright, Ph.D, and Douglas Goff, Ph.D. MSc. student coordinators (Bonnie) Hau Ming Kung and Alexander Lazier will be involved in day-to-day study activities and interactions. HNRU Manager Dr. Amy Tucker will support the study activities.

This handbook outlines study protocol and schedules for study visits, HNRU visits, and all data collection. We really appreciate your participation in the Milk Study and your contribution to education and research at the University of Guelph. We assert that all information and data collected throughout this study process will remain confidential to the best of our ability, and you have the option to withdraw from the study at any point. We hope that you enjoy your participant experience in this study. Please do not hesitate to contact the study coordinators if you have any questions, comments or concerns.

Sincerely,

Amanda J. Wright, Ph.D.  
Associate Professor  
Ph: (519) 824-4120 x 54697  
[ajwright@uoguelph.ca](mailto:ajwright@uoguelph.ca)

H. Douglas Goff, Ph.D.  
Professor  
Ph: (519) 824-4120 x 53878  
[dgoff@uoguelph.ca](mailto:dgoff@uoguelph.ca)

Amy Tucker, Ph.D.  
HNRU Manager  
Ph: (519) 824-4120 x 53749  
[aborland@uoguelph.ca](mailto:aborland@uoguelph.ca)

(Bonnie) Hau Ming Kung,  
MSc. Student  
Ph: (519) 824-4120 x 56314  
[kungh@uoguelph.ca](mailto:kungh@uoguelph.ca)

Alexander Lazier, BSc.H.  
MSc. Student  
Ph: (519) 824-4120 x 56314  
[laziera@uoguelph.ca](mailto:laziera@uoguelph.ca)

## **Study Visits at the HNRU**

On 5 occasions you will be asked to visit the HNRU for a 3.5 hour period. At each visit you will consume a different milk product with Honey Nut Cheerios for breakfast, a pizza lunch and a snack. You will have fingerprick blood sampled and complete satiety questionnaires. All participants will have blood sampled by fingerprick, simultaneously a subset of 12 participants

have also consented to having venous blood sampled from a catheter inserted into a forearm for the duration of each visit. Food intake will be calculated, and you will be asked to fill out other questionnaires related to the milk product and your wellbeing.

Visit 1 represents the 1<sup>st</sup> day of the “study period” and Visit 5 represents the last day of the “study period”. Please refer to pages 4-5 of the handbook for a detailed outline of how to arrive prepared to these study visits. For a summary of complete study tasks by day, please refer to page 6.

## You may be asking yourself:

### 1) *What should I bring with me to a study visit?*

You can bring quiet activities to work on (Refer to “Study Design- Detailed Instructions” on page 6 for suggested activities) between breakfast (test products), lunch and blood sampling. Please come prepared with your completed gastrointestinal questionnaire for visits #2-5, and get into the habit of bringing your Study Handbook with you to each visit.

### 2) *Where should I go?*

Please meet us in the HNRU classroom, Food Science room 146, located at 88 McGilvray St., University of Guelph campus. This is the same location where the orientation visits were completed.

### 3) *What should I do before the HNRU visits?*

Please refer to the “Preparing for Study Visits” section on Page 5 and “Study Design- Detailed Instructions” section on Page 6. These sections will provide you with information regarding how to prepare for the study period.

### 4) *What time should I arrive?*

Please meet us at 8:15am, and plan to stay for a 3.5 hour period.

### 5) *What should I do between study periods?*

We ask that you maintain your usual dietary habits, **medication use and lifestyle** between study visits. Please notify us if you have any changes to your medications or unusual circumstances that may interfere with your participation.

## Preparing for Study Visits

### **1) Consume habitual diet**

We ask that you consume a standard dinner meal, which will be created from your **1-day food record**. We will provide a copy of your food record for you to follow.

### **2) Fasting**

Please come to all study sessions after fasting overnight for 12 hours. This means that you:

- **Should not** consume any food after approximately 8:30 PM the night before each study session.

- **Should not** consume any breakfast the morning of each study session. You may drink water up until one hour before your study session (i.e., 7:15am).

***Breakfast, lunch and a snack will be provided for the study sessions.***

### **3) Exercise**

Please avoid strenuous physical activity within 24 hours of each study session. This includes exercising at the gym, yoga/pilates, playing sports, running/jogging and other strenuous activities. Walking to school or work is OK, as long as it is consistent.

### **4) Alcohol**

Please do not consume any alcoholic beverages (beer, wine, liquor, etc.) within 24 hours of each study session.

### **5) Medication/Unusual Event Tracking**

Please notify the study coordinator if you are currently taking, or have been recently prescribed medications of any kind. Unusual events or unfortunate circumstances that arise prior to study sessions that may affect participation in the study should be recorded on the at-home daily study dairy. A study coordinator can decide if your visit should be rescheduled for a future date.

#### **List of product trade names to avoid that typically contain acetaminophen:**

Alka-Seltzer Plus Liquid Gels®  
Dayquil®  
Dimetapp®  
Excedrin®  
Midol®  
Nyquil®  
Robitussin®  
Sinutab®  
Sudafed®  
Tylenol® and Tylenol Cold®

#### **List of Medical and Natural Health Products to avoid:**

Acetylsalicylic acid (ASA) (E.g. Aspirin)  
Alcohol (E.g. beer, wine, liquor)  
Blood thinners (E.g. warfarin, Coumadin)  
Carbamazepin (E.g. Tegretol- mood)  
Cholestyramine (E.g. Colestyr- bile acids)  
Phenobarbital (E.g. Luminal- insomnia)  
Phenytoin (E.g. Dilantin- anti-anxiety)

## **Study Design – Detailed Instructions**

### **Study Sessions**

When you arrive for your study visit at 8:15 am, a student coordinator will ask you to complete a Sleep Habits and Stress Factors Questionnaire and a Satiety Questionnaire. The Sleep Habit and Stress Factors Questionnaire is to ensure you complied with the pre-study protocol above. A Gastrointestinal Questionnaire will also be collected for visits 2-5. We will then obtain your fasting fingerprick blood sample or venous blood sample for the subset group. At 8:30am you will receive your breakfast meal of a milk product and Honey Nut Cheerios. The milk product you receive will be

randomized for each participant. After you have consumed the milk product, we ask that you fill out a palatability questionnaire.

Over the next 3 hours participants are to remain seated and can participate in quiet activities throughout the visit (i.e. read, listen to music with headphones, usage of personal laptops). You will have fingerpick blood samples obtained at 15, 30, 45, 60, 90, 120, 140, 155, 170, 185, and 200 minutes. A subset group will have intravenous blood samples obtained at 0, 30, 60, 120, 140, 170, and 200 minutes. We will ask you to fill out a Satiety Questionnaire 5 minutes prior to each fingerpick blood sample measurement. At 120 minutes, a pizza buffet lunch will be served. Please eat until you feel comfortably full.

After the 200 minute blood sample is obtained, you will be properly bandaged and a light snack will be served to you.

## **Study Design – Quick Summary by Time**

<b>Time Point</b>	<b>Activities</b>
<b>Pre-study visit</b>	<ul style="list-style-type: none"> <li>• Have a standard dinner based on food records provided</li> <li>• Start fasting after 8:00pm</li> <li>• Avoid vigorous exercise and alcohol intake</li> <li>• Drink water up until 1 hour prior to study visit</li> </ul>
<b>8:15 am</b>	<ul style="list-style-type: none"> <li>• Come fasted to the HNRU for the 3.5 hour study visit</li> <li>• Return Gastrointestinal Questionnaire from previous study day</li> <li>• Complete Sleep Habits and Stress Factors Questionnaire</li> <li>• Complete Fasting Satiety VAS Questionnaire</li> <li>• Fasting Fingerpick Blood Sample</li> <li>• Subset: Fasting Venous Blood Sample</li> </ul>
<b>8:30 am</b>	<ul style="list-style-type: none"> <li>• Consume milk product with Honey Nut Cheerios within 5 minutes</li> <li>• Fill out Palatability Questionnaire</li> </ul>
<b>8:45 am</b>	<ul style="list-style-type: none"> <li>• (8:40) First Satiety VAS Questionnaire</li> <li>• First Fingerpick Blood Sample</li> </ul>
<b>9:00 am</b>	<ul style="list-style-type: none"> <li>• (8:55) Second Satiety VAS Questionnaire</li> <li>• Second Fingerpick Blood Sample</li> <li>• Subset: First Venous Blood Sample</li> </ul>
<b>9:15 am</b>	<ul style="list-style-type: none"> <li>• (9:10) Third Satiety VAS Questionnaire</li> <li>• Third Fingerpick Blood Sample</li> </ul>
<b>9:30 am</b>	<ul style="list-style-type: none"> <li>• (9:25) Fourth Satiety VAS Questionnaire</li> <li>• Fourth Fingerpick Blood Sample</li> <li>• Subset: Second Venous Blood Sample</li> </ul>
<b>10:00 am</b>	<ul style="list-style-type: none"> <li>• (9:55) Fifth Satiety VAS Questionnaire</li> <li>• Fifth Fingerpick Blood Sample</li> </ul>
<b>10:30 am</b>	<ul style="list-style-type: none"> <li>• (10:25) Sixth Satiety VAS Questionnaire</li> <li>• Sixth Fingerpick Blood Sample</li> </ul>

	<ul style="list-style-type: none"> <li>Subset: Third Venous Blood Sample</li> <li>Pizza Buffet Lunch</li> <li>Palatability Questionnaire at the end of the lunch</li> </ul>
<b>10:50 am</b>	<ul style="list-style-type: none"> <li>(10:50) Seventh Satiety VAS Questionnaire</li> <li>Seventh Fingerprick Blood Sample</li> <li>Subset: Fourth Venous Blood Sample</li> </ul>
<b>11:05 am</b>	<ul style="list-style-type: none"> <li>(11:00) Eighth Satiety VAS Questionnaire</li> <li>Eighth Fingerprick Blood Sample</li> </ul>
<b>11:20 am</b>	<ul style="list-style-type: none"> <li>(11:15) Ninth Satiety VAS Questionnaire</li> <li>Ninth Fingerprick Blood Sample</li> <li>Subset: Fifth Venous Blood Sample</li> </ul>
<b>11:35 am</b>	<ul style="list-style-type: none"> <li>(11:30) Tenth Satiety VAS Questionnaire</li> <li>Tenth Fingerprick Blood Sample</li> </ul>
<b>11:50 am</b>	<ul style="list-style-type: none"> <li>(11:45) Eleventh Satiety VAS Questionnaire</li> <li>Eleventh Fingerprick Blood Sample</li> <li>Subset: Sixth Venous Blood Sample</li> <li>A snack is served after the blood sample measurements</li> </ul>
<b>24 hours later</b>	<ul style="list-style-type: none"> <li>Fill out Gastrointestinal Questionnaire to be returned at the next study date</li> </ul>

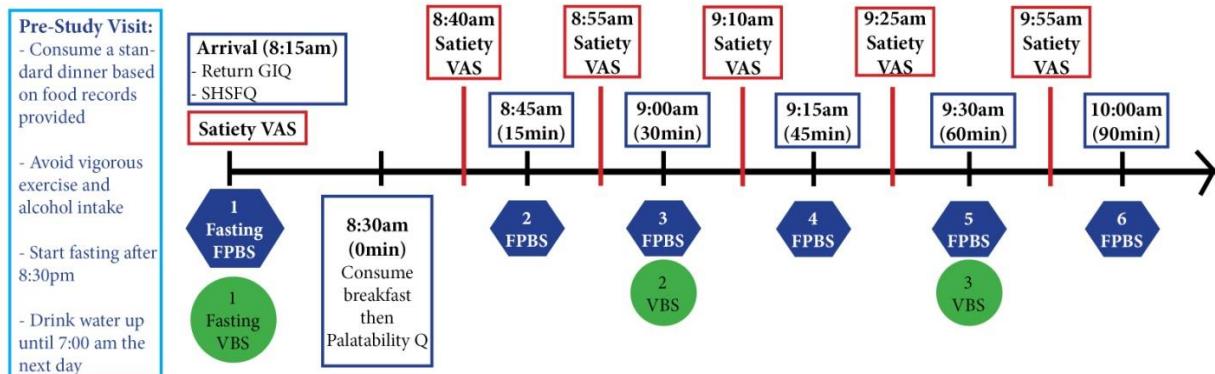
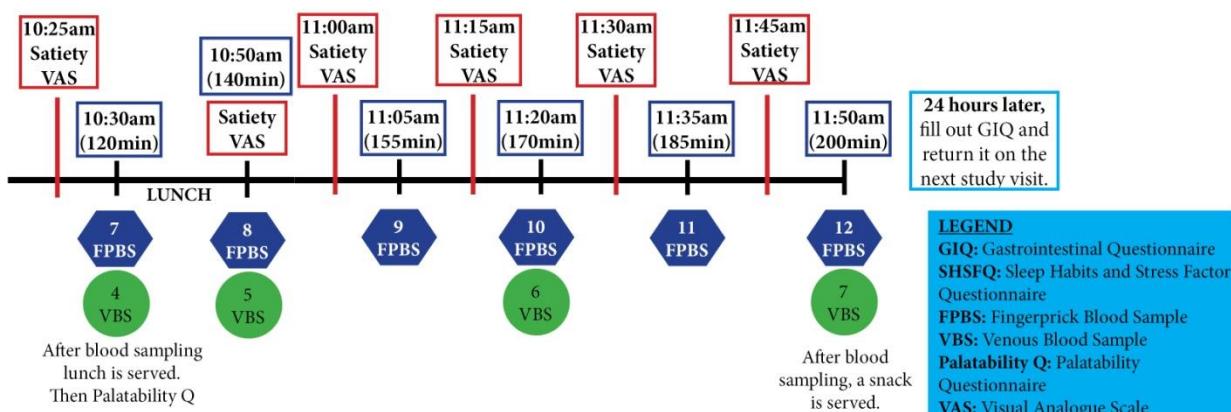


Figure 1: Overview of a study visit in The Milk Study



## How to Use the Rating Scales

In this study, you will be asked to complete rating scales in the HNRU. It is very important that you understand the scale you will be using and how to appropriately score based on your experiences.

**Visual Analog Scales (VAS):** These types of scales are commonly used in research to indicate participant perceptions. In this study we will ask a variety of questions through the use of VAS scales. This type of scale helps you to describe the intensity of different characteristics that may influence you. We will ask questions directly related to the milk product and your wellbeing i.e. texture, taste, hunger, thirst etc.

Please indicate how tired you feel right now:



Each VAS line is 100mm. At either end are “anchors” that hold the extremes of a particular characteristic. It is very important to read each VAS carefully because the characteristics may change question to question but the scales will look very similar. It is important to reflect on your perception to each VAS but you should remember there are no right or wrong answers. Try to answer truthfully and accurately as possible without spending too much time on each question. You will be asked to indicate where your perception is reflected best, using a vertical strike perpendicular to the horizontal VAS line, as shown in the example above. Your score is determined by measuring the distance (mm) from the left anchor to the perpendicular strike you have made.

## Take-Home Questionnaire

We ask that you take home a Gastrointestinal Questionnaire to be filled out 24 hours after your study visit at the HNRU. The purpose of this questionnaire is to gather information about your gastrointestinal function within a 24-hour period. Please bring your filled Gastrointestinal Questionnaire to the next study visit.

## Appendix H: Ingredient specifications

**Table 1.** Nutrition analyses for milk liquid and powders, permeate and cereal (Maxxam Analytics Int. Corp., Mississauga, Ontario)



Success Through Science®

Maxxam Job #: B4E3384  
Report Date: 2014/08/21

University of Guelph  
Client Project #: THE MILK STUDY  
Your P.O. #: 14JN004

### RESULTS OF ANALYSES OF FOOD

Maxxam ID		XB3182		XB3183		XB3184		
Sampling Date								
	Units	PRODEL 35 (WHEY)	QC Batch	SKIM MILK POWDER	QC Batch	DARISWEET 200	RDL	QC Batch
<b>General Food Parameters</b>								
Professional Services	N/A	*	3720317	*	3720317	*	N/A	3720317
<b>Nutritional Parameters</b>								
KJ	/100g	1544	3706402	1504	3706402	1513	1	3706402
Ash	g/100g	6.9	3709189	7.9	3709189	7.9	0.1	3711376
Fat (gravimetric)	g/100g	2.26	3710617	0.67	3712423	0.35	0.10	3710617
Calories	/100g	369	3706400	359	3706400	362	1	3706400
Protein	g/100g	33.54	3711891	35.15	3711891	3.36	0.10	3711891
Carbohydrates	g/100g	53.6	3706401	53.2	3706401	86.3	0.1	3706401
Total Sugars	g/100g	46.7	3706421	49.9	3706421	73.4	0.4	3706421
Lactose	g/100g	46.7	3711169	49.9	3711169	73.4	0.4	3711169
Moisture	g/100g	3.7	3709124	3.1	3709124	2.2	0.1	3709124

RDL = Reportable Detection Limit

QC Batch = Quality Control Batch

N/A = Not Applicable

Maxxam ID		XB3185		XB3186		
Sampling Date						
	Units	HONEY NUT CHEERIOS	QC Batch	SKIM MILK (LIQUID)	RDL	QC Batch
<b>General Food Parameters</b>						
Professional Services	N/A	*	3720317	*	N/A	3720317
<b>Nutritional Parameters</b>						
KJ	/100g	1678	3706402	143	1	3706402
Ash	g/100g	3.0	3707760	0.7	0.1	3709189
Fat (gravimetric)	g/100g	4.55	3710617	0.17	0.10	3712423
Calories	/100g	401	3706400	34	1	3706400
Protein	g/100g	8.28	3711891	3.12	0.10	3711891
Carbohydrates	g/100g	81.8	3706401	5.0	0.1	3706401
Total Sugars	g/100g	33.6	3706421	4.5	0.4	3706421
Lactose	g/100g	ND	3711169	4.5	0.4	3711169
Moisture	g/100g	2.4	3709600	91.0	0.1	3716330

RDL = Reportable Detection Limit

QC Batch = Quality Control Batch

N/A = Not Applicable

ND = Not detected

**Table 2.** AA profiling of skim milk powder (SMP), whey protein concentration (WPC) and cereal (Agricultural Experiment Station Chemical Laboratories (University of Missouri-Columbia, Columbia, MO)

Sender: Douglas Goff  
 Address: Dept of Food Science (Building 38), University of Guelph  
 50 Stone Road East, Guelph, Ontario N1G 2W1 Canada  
 Phone: 519-824-4120 X 53878  
 Purchase Order #: POL032612017LV Date of Report: December 12, 2016

Description: Dairy Products		Page 1 of 1	
ESCL #	19889	19890	1104
Units	W/W%	W/W%	W/W%
Guelph ID	SMP	WPC	Cheerios
<b>Essential</b>			
Histidine	0.95	0.62	0.20
Isoleucine	1.84	2.13	0.34
Leucine	3.41	3.55	0.66
Lysine	2.80	3.10	0.35
Hydroxylysine	0.01	0.04	0.13
Methionine	0.80	0.59	
Phenylalanine	1.67	1.14	0.47
Threonine	1.41	2.18	0.29
Tryptophan	0.54	0.68	0.11
Valine	2.25	2.02	0.48
<b>Total BCAA</b>	<b>7.50</b>	<b>7.70</b>	<b>1.48</b>
<b>Total EAA</b>	<b>15.68</b>	<b>16.02</b>	<b>3.01</b>
<b>Non-Essential</b>			
Alanine	1.10	1.62	0.42
Arginine	1.17	0.83	0.54
Aspartic Acid	2.58	3.49	0.68
Serine	1.57	1.42	0.38
Cysteine	0.24	0.68	0.22
Glutamic Acid	7.31	5.80	1.86
Glycine	0.64	0.64	0.44
Proline	3.21	1.98	0.51
Tyrosine	1.54	0.94	0.23
<b>Total NEAA</b>	<b>19.35</b>	<b>17.40</b>	<b>5.26</b>
<b>Total AA</b>	<b>35.49</b>	<b>33.99</b>	<b>8.38</b>
Crude protein*	35.06	33.38	9.50
Corr. protein (subtract NF)	34.74	32.98	9.44

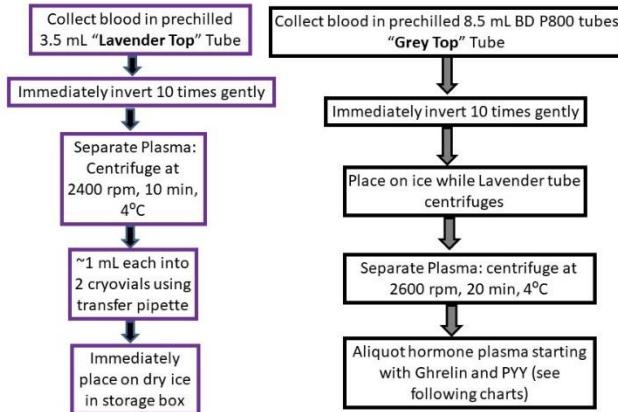
W/W% = grams per 100 grams of sample.

Results are expressed on an "as is" basis unless otherwise indicated.

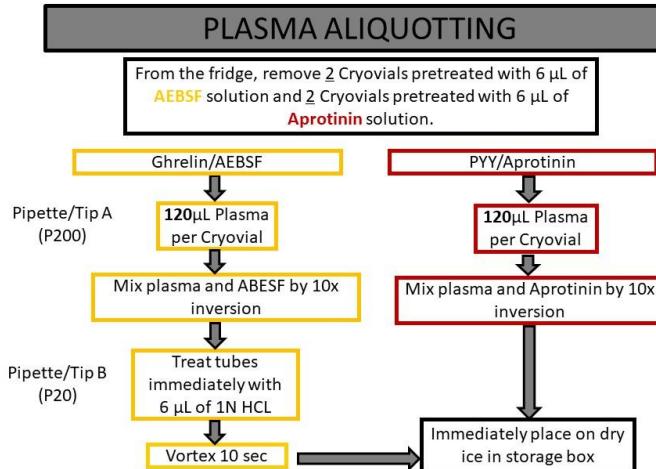
Crude protein\* = %N x 6.38. § Non-proteinogenic amino acids.

## Appendix I: Subset participant blood sample collection and processing

### MILK STUDY – PARTICIPANT SAMPLE COLLECTION AND PROCESSING PLASMA ALIQUOTTING

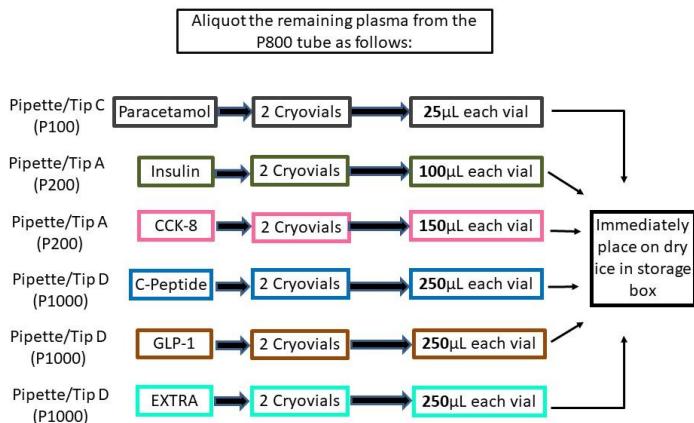


### MILK STUDY – PARTICIPANT SAMPLE COLLECTION AND PROCESSING

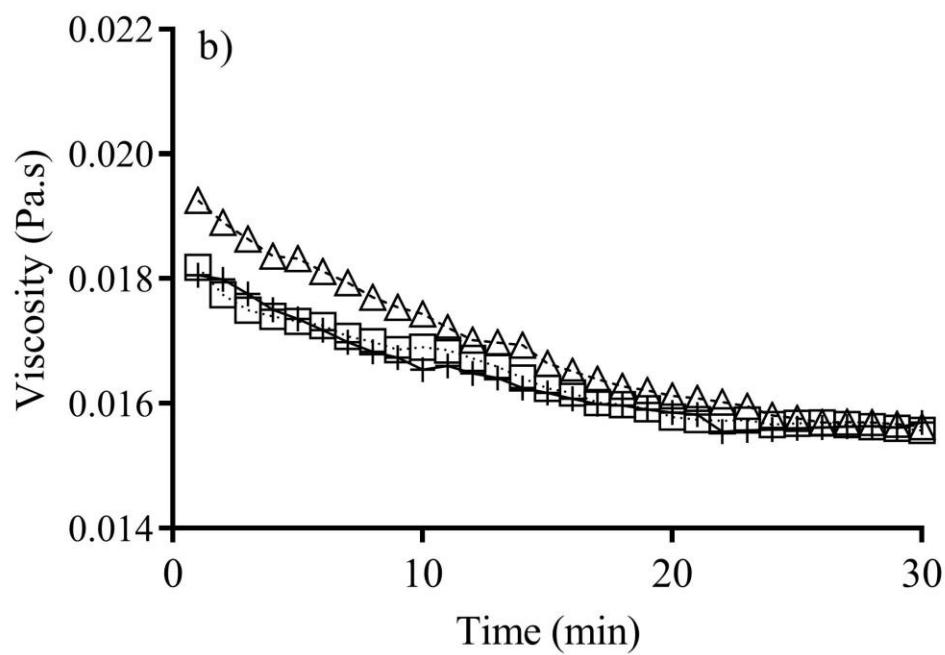
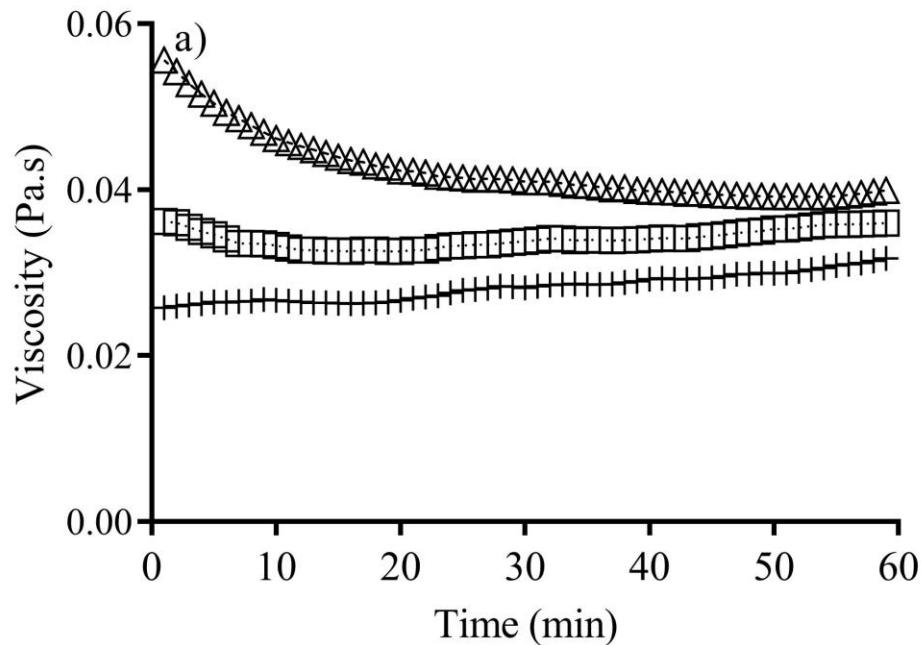


### MILK STUDY – PARTICIPANT SAMPLE COLLECTION AND PROCESSING

### PLASMA ALIQUOTTING



**Appendix J:** *In vitro* digestion viscosity (Pa.s) for a) gastric (0 - 60 min) and b) duodenal (0 – 30 min) for the control (+), 9.3 wt% (80:20) ( $\triangle$ ), and 9.3 wt% (40:60) ( $\square$ ).



**Appendix K:** Mean ( $\pm$  SEM) buffering capacity for gastric and duodenal phase at pH 0.5 interval

	<b>Treatments</b>			
	<b>Control<sup>1</sup></b>	<b>9.3 wt% (80:20)<sup>2</sup></b>	<b>9.3 wt% (40:60)<sup>3</sup></b>	<b>P-value</b>
<b>Gastric digestion (pH)<sup>4</sup></b>				
5.0	-	0.013 $\pm$ 0.000 <sup>a</sup>	0.012 $\pm$ 0.000 <sup>b</sup>	0.01
4.5	-	0.011 $\pm$ 0.000 <sup>a</sup>	0.012 $\pm$ 0.000 <sup>b</sup>	0.02
4.0	0.011 $\pm$ 0.000 <sup>ab</sup>	0.010 $\pm$ 0.000 <sup>a</sup>	0.012 $\pm$ 0.000 <sup>b</sup>	0.02
3.5	0.013 $\pm$ 0.001	0.011 $\pm$ 0.001	0.013 $\pm$ 0.001	0.12
3.3	0.012 $\pm$ 0.001 <sup>b</sup>	0.017 $\pm$ 0.001 <sup>a</sup>	0.016 $\pm$ 0.001 <sup>a</sup>	0.04
<b>Duodenal digestion (pH)<sup>5</sup></b>				
3.7	0.017 $\pm$ 0.001 <sup>c</sup>	0.022 $\pm$ 0.000 <sup>a</sup>	0.025 $\pm$ 0.000 <sup>b</sup>	<0.01
4.0	0.017 $\pm$ 0.001 <sup>c</sup>	0.022 $\pm$ 0.000 <sup>a</sup>	0.025 $\pm$ 0.000 <sup>b</sup>	<0.01
4.5	0.015 $\pm$ 0.000 <sup>c</sup>	0.021 $\pm$ 0.000 <sup>a</sup>	0.022 $\pm$ 0.000 <sup>b</sup>	<0.01
5.0	0.012 $\pm$ 0.001 <sup>b</sup>	0.021 $\pm$ 0.000 <sup>a</sup>	0.020 $\pm$ 0.000 <sup>a</sup>	<0.01
5.5	0.011 $\pm$ 0.001 <sup>b</sup>	0.019 $\pm$ 0.000 <sup>a</sup>	0.018 $\pm$ 0.000 <sup>a</sup>	0.02
6.0	0.011 $\pm$ 0.000 <sup>c</sup>	0.020 $\pm$ 0.000 <sup>a</sup>	0.018 $\pm$ 0.000 <sup>b</sup>	<0.01
6.5	0.011 $\pm$ 0.000 <sup>c</sup>	0.023 $\pm$ 0.000 <sup>a</sup>	0.018 $\pm$ 0.000 <sup>b</sup>	<0.01
7.0	0.012 $\pm$ 0.001 <sup>b</sup>	0.022 $\pm$ 0.000 <sup>a</sup>	0.018 $\pm$ 0.000 <sup>b</sup>	<0.01

<sup>1</sup> Control (water with whey permeate)

<sup>2</sup> High MP 9.3 wt% (80 casein : 20 whey protein)

<sup>3</sup> High MP 9.3 wt% (40 casein : 60 whey protein)

<sup>4</sup> Gastric digestion from 2 to 62 min

<sup>5</sup> Duodenal digestion from 62 to 92 min

<sup>a,b</sup>Mean buffering capacity within a row with superscripts differ according to LS Means multiple comparison for treatment x time interactions (P < 0.05).

**Appendix L:** Increased milk protein content and whey to casein ratio in milk served with breakfast cereal reduced postprandial glycemia in healthy adults

***Under Review***

Dalia El Khoury, Shirley Vien, Diana Sanchez-Hernandez, Bonnie Kung, Amanda Wright, H. Douglas Goff, G. Harvey Anderson

**ABSTRACT**

This study describes the effects of increasing protein concentration and increasing the whey to casein ratio in milk on glycemic response when consumed with a high glycemic breakfast cereal. Twelve healthy men and women, between the ages of 18 and 30 years and with a body mass index (BMI) of 20–24.9 kg/m<sup>2</sup>, consumed, in random order, milk beverages (250 mL) containing either 3.1% or 9.3% protein with casein to whey ratios of either 80:20, or 40:60. Postprandial appetite, glucose, regulatory hormones and stomach emptying rate were measured over 200 min, and food intake at an *ad libitum* meal at 120 min. Milk beverages with increased protein content, and to a lesser extent with a decreased casein to whey ratio, when consumed with carbohydrate, lowered postprandial glycemia through insulin-independent mechanisms, primarily associated with delayed stomach emptying.

**INTRODUCTION**

Dietary food patterns are advocated based on associations that show promising benefits for the prevention and/or management of obesity, T2D and related disorders (Erber et al., 2010; Villegas et al., 2007). Dairy is of interest because its frequent consumption associates with healthy body weights and lower rates of diabetes (Pasiakos, 2015; Hirahatake et al., 2014; Chen et al., 2014). These benefits are often attributed to its macronutrient content and the unique composition of its proteins (Anderson et al., 2011).

Proteins are well-known to contribute to the regulation of postprandial glycemia, appetite (Jahan-Mihan et al., 2011; Anderson and Moore, 2004) and body weight (Zemel, 2004). Milk proteins are a heterogeneous group of proteins, which mainly consist of casein (80%) and whey protein (20%) (Jahan-Mihan et al., 2011). Within the gastrointestinal tract, casein and whey proteins and their bioactive peptides affect several regulatory functions by interacting with receptors releasing hormones, affecting stomach emptying and gastrointestinal nutrient transport and absorption, transmitting neural signals to the brain and modifying the microflora (Jahan-Mihan et al., 2011). Based on their digestion and absorption rates, measured by appearance of amino acids in plasma, and their effects on protein synthesis, casein has been referred to as a “slow” protein, and whey as a “fast” protein (Boirie et al., 1997).

The physiological role of dairy proteins starts with their synergistic effects in the gastrointestinal tract (Luhovyy et al., 2007). When consumed alone, whey protein lowers post-meal blood glucose (Akhavan et al, 2010), enhances satiety and reduces food intake (Akhavan et al, 2010; Luhovyy et al, 2007) by both insulin-dependent and insulin-independent mechanisms (Akhavan et al, 2014). Whey protein stimulates a quick release of gut hormones, including glucagon-like peptide 1 (GLP-1), cholecystokinin (CCK), peptide tyrosine-tyrosine (PYY) and glucose-dependent insulinotropic peptide, in parallel to inducing greater effects on satiety than casein (Hall et al, 2003). Whey proteins are initially more satiating than casein proteins in normal weight as well as in overweight and obese individuals (Veldhorst et al., 2009; Anderson and Moore, 2004; Hall et al., 2003). This has been attributed to the clotting properties of casein in the stomach that slow the rate of digestion and prolong stimulation of gastrointestinal hormones, which in turn slow stomach emptying (Anderson and Moore, 2004; Dangin et al., 2001; Boirie et al., 1997). Although the physiological characteristics of whey and casein are well

described, the physiological significance of their ratio (20:80) and concentration (3.1%) in cow's milk are not. In contrast, human milk has a whey to casein ratio of 60:40, and a protein content of 1 wt% (Fox and McSweeney, 1998). Current infant milk formulas often approximate this ratio, inspired by breast milk composition (Klein, 2002), but are higher in protein (2-2.5%) (Martin et al., 2016). Thus the introduction of cow's milk leads to a large change in the ratio and protein concentration in the baby's diet.

Many studies have shown that consumption of milk, with the usual protein content of 3.1% and 80:20 casein to whey ratio, contributes to reduction of postprandial glycemia, appetite and food intake (Law et al., 2017a,b; Eussen et al., 2016; Anderson et al., 2011). However, there are no reports of the effect of increasing protein content or reducing the casein-to-whey ratio in milk on responses of and associations among glucoregulatory or appetite hormones. Therefore, the objective of the current study was to describe the effects of milk beverages with increased protein content (9.3% vs 3.1%) and whey to casein ratio (60:40 vs 20:80) when consumed with a high glycemic breakfast cereal on postprandial appetite, food intake, glucose and regulatory hormones in healthy men and women. We hypothesized that both the protein content and the whey to casein ratio in milks are factors in reduction of postprandial glycemia and do so through insulin independent mechanisms.

## MATERIALS AND METHODS

The data reported herein are derived from a subset of 12 of 32 participants in a study focused on the effects of the same treatments on subjective appetite, food intake and postprandial glycemia (Chapter 2, Kung et al., 2018). This trial was registered at <https://clinicaltrials.gov>, with clinical trial registry number of NCT02471092.

### *Participants*

Twelve healthy men and women, between the ages of 18 and 30 years and with a body mass index (BMI) of 20–24.9 kg/m<sup>2</sup>, participated. They were recruited through advertisements posted on the University of Guelph campus and surrounding areas (Chapter 2). University of Guelph Research Ethics Board approved the study protocol before commencement (#14JN004). A brief phone screening was used initially to assess the eligibility requirements of potential participants. Exclusion criteria consisted of the following: presence of any medical condition, including gastrointestinal disorders, regular medication use (besides hormonal contraceptives), any food allergy and/or anaphylactic allergy, smoking and recreational drug use. Elite or training athletes were excluded, along with persons trying to lose weight or whose body weight had changed >5 kg within the previous 6 months. Participants were excluded if they scored > 16 on the Cognitive Restraint scale on the Three-Factor Eating Questionnaire (Stunkard and Messick, 1985). Additionally, persons regularly consuming a high number of caffeinated (>4 drinks/day) or alcoholic drinks (>14 drinks/weeks), taking protein supplements, or who were pregnant or breastfeeding were also excluded. Qualified participants were invited to participate in the study and to read and sign the consent form. Their height and weight were measured and BMI calculated.

Based on previous experimental studies of treatment effects on blood glucose and glucoregulatory and appetite hormones (Akhavan et al., 2014; Panahi et al., 2014), a sample size calculation using power analysis for within-subject design estimated 12 participants to detect treatment differences.

#### *Treatments*

Five beverages were provided in a repeated measures design and assigned in random order once per week for all participants (Chapter 2). The four milks contained either regular (3.1%) or high (9.3%) milk protein (MP) concentrations with either 80:20 or 40:60 casein to

whey ratio. Milk permeate provided the control beverage and was added to all milks to standardize their lactose content. The 250 mL drinks were formulated based on whey permeate (DariSweet 200, #215503), skim milk (Neilson Dairy – Saputo Dairy Products Canada G.P., St-Laurent, Quebec), skim milk powder (Low Temp, #202001), and whey protein concentrate (Prodel 35, #33703) (Parmalat Canada, London, ON) to increase the concentration of commercial milk protein (3.1 wt%) three-fold (9.3 wt%) and modify the casein:whey protein ratio from (normal) 80:20 to high whey, 40:60, while holding the lactose (34.7 g) content constant (Chapter 2, Table 2.1). The beverages were cold mixed and served chilled (at a volume of 250 mL), with 58 g of Honey Nut Cheerios (Honey Nut Cheerios, General Mills, Mississauga, Canada) and with 100 mL of water; and consumed by participants in isolation at a constant pace within 5 min. All breakfast meals were standardized to provide a total available carbohydrate content of 76.7 g, approximating 48 g of glucose provided from the oat flour (21.2 g), sucrose (18 g) and lactose (34.7 g). 1.5 g of crushed acetaminophen (Tylenol; McNeil Consumer Healthcare, Markham, ON) was added to each 250-mL serving of breakfast meal for the purpose of measuring plasma paracetamol as a marker of gastric emptying.

#### *Protocol*

A double-blinded, controlled, crossover randomized design was used (Chapter 2). Participants randomly consumed one of each of the five allocated 3-digit number coded treatments at study visits separated by 1-week. Experimental sessions took place at the Human Nutraceutical Research Unit at the University of Guelph, Guelph, Ontario. As in previous studies, participants were asked not to consume any food within 12 h but were allowed to drink water until 1 h before their session (El Khoury et al., 2014a; Panahi et al., 2014; Panahi et al., 2013a). Each subject arrived at the same chosen time for each session in order to minimize within-subject variability. They were instructed to refrain from alcohol consumption and any

unusual exercise and activity 24 h before the study sessions. In addition, participants completed a 24 hour-food recall and were asked to eat according to the recall the day before each study visit. To ensure that these instructions were followed, participants filled out a questionnaire on sleep habits and stress factors (Chapter 2). Participants who indicated feelings of illness, atypical sleep, or stress were asked to reschedule. Baseline fingerprick blood samples for glucose concentration were obtained in duplicate and analyzed using a glucose meter (HemoCue Glucose 201+ Analyzer, HemoCue, Angelholm, Sweden). If participants reported significant deviations from their usual patterns, or if fasting blood glucose was above 5.5 mmol/L, sessions were rescheduled.

At the beginning of each session, satiety visual analogue scale (VAS) questionnaires and finger prick samples (for the analysis of blood glucose) were obtained and then an indwelling intravenous catheter was placed in an antecubital vein by a phlebotomist. Immediately after, participants were given the treatment and asked to consume it within 5 min. They then completed a palatability VAS (at 5 min) (El Khoury et al., 2014a,b), and continued to do so at 15 min intervals concurrently prior to finger prick blood samples at 15-30 min intervals thereafter (i.e. 0, 15, 30, 45, 60, 90, 120, 140, 155, 170, 185, 200 min). Venous blood samples were collected at 30, 60 and 120 min prior to the consumption of an *ad libitum* pizza lunch meal, and then at 140, 170 and 200 min post-lunch meal consumption. Gastrointestinal discomfort was measured.

At 120 min, participants, in isolation, were served an *ad libitum* pizza lunch and instructed to eat until comfortably full. The four-cheese 5-inch pizzas (McCain Foods, Florenceville, NB) were prepared by baking in an oven at 425 °F for 10 min. Pizzas were cut into quarter slices and four pizzas were served (approximately 81 g/plate) on each tray and served with a 500-mL glass of cool water. Participants were given 6-7 min with each tray up to 3 trays and a maximum total lunch period of 20 min. The pizza and water were weighed before and after serving to determine

food and energy intakes, which were calculated based on the product label nutritional information. Satiety as well as blood glucose and gastrointestinal hormone measurements continued for 1-hour after the pizza lunch meal (i.e. at 140, 155, 170, 185, and 200 min). Satiety was measured using a 100 mm VAS paper self reported questionnaire, with Hunger, Fullness, Desire to Eat and Prospective Food Consumption horizontal anchored scales (i.e. ‘not at all hungry’ versus ‘extremely hungry’) (Poortvliet et al., 2007). Appetite score was calculated based on the average of individual scales, i.e. (Hunger + (100-Fullness) + Desire to Eat + Prospective Food Consumption)/4. Participants were also asked to complete questionnaires related to palatability of the breakfast and lunch meals and gastrointestinal symptoms during the 24 h period following each study visit, using an anchored 100 mm horizontal VAS line scale (Guyonnet et al., 2013).

#### *Blood parameters*

Blood was collected in 8.5 mL BD P800 tubes (BD Diagnostics, Franklin Lakes, NJ, USA), containing spray-dried K<sub>2</sub>EDTA anticoagulant and proprietary additives to prevent their immediate proteolytic activity. The tubes were centrifuged at 1300 RCF for 20 min at 4°C. Collected plasma samples were aliquoted in Eppendorf tubes and stored at -80°C for analyses. Blood glucose, and plasma concentrations of insulin, active GLP-1, active ghrelin, PYY, CCK, C-peptide and paracetamol (acetaminophen) were measured. Glucose was measured through fingerprick blood using a glucose meter (HemoCue Glucose 201+Analyzer, HemoCue, Angelholm, Sweden). Insulin (intra-CV: <4%; inter-CV: <5%; CAT# 80-INSHU-E01.1, E10.1), active GLP-1 (intra-CV: <5%; inter-CV: <1%; CAT# EGLP-35K), active ghrelin (intra-CV: <5%; inter-CV: <5%; CAT# EZGRA-88K), total PYY (intra-CV: <3%; inter-CV: <3%; CAT# EZHPYYT66K), CCK (intra-CV: <4%; inter-CV: <7%; CAT# EKE-069-04), C-peptide (intra-CV: <4%; inter-CV: <4%; CAT# 80-CPTHU-E01.1) and paracetamol (CAT# K5055.02) were

measured with ELISA kits (ALPCO, Salem, NH, USA; Cambridge Life Sciences, Cambridgeshire, UK; Millipore, Billerica, MA, USA; Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA). These biomarkers were measured at the Department of Nutritional Sciences, University of Toronto, Toronto, ON.

*Data and statistical analysis*

SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. *A priori*, it was decided to analyze only the four protein treatments and exclude the permeate (no protein) treatment reported in the previous study (Kung et al 2018) for the purpose of determining the effect of protein concentration (regular 3.1% vs. high 9.3%) and ratio (80:20 vs. 40:60 casein to whey ratio). Therefore, the source of variation was divided into four components: variability due to concentration, variability due to ratio, variability due to the interaction between concentration and ratio and the residual variation. All analyses initially included sex as a factor but results were pooled when no significant sex effect or interactions were found.

Three-factor ANOVA was followed by Tukey's post hoc test using SAS PROC MIXED procedure to analyze the effects of protein concentration, protein ratio, sex and their interaction on food intake (pizza (kcal)) and total food intake (pizza (kcal) + breakfast (kcal)). Three-factor repeated measures ANCOVA was followed by Tukey's post hoc test using SAS PROC MIXED procedure to analyze appetite, glucose, insulin, GLP-1, PYY, ghrelin, C-peptide, CCK and paracetamol concentrations to treatments during the pre-meal period (0–120 min) with baseline (0 min) as a covariate. Three-factor repeated measures ANOVA were applied to describe treatments effects on the absolute plasma concentrations over the post-meal period (140–200 min). When there was a significant interaction between time and protein concentration or time and protein ratio, a one-way ANOVA was performed at all time points.

Pearson correlation coefficients were used to detect associations among the means, in the pre and post-meal periods, for all dependent variables. All data were checked for normality.

When data were not normally distributed, SAS PROC GLIMMIX for all ANCOVAs, ANOVAs and Spearman's rank-order for correlations were conducted. Significance was set at  $p \leq 0.05$ .

## RESULTS

### *Participants*

Twelve participants completed the study, including 6 male and 6 female participants. The 12 participants had a mean age of  $22.8 \pm 3.0$  years, body weight of  $70.0 \pm 11.21$  kg, height of  $1.7 \pm 0.1$  m, BMI of  $23.2 \pm 2.7$  kg/m<sup>2</sup>, waist circumference of  $79.3 \pm 8.7$  cm, blood pressure of  $124.5 \pm 13.8 / 71.33 \pm 10.0$  mmHg and fasting blood glucose of  $5.0 \pm 0.4$  mmol/L. A summary of the baseline characteristics of men and women is shown in Chapter 3, Table 3.2. Men had significantly higher body weight, height and systolic blood pressure at baseline compared to women ( $p < 0.05$ ).

### *Food intake*

Food intake (pizza (kcal)) and total food intake (pizza (kcal) + breakfast (kcal)) were similar after all four treatments (Table 3). However, caloric intakes were higher in men in comparison to women from the pizza meal alone and the pizza and breakfast meals combined ( $p=0.0005$ ).

**Table 3.** Food Intake<sup>1</sup>

Sex	Treatment	Pizza intake (kcal) <sup>2</sup>	Total food intake (kcal) <sup>3</sup>
Females	3.1% MP (80:20)	$451.81 \pm 78.80$	$884.91 \pm 78.80$
	3.1% MP (40:60)	$464.86 \pm 67.65$	$898.96 \pm 67.65$
	9.3% MP (80:20)	$431.42 \pm 77.12$	$917.02 \pm 77.12$
	9.3% MP (40:60)	$376.41 \pm 80.46$	$875.51 \pm 80.46$
Males	3.1% MP (80:20)	$963.78 \pm 99.46$	$1396.88 \pm 99.46$
	3.1% MP (40:60)	$1053.38 \pm 90.15$	$1487.48 \pm 90.15$
	9.3% MP (80:20)	$1096.54 \pm 108.79$	$1582.14 \pm 108.79$
	9.3% MP (40:60)	$1037.64 \pm 130.21$	$1536.74 \pm 130.21$

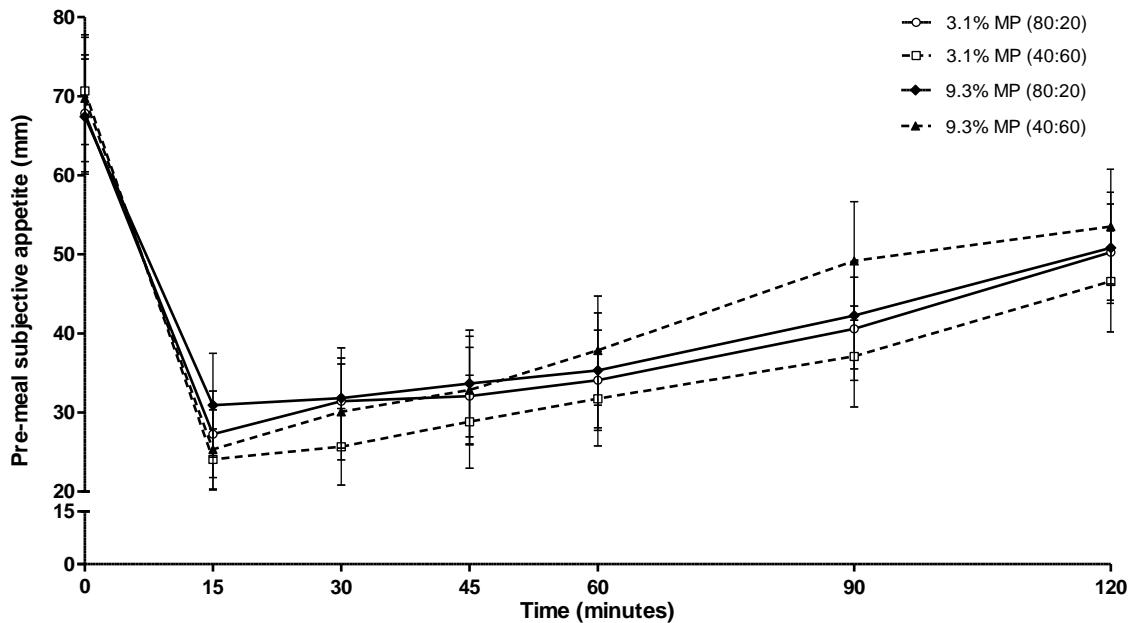
<sup>1</sup>All values are means $\pm$ S.E.M.s; Total n=12.

<sup>2</sup>Effect of treatments on pizza intake (kcal). Concentration:  $p = 0.9509$ , Ratio:  $p = 0.9423$ , Sex:  $p = 0.0005$ , Concentration\*Ratio:  $p = 0.0981$ , by three-way ANOVA.

<sup>3</sup>Effect of treatments on total food intake pizza ((kcal) + breakfast (kcal)). Concentration:  $p = 0.0901$ , Ratio:  $p = 0.9094$ , Sex:  $p = 0.0005$ , Concentration\*Ratio:  $p = 0.1377$ , by three-way ANOVA.

### *Subjective appetite*

In the pre-meal period, appetite was affected by time ( $p<0.0001$ ) and high MP concentration. Higher appetite (less satiety) scores were reported after high compared to regular MP treatments ( $p=0.0263$ ) (Figure 1). However, there were no differences in concentration or ratio during the post-meal period (Table 4).



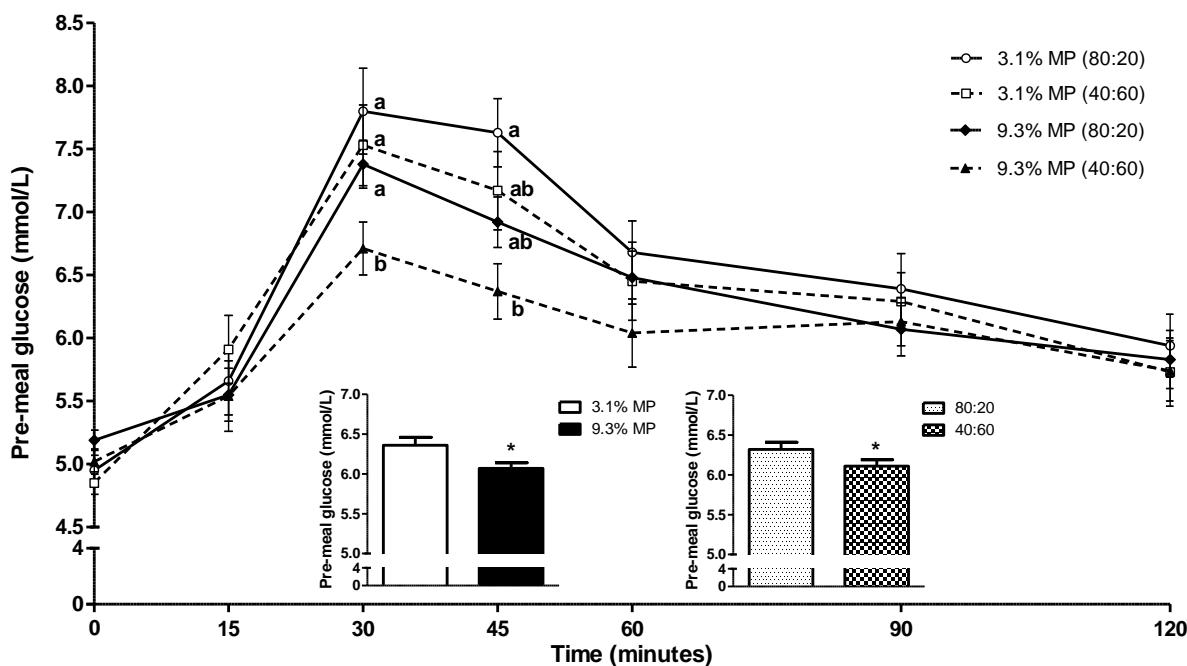
**Figure 1.** Effect of treatments on pre-meal appetite over time.

Concentration:  $p = 0.0263$ , Ratio:  $p = 0.3469$ , Time:  $p < 0.0001$ , Concentration\*Ratio:  $p = 0.2452$ , Concentration\*Time:  $p = 0.7365$ , Ratio\*Time:  $p = 0.7295$ , by three-way ANCOVA. All values are means $\pm$ S.E.M.s; n=12. Embedded panel shows mean appetite suppression by concentration.

### *Blood glucose*

Pre-meal blood glucose was affected by time ( $p<0.0001$ ), MP concentration ( $p=0.0022$ ), ratio ( $p=0.0409$ ), and concentration by time interaction ( $p=0.0306$ ) (Figure 2). Pre-meal mean blood glucose was lower after high compared to regular MP treatments. These effects can be explained by differences observed at 30 and 45 min. Blood glucose peaked at 30 min after all treatments, but was lowest after the high MP treatment with 40:60 casein to whey ratio. It remained lower for only the high MP with 40:60 casein to whey ratio treatment compared to

regular MP with 80:20 ratio at 45 min (Figure 2). In the post-meal period, blood glucose was only affected by time ( $p=0.0030$ ).



**Figure 2.** Effect of treatments on pre-meal glucose over time.

Concentration:  $p = 0.0022$ , Ratio:  $p = 0.0409$ , Time:  $p < 0.0001$ , Concentration\*Ratio:  $p = 0.5753$ , Concentration\*Time:  $p = 0.0306$ , Ratio\*Time:  $p = 0.2620$ , by three-way ANCOVA. All values are means $\pm$ S.E.M.s; n=12. Embedded panels show mean glucose by concentration and ratio.

#### Plasma insulin

Pre-meal plasma insulin concentration was affected by time ( $p < 0.0001$ ) but not by MP concentration or ratio (Table 4). Pre-meal insulin was significantly higher in female than male participants ( $47.8 \pm 4.2$  vs.  $29.5 \pm 4.2$   $\mu$ IU/mL,  $p=0.0112$ ). Post-meal insulin was increased by meal consumption but only affected by time post-meal ( $p=0.0065$ ).

**Table 4. LS means for pre- and post-meal appetite, glucose, insulin, C-peptide, ghrelin, GLP-1, PYY, CCK and paracetamol<sup>1</sup>**

Biomarker	Period	Treatments				p-values		
		3.1% MP (80:20)	3.1% MP (40:60)	9.3% MP (80:20)	9.3% MP (40:60)	Concentration	Ratio	Concentration X Ratio
Appetite (mm)	Pre-meal	40.36 ± 4.62	37.41 ± 4.62	42.14 ± 4.62	42.46 ± 4.62	0.0263	Ns	ns
	Post-meal	14.00 ± 2.47	14.07 ± 2.47	12.56 ± 2.47	12.98 ± 2.47	ns	Ns	ns
Glucose (mmol/L)	Pre-meal	6.45 ± 0.14 <sup>a</sup>	6.31 ± 0.14 <sup>a</sup>	6.16 ± 0.14 <sup>ab</sup>	5.93 ± 0.14 <sup>b</sup>	0.0022	0.0409	ns
	Post-meal	6.09 ± 0.18	6.15 ± 0.18	5.98 ± 0.18	6.10 ± 0.18	ns	Ns	ns
Insulin (μIU/mL)	Pre-Meal	39.08 ± 3.60	39.83 ± 3.58	36.71 ± 3.59	38.92 ± 3.65	ns	Ns	ns
	Post-meal	59.18 ± 11.67	59.34 ± 11.69	50.44 ± 11.67	59.92 ± 11.67	ns	Ns	ns
C-Peptide (pmol /L)	Pre-Meal	907.33 ± 77.65	908.78 ± 77.22	865.33 ± 77.36	905.67 ± 77.26	ns	Ns	ns
	Post-meal	1246.67 ± 216.71	1410.16 ± 216.71	1240.27 ± 216.71	1344.07 ± 216.71	ns	0.0147	ns
GLP-1 (pmol /L)	Pre-Meal	4.82 ± 0.63 <sup>b</sup>	5.97 ± 0.63 <sup>ab</sup>	6.19 ± 0.63 <sup>ab</sup>	6.97 ± 0.63 <sup>a</sup>	0.0075	0.0245	ns
	Post-meal	8.06 ± 1.59	8.24 ± 1.59	8.92 ± 1.59	8.61 ± 1.59	ns	Ns	ns
PYY (pg/mL)	Pre-Meal	107.51 ± 5.24	111.19 ± 5.19	108.00 ± 5.18	110.82 ± 5.21	ns	Ns	ns
	Post-meal	149.63 ± 5.99	147.43 ± 6.00	152.57 ± 6.00	141.47 ± 5.99	ns	Ns	ns
CCK (ng/mL)	Pre-Meal	0.206 ± 0.006	0.213 ± 0.006	0.214 ± 0.006	0.225 ± 0.006	0.0443	Ns	ns
	Post-meal	0.278 ± 0.025	0.292 ± 0.025	0.326 ± 0.025	0.285 ± 0.025	ns	Ns	ns
Ghrelin (pg/mL)	Pre-Meal	351.89 ± 15.18	344.87 ± 15.18	334.18 ± 15.14	321.55 ± 15.14	ns	Ns	ns
	Post-meal	275.17 ± 34.06 <sup>a</sup>	241.26 ± 33.97 <sup>ab</sup>	288.42 ± 33.90 <sup>a</sup>	218.08 ± 34.06 <sup>b</sup>	ns	0.0012	ns
Paracetamol (mmol/L)	Pre-Meal	0.164 ± 0.007 <sup>a</sup>	0.153 ± 0.007 <sup>ab</sup>	0.145 ± 0.007 <sup>ab</sup>	0.135 ± 0.007 <sup>b</sup>	0.0135	Ns	ns
	Post-meal	0.171 ± 0.008	0.177 ± 0.008	0.174 ± 0.008	0.149 ± 0.008	ns	Ns	ns

<sup>1</sup>All values are Means ± S.E.M. n=12. Values in the same row with different superscript letters are significantly different, p<0.05 by three-factor ANCOVA (pre-meal) and three-factor ANOVA (post-meal) with proc mixed procedure, Tukey's post hoc. Abbreviations: NS=Nonsignificant.

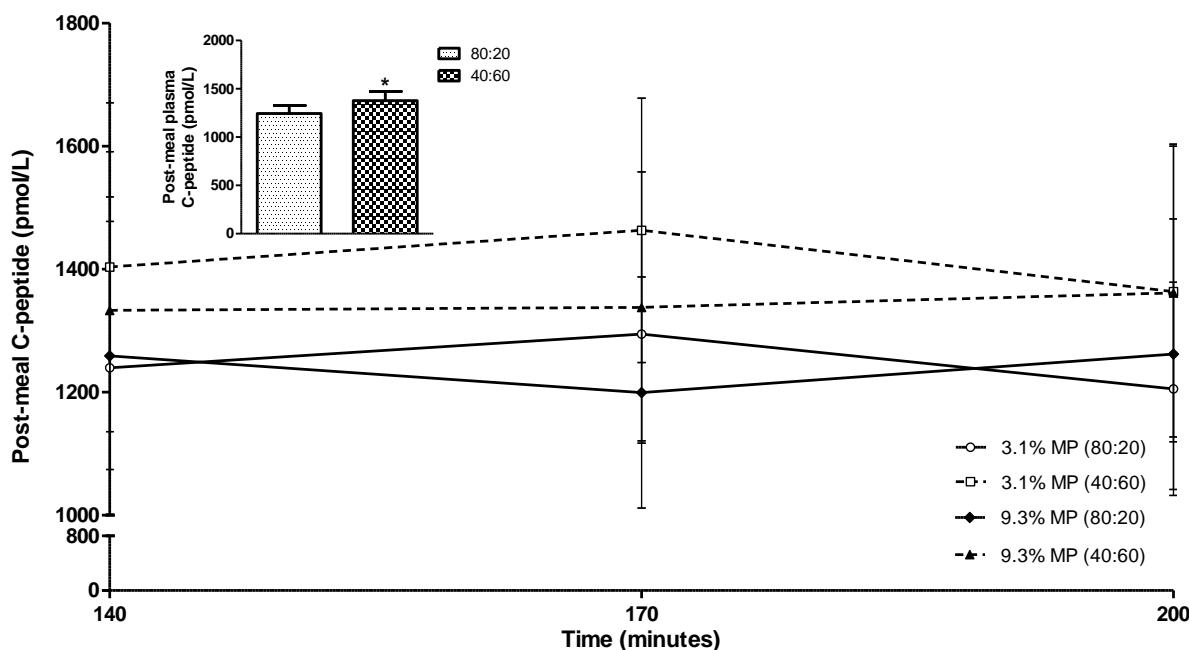
**Table 5. Relations between dependent pre- and post-meal means<sup>1</sup>**

Dependent Variables	Subjective appetite (mm)	Glucose (mmol/L)	Insulin ( $\mu$ IU/mL)	C-Peptide (pmol/L)	GLP-1 (pmol/L)	PYY (pg/mL)	CCK (ng/mL)	Ghrelin (pg/mL)
Pre-meal (0-120 min)								
Subjective appetite (mm)	-							
Glucose (mmol/L)	NS	-						
Insulin ( $\mu$ IU*min /mL)	r = -0.36	NS	-					
C-Peptide (pmol/L)	NS	NS	r = 0.60	-				
GLP-1 (pmol /L)	NS	r = -0.32	NS	NS	-			
PYY (pg/mL)	NS	NS	NS	NS	r = 0.32	-		
CCK (ng/mL)	NS	NS	r = 0.31	r = 0.40	NS	NS	-	
Ghrelin (pg/mL)	NS	NS	r = -0.57	NS	NS	NS	NS	-
Paracetamol (mmol/L)	NS	NS	r = -0.32	NS	NS	NS	r = -0.30	NS
Post-meal (140-200)								
Subjective appetite (mm)	-							
Glucose (mmol/L)	r = 0.32	-						
Insulin ( $\mu$ IU*min /mL)	r = 0.38	r = 0.63	-					
C-Peptide (pmol/L)	NS	r = 0.54	r = 0.67	-				
GLP-1 (pmol /L)	r = 0.54	NS	NS	NS	-			
PYY (pg/mL)	NS	NS	NS	NS	NS	-		
CCK (ng/mL)	NS	NS	r = 0.40	r = 0.58	NS	NS	-	
Ghrelin (pg/mL)	NS	r = -0.39	r = -61	r = -0.33	NS	NS	NS	-
Paracetamol (mmol/L)	NS	NS	NS	NS	NS	NS	NS	NS

<sup>1</sup> Correlation coefficients (r) indicate significant differences between dependent measures,  $p < 0.05$ . NS=nonsignificant.

### Plasma C-peptide

Pre-meal plasma C-peptide concentrations were affected by time ( $p<0.0001$ ), but not by MP concentration or ratio (Table 4). Pre-meal C-peptide was higher in female than male participants ( $1070.8 \pm 99.6$  vs.  $722.7 \pm 99.6$  pmol/L,  $p=0.0345$ ). Post-meal plasma C-peptide was only affected by MP ratio where C-peptide was higher after treatments containing 40:60 compared to 80:20 casein to whey ratio ( $1377.1 \pm 214.2$  vs.  $1243.5 \pm 214.2$ ,  $p=0.0147$ ) (Figure 3).



**Figure 3.** Effect of treatments on post-meal C-peptide over time.

Concentration:  $p = 0.4498$ , Ratio:  $p = 0.0147$ , Time:  $p = 0.9020$ , Concentration\*Ratio:  $p = 0.5319$ , Concentration\*Time:  $p = 0.4849$ , Ratio\*Time:  $p = 0.9517$ , by three-way ANOVA. All values are means $\pm$ S.E.M.s; n=12. Embedded panels show mean C-peptide by ratio.

### Plasma GLP-1

Pre-meal plasma GLP-1 was affected by time ( $p<0.0001$ ), MP concentration ( $p=0.0075$ ) and ratio ( $p=0.0245$ ) (Table 4). GLP-1 was higher after treatments containing high MP with the 40:60 casein to whey ratio compared to regular MP concentration with the 80:20 ratio ( $p=0.0081$ ). Post-meal GLP-1 was only affected by time ( $p<0.0001$ ) but not by MP concentration or ratio.

#### *Plasma PYY*

Pre-meal PYY concentrations were only affected by time ( $p<0.0001$ ). In the post-meal period, PYY was only affected by sex where PYY was greater in male than female participants ( $160.8 \pm 7.6$  vs.  $134.7 \pm 7.6$  pg/mL,  $p=0.0363$ ).

#### *Plasma CCK*

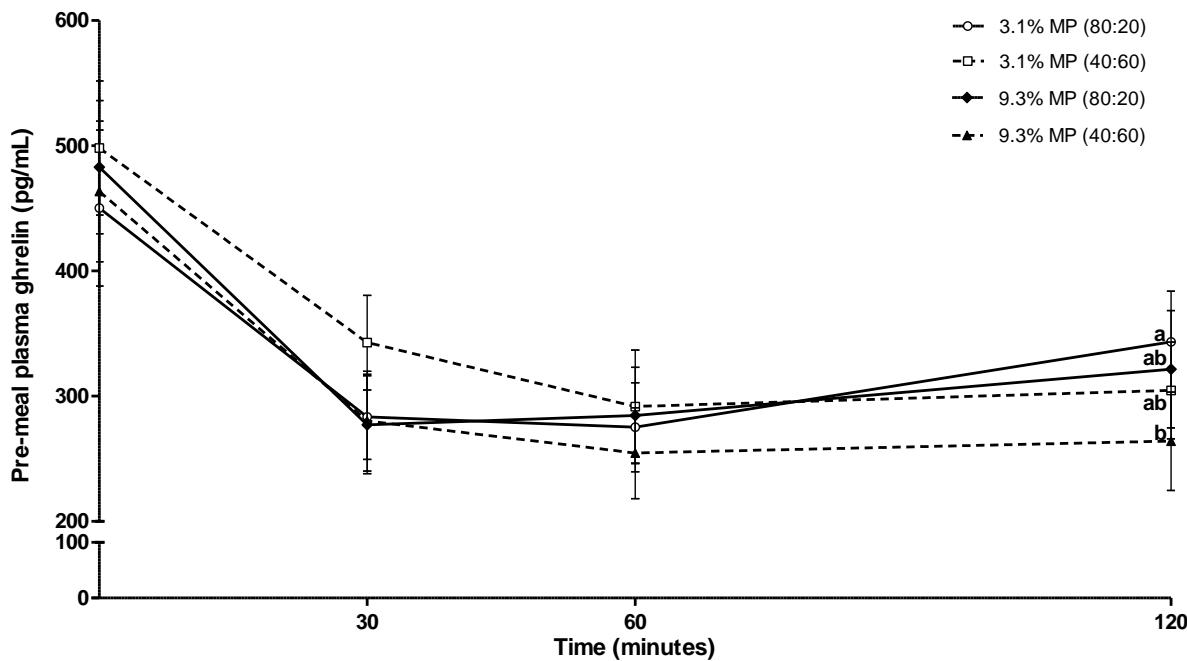
Pre-meal CCK concentrations were affected by time ( $p<0.0001$ ), MP concentration and sex. Pre-meal CCK was greater after the high than regular MP treatments ( $0.219 \pm 0.004$  vs.  $0.210 \pm 0.004$  ng/mL,  $p=0.0443$ ) and greater in female than male participants ( $0.226 \pm 0.005$  vs.  $0.203 \pm 0.005$  ng/mL,  $p=0.0066$ ). A MP concentration by sex interaction was also significant. The interaction can be explained by higher plasma concentrations in female participants after treatments containing high compared to regular MP ( $p=0.0201$ ), but responses were similar in their male counterparts after both treatments. Post-meal CCK did not reveal any differences.

#### *Plasma ghrelin*

Pre-meal ghrelin concentrations were affected by time ( $p<0.0001$ ). There was an interaction between MP ratio and time ( $p=0.0388$ , Figure 4) but no main effect of ratio. At 120 min ghrelin was suppressed more after treatments containing 40:60 compared to 80:20 casein to whey ratio ( $p=0.0078$ , Figure 4). Post-meal ghrelin concentrations were affected by time ( $p=0.0012$ ) and MP ratio, where ghrelin was suppressed more after treatments containing 40:60 compared to 80:20 casein to whey ratio ( $p=0.0012$ , Table 4).

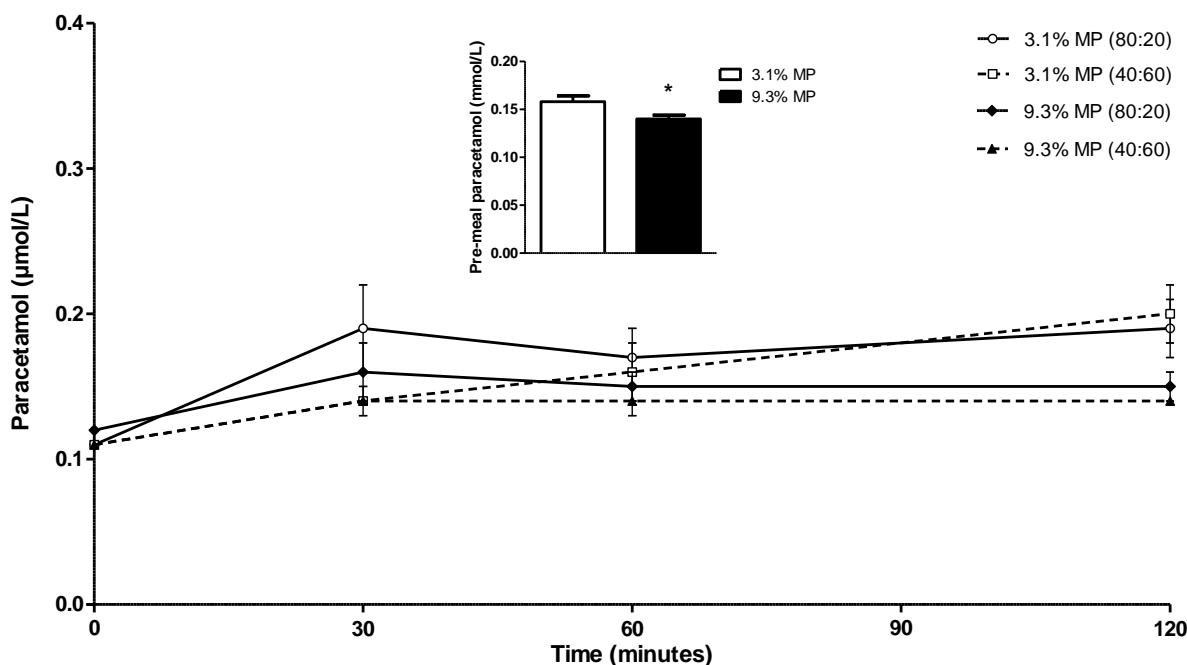
#### *Plasma paracetamol*

Pre-meal paracetamol concentrations were affected by time ( $p<0.0001$ ) and MP concentration ( $p=0.0135$ , Table 4). Plasma paracetamol response was lower after the treatments containing high compared to regular MP (Figure 5). The post-meal period did not reveal any differences.



**Figure 4.** Effect of treatments on pre-meal ghrelin over time.

Concentration:  $p = 0.0690$ , Ratio:  $p = 0.2888$ , Time:  $p < 0.0001$ , Concentration\*Ratio:  $p = 0.7054$ , Concentration\*Time:  $p = 0.5885$ , Ratio\*Time:  $p = 0.0388$ , by three way ANCOVA. All values are means $\pm$ S.E.M.s; n=12. Different letters are significantly different,  $p < 0.05$  by Tukey's post hoc.



**Figure 5.** Effect of treatments on pre-meal paracetamol over time.

Concentration:  $p = 0.0135$ , Ratio:  $p = 0.1203$ , Time:  $p < 0.0001$ , Concentration\*Ratio:  $p = 0.9190$ , Concentration\*Time:  $p = 0.1139$ , Ratio\*Time:  $p = 0.1552$ , by three way ANCOVA. All values are means $\pm$ S.E.M.s; n=12. Embedded panels show mean paracetamol by concentration.

### *Correlations*

Correlations between pre-meal means (Table 5) showed that subjective appetite was negatively associated with insulin ( $r=-0.36$ ). Glucose was negatively associated with GLP-1 ( $r=-0.32$ ). Insulin was positively associated with C-peptide ( $r=0.60$ ) and CCK ( $r=0.31$ ) but negatively with ghrelin ( $r=-0.57$ ) and paracetamol ( $r=-0.32$ ). C-peptide was positively correlated with CCK ( $r=0.40$ ). GLP-1 was positively correlated with PYY ( $r=0.32$ ). CCK was negative associated with paracetamol ( $r=-0.30$ ).

For the post-meal means (Table 5), subjective appetite was positively associated with glucose ( $r=0.32$ ), insulin ( $r=0.38$ ), and GLP-1 ( $r=0.54$ ). Glucose was positively associated with insulin ( $r=0.63$ ) and C-peptide ( $r=0.54$ ) but negatively with ghrelin ( $r=-0.39$ ). Insulin was positively associated with C-peptide ( $r=0.67$ ) and CCK ( $r=0.40$ ) but negatively with ghrelin ( $r=-0.31$ ). C-peptide was positively correlated with CCK ( $r=0.58$ ) but negatively with ghrelin ( $r=-0.33$ ).

## **DISCUSSION**

The hypothesis that the amount of milk protein, and to a lesser extent the casein to whey ratios, of milk beverages affect post-consumption responses of blood glucose and glucoregulatory and appetite hormones is supported by a number of findings from this study: 1) blood glucose responses were lower following the consumption of 9.3% MP compared to 3.1% MP preloads, during the entire postprandial as well as the pre-lunch meal periods. In particular, the preload with increased protein content (9.3% MP) and reduced casein to whey ratio (40:60) resulted in lower peak glucose responses in the pre-meal period, at 30 and 45 min; 2) these beneficial effects on blood glucose were not explained by insulin-dependent mechanisms; insulin

and C-peptide responses did not differ among treatments; 3) on the other hand, they were explained by insulin-independent mechanisms, including delayed gastric emptying. In fact, preloads with increased MP content (9.3%) resulted in higher GLP-1 responses in the total and pre-meal periods, higher CCK levels in the pre-meal period and lower paracetamol concentrations in the total, pre-meal and within-meal periods. In addition, mean plasma ghrelin levels were lower after preloads with lower casein to whey ratio 40:60, compared to 80:20 ratio, during lunch meal and in the post-meal period.

The beneficial effect of increased protein content of the milks on blood glucose is consistent with other reports that proteins, in general (Karamanlis et al., 2007; Gannon et al., 1988), and milk proteins, specifically (Petersen et al., 2009; Frid et al., 2005), reduce glycemic responses compared with carbohydrate alone. Several studies from our lab have reported improved glycemic regulation following the consumption of different forms of dairy products, when consumed prior to or during an *ad libitum* meal (El Khoury et al., 2014a,b; Panahi et al., 2013a,b) or with glycemic carbohydrate at a breakfast meal (Law et al., 2017a,b). This effect has been attributed to milk proteins (Akhavan et al., 2009; Luhovyy et al., 2007), and more specifically to whey protein (Jakubowicz and Froy, 2013).

Milks not only with increased MP content but also with decreased casein to whey ratio resulted in lower glucose peaks than the other preloads during the pre-meal period. At glucose peak time, 30 min, in particular, mean glucose peak levels were significantly lower following preloads with reduced casein to whey ratio (40:60) compared to those with a regular ratio (80:20) ( $P = 0.0021$ ). These findings are consistent with the known effects of whey proteins on metabolic control. Observations in animal models as well as humans show that whey is efficacious for glycemic control. Increasing the dietary protein to carbohydrate ratio with whey protein isolate

(40%) reduced plasma glucose levels in high fat diet-fed mice (McAllan et al., 2014). Improvements in glucose tolerance, tested in obese rats fed whey, casein or whey plus casein treatments, were greater in whey-fed than in whey plus casein-fed rats (Pezeshki et al., 2015; Nilsson et al., 2007). Additionally, 50 g of whey protein in a meal lowered glycemia more than a similar amount of protein from turkey or egg albumin over 240 min in lean men (Pal and Ellis, 2010). Even in patients with T2D, 55 g whey isolate consumed orally as a preload to or co-ingested with a carbohydrate meal has been shown to slow gastric emptying of, and to reduce the glycemic response to, that meal (Ma et al., 2009). To our knowledge, there are no studies comparing the metabolic effects of human milk or infant formulas to cow's milk, being of different casein to whey ratios. However, human milk (high whey to casein ratio) and a typical infant formula with the low whey to casein ratio and higher protein content elicited similar postprandial glycemic and insulinemic responses in healthy breastfeeding mothers (Wright et al., 2015). In that study, the protein to carbohydrate ratios in the milk and formula averaged 1:6 as in the present low protein milks, which also failed to show an effect of the ratio, which was only apparent in the high protein milks with the high whey to casein ratio.

Insulin was not found to be higher after the high protein milks, which contrasts with the suggestion that the lower glucose is due to a rise in blood insulin stimulated by branched chain amino acids (Anderson et al., 2011). In lean men, the intra-duodenal infusion of hydrolyzed whey protein alone was found to stimulate insulin and glucagon release in a load-dependent fashion, associated with a modest reduction in blood glucose (Ryan et al., 2012). In another study on lean individuals, the oral ingestion of 18 g intact whey protein with 25 g glucose increased insulin release and reduced blood glucose compared with 25 g glucose alone (Ryan et al., 2013). In the present study, the lower postprandial glucose concentrations reported after the

consumption of beverages with increased MP content and reduced casein to whey ratio consumed with 77 g carbohydrate, cannot be attributed to increased insulin secretion for two reasons. First, no differences in insulin concentrations were affected by treatments. Second, the postprandial levels of C-peptide were not affected by protein concentration. C-peptide is the part of proinsulin which is cleaved prior to co-secretion with insulin from the pancreatic beta cells; thus, it is produced in equimolar amounts to endogenous insulin (Leighton et al., 2017). These findings support previous studies showing modulation of postprandial glycemia by milk proteins is through insulin-independent mechanisms (Akhavan et al., 2014; Panahi et al., 2014). Others have also reported that reduced glycemia after protein consumption either with carbohydrates (Karamanlis et al., 2007) or alone (Hall et al., 2003; Boirie et al., 1997) is not solely due to increased insulin release but may be related to the release of gut hormones that delay stomach emptying or to the release of incretins that increase the efficacy of insulin (Jakubowicz and Froy, 2013).

In this study, the treatment effects that might explain the decrease in postprandial glycemia may be responses in GLP-1 and stomach emptying as measured by paracetamol appearance in blood. In the pre-meal periods, increased GLP-1 concentrations were found following consumption of milk beverages with increased protein content, and glucose negatively correlated with GLP-1. Previous studies have shown increased GLP-1 (Akhavan et al., 2014; Ryan et al., 2012; Akhavan et al., 2010) and delayed stomach emptying (Ma et al., 2009; Akhavan et al., 2014) in response to whey protein consumption. In mice with insulin resistance induced by a high-fat diet, GLP-1 improved hepatic insulin sensitivity (Khound et al., 2017). GLP-1 also slows gastric emptying by crossing the blood–brain barrier and directly transmitting signals that inhibit gastric emptying (Nauck et al., 1997). There was a negative correlation

between paracetamol with AUCs for GLP-1 and insulin levels throughout the whole postprandial period. Paracetamol responses were lower after the preloads with increased (9.3%) in comparison to those with regular (3.1%) MP concentrations, during the pre-meal and the total time periods. Additionally, at glucose peak time, 30 min, paracetamol mean absolute levels were lower with 40:60 whey to casein preloads compared to 80:20 ( $P = 0.0358$ ), in parallel to the reported lower mean glucose peak levels. In healthy individuals, the rate of gastric emptying acts as a major factor in blood glucose homeostasis by controlling the delivery of carbohydrate to the small intestine (Horowitz et al., 1993). Although correlations cannot show cause and effect, many of the associations among the hormonal responses, including PYY, CCK, and ghrelin in addition to GLP-1 and insulin, participate in feedback loops that affect stomach emptying (Panahi et al., 2014).

Although the sample size was expected to be insufficient to show treatment effects on subjective appetite and food intake, the absence of effect of treatments on appetite and food intake were also reported for the complete sample of 32 and, as suggested, may have been compromised by stomach discomfort reported due to the high lactose content of the treatments (Chapter 2). Nevertheless, it is surprising as the appetite regulatory hormones were strongly affected by the treatments and showed coordinated responses that would be expected to decrease food intake. In the present study, pre-meal glucose was lowest following the milk with 9.3% MP and 40:60 casein to whey ratio, significantly at 30 and 45 min, whereas post-meal was lowest after the milk with 9.3% MP and 80:30 casein to whey ratio, without reaching statistical significance (Figure 1). Although difficult to explain, the results emphasize the importance of the ratio.

There are some limitations in the study. First, the menstrual cycle for women was not controlled for and is known to affect appetite and food intake (Buffenstein et al., 1995). Second, the sample size was too small for meaningful comparisons between men and women. Third, this study assessed only the acute and short-term effects of these beverages in healthy young men and women. Thus, their application to those with insulin resistance and on long-term glycemic control is unclear. However, the results of this study add to the accumulative evidence that consumption of dairy protein quantity and composition has potential to aid in the dietary management of obesity and type 2 diabetes mellitus (Jakubowicz and Froy, 2013).

In conclusion, milk beverages with increased protein content, and to a lesser extent decreased casein to whey ratio, when consumed with carbohydrate, lower postprandial glucose responses through insulin-independent mechanisms in healthy adult men and women.

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