Novel nutrient based approaches for appetite regulation and obesity

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Abstract

Obesity is a global epidemic, with 1 in 8 people being affected and facing greater risks of morbidity and mortality. Increased protein intake in the daily diet has been shown to contribute to appetite management and thus has the potential to help prevent obesity. These benefits have contributed to the increasing protein consumption observed globally over the past few decades. Plant-based proteins may serve as a more environmentally sustainable alternative to animal-derived proteins; however, research on the appetite regulatory effects of plant-based proteins is still in its early stages.

This thesis therefore focused on assessing the effects of plant and animal-derived proteins (potato, PP; rice, RP; pea, PeP and whey, WP) in the context of appetite regulation and obesity, developing and utilising novel cell-based models employing the Caco-2 and STC-1 cell lines to assess the mechanisms underlying the whole-body effects observed in complementary human studies. The study is the first to demonstrate gastric inhibitory peptide (GIP) expression and secretion in the Caco-2 cell line, while observing significantly higher GIP, glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY) secretion with RP treatment vs all other conditions at 6.25 mg/mL protein digestate exposure following four hours of treatment. Appetite hormone secretion with PP treatment (6.25 mg/mL) was also significantly higher vs WP. However, treatment with lower protein concentration (4.17 mg/mL) for the same duration led to significantly higher GLP-1 and PPY secretion with PP vs RP, with no increases observed with RP. Identical trends were observed in both Caco-2 and STC-1 cells.

Additionally, for the first time WP was observed to lead to significantly higher postprandial plasma GIP levels compared to PP at 30 min (22.67 ± 10.73 pg/mL, $p = 0.0159$), 60 min (18.85 ± 9.69 pg/mL, $p = 0.0041$) and 120 min (15.33 ± 6.16 pg/mL, $p = 0.0012$) when consumed as part of a mixed macronutrient meal, resulting in insulin-independent glycaemic responses in healthy male participants (n = 10, mean age 28.4 years). Circulatory GLP-1 levels were significantly higher with both WP and PP vs control, with mean differences of 27.42 pmol/L (± 9.31, $p = 0.0315$) and 27.05 pmol/L (± 8.53, $p = 0.0178$), respectively, 180 min following meal consumption.

Taken together, the results demonstrate that differences in concentration, digestion rate, as well as the levels of available amino acid (AA) and bioactive peptides with potential appetite-suppressive effects (e.g. potato protease 2) or calcium-binding and -chelating characteristics (e.g. peptide AHV) present in the digested fractions of proteins may have influenced the observed appetite regulatory effects *in vitro* in either cell line. These data highlight the suitability of Caco-2 cells as an alternative cell line model for nutrient-induced appetite hormone regulatory studies. The metabolic responses evoked by WP as part of a mixed meal indicate that it may elicit potential benefits for populations where tighter control of glycaemic and insulinemic regulation is required, while the effects of plant-based PP on GLP-1 secretion indicate that it could be as effective for appetite regulation as WP, when combined with an appropriate carrier. This study thus provides novel insights on the relative appetite regulatory effects of dietary proteins both *in vitro* and *in vivo* and paves the way for further investigation into developing non-pharmacological approaches to managing obesity.

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Abstracts from oral presentations:

Novel nutrient based approaches for appetite regulation and obesity - University of Westminster, School of Life Sciences Conference, 2024, January, University of Westminster, London, United Kingdom.

Dairy or plant-based proteins for appetite regulation - does it matter? - Vitafoods Europe 2024 "Nutraceutical Pioneers: Insights from Rising PhD Innovators" at Palexpo in Geneva, Switzerland.

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Tiekou Lorinczova, H., Gámez Fernández, M.M., Schiano, E., Patel, V.B. and Gulrez, Z.M., The effects of protein digest treatments on Caco-2 and STC-1 cells

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Author's declaration

I declare that all the material contained in this thesis is my own work.

Glossary of abbreviations

5-HT	5-hydroxytryptamine
a-MSH	a-melanocyte stimulating hormone
AA	amino acid
AgRP	agouti-related protein
AHV	peptide AHVGMSGEEPE
ANF	antinutritional factor
ANOVA	analysis of variance
ANS	autonomic nervous system
ATCC	American Type Culture Collection
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCAA	branched-chain amino acid
BCFA	branch-chained fatty acids
BD	blank digestion
BMI	body mass index
BSA	bovine serum albumin
CART	cocaine and amphetamine-regulated transcript
CASR	calcium-sensing receptor
CCK	cholecystokinin
CHD	coronary heart disease
CI	confidence intervals
CNS	central nervous system
DHSC	Department of Health and Social Care
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DPP-IV	dipeptidyl peptidase IV

EAA	essential amino acid
EEC	enteroendocrine cell
FBS	foetal bovine serum
FDA	Food and Drug Administration
FFA2	free fatty acid receptor 2
GABA	gamma-aminobutyric acid
GDPR	general data protection regulation
GHS-R1	growth hormone secretagogue receptor
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide 1
GLP-1A	GLP-1 analogue
GLP-1R	GLP-1 receptor
GLP-1Ra	GLP-1 receptor agonist
GMP	glycomacropeptide
GPCR	G-protein coupled receptors
GSH	glutathione
IEC	ion exchange chromatography
Ikkb	I kappa B kinase beta
IL-6	interleukin 6
InsR	insulin receptors
IR	insulin resistance
IRS	insulin receptor substrate
JAK/STAT	Janus kinase-signal transducer and activator of transcription
LCD	low-calorie diet
LEP-R	leptin receptors
LEU	leucine
LYS	lysine

MC4R	melanocortin 4 receptor
MD	mean difference
MEM	minimum essential medium
MFI	mean fluorescence intensity
MHRA	Medicines and Healthcare products Regulatory Agency
mTORC1	mechanistic target of rapamycin complex 1
MTT	thiazolyl blue tetrazolium bromide
MWCO	molecular weight cut-off
NF-κB	nuclear factor-kappaB
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
Nos2	nitric oxide synthase 2
NST	nucleus of the solitary tract
NYP	neuropeptide Y
OPA	ophthaldialdehyde
PeP	pea protein
PEPT1	proton-coupled peptide transporter 1
PFJ	potato fruit juice
PHE	Public Health England
PI2	potato protease inhibitor II
POMC	proopiomelanocortin
PP	potato protein
PYY	peptide tyrosine tyrosine
RP	rice protein
RP-HPLC-MS/MS	reverse-phase high-performance liquid chromatography with mass spectrometry
RYGB	Roux-en-Y gastric bypass

SCFA	short-chain fatty acids
SD	standard deviation
SDS	sodium dodecyl sulphate
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SSF	simulated salivary fluid
STC-1	secretin tumour cell line
T2D	type 2 diabetes mellitus
TAME	p-toluene-sulfonyl-L-arginine methyl ester
TCA	trichloroacetic acid
TGR5	Takeda GPCR 5
TNF	tumour necrosis factor-alpha
TRYP	tryptophan
UK	United Kingdom
VAS	visual analogue scales
VLCD	very low-calorie diet
VSG	vertical sleeve gastrectomy
WHO	World Health Organization
WP	whey protein
WPHd	WP hydrolysate digest
WPId	WP isolate digest
αlac	α-lactalbumin
β-lg	β-lactoglobulin

1. CHAPTER 1 – Background

1.1. Obesity

Obesity is a global epidemic, with 2.5 billion adults being classified as overweight and 890 million of these classified as obese. This indicates a nearly threefold increase since 1990 (World Health Organization (WHO, 2024). The increasing rate of obesity is predominantly related to the increased availability and affordability of highly palatable, energy-dense food items in combination with reduced energy expenditure due to more sedentary urban lifestyles (Romieu et al., 2017). Click or tap here to enter text. As per WHO, overweight and obesity are the 5th cause of mortality globally, with an estimated 2.8 million adult deaths being related to overweight or obesity (WHO, 2015). Obesity and overweight are classified as the accumulation of excessive body fat to the degree that is likely to have detrimental effects on health (WHO, 2015).

Body mass index (BMI) is the most used indicator, with a BMI ≥ 25 indicating overweight and a BMI ≥ 30 indicating obesity. BMI is calculated as a weight-to-height ratio where weight is expressed in kilograms (kg), height is expressed in meters (m), and the unit is kg/m^2 (WHO, 2015). High BMI is a predictor of obesity-related comorbidities such as metabolic syndrome, insulin resistance (IR), type 2 diabetes mellitus (T2D), coronary heart disease (CHD), stroke and certain types of cancer (e.g., bowel cancer and breast cancer) (Kyle, Dhurandhar and Allison, 2016; Powell-Wiley et al., 2021; Lopez-Jimenez et al., 2022). However, when assessing for metabolic health alongside BMI, body fat distribution should also be considered, as fat accumulation around the body's midsection has been associated with metabolic conditions such as insulin resistance, a predictor of T2D. In contrast, peripheral fat deposits (subcutaneous fat accumulation around the upper and lower extremities of the body) are observed to have a negligible effect on metabolic health (Dhokte and Czaja, 2024; Linge et al., 2024; Qiu et al., 2024). Metabolic health may be characterised by central obesity, high blood pressure, decreased high-density lipoprotein cholesterol, elevated low-density lipoprotein cholesterol, serum triglycerides and plasma glucose, as well as the pro-inflammatory and prothrombotic state (Grundy, 2020). Chronic obesity-inducing inflammation, most prominently via increased macrophage recruitment,

has been identified as a key contributor to IR and T2D and metabolic syndrome development (Lee, Korf and Vidal-Puig, 2023). Macrophages constitute up to half the total adipose tissue cell content in obese individuals compared to only 5% in lean individuals (Weisberg et al., 2003; de Luca and Olefsky, 2008). The macrophages recruited during obesity, namely M1-polarised macrophages, secrete pro-inflammatory cytokine tumour necrosis factor-alpha (TNF) (Weisberg et al., 2003). TNF can affect insulin signalling via insulin receptor substrate (IRS) mediated by activating I kappa B kinase beta (Ikkb), a serine kinase in the nuclear factor NF-κB pathway. Ikkb, in turn, phosphorylates IRS-1 on serine 307, leading to the inactivation of the insulin receptor tyrosine kinase substrate (Laakso et al., 1994; Almind et al., 1996; Hotamisligil et al., 1996; Rondinone et al., 1997; Aguirre et al., 2002). Macrophages in the adipose tissue are also primarily responsible for interleukin 6 (IL-6) and nitric oxide synthase 2 (Nos2) expression (Weisberg et al., 2003). Notably, Nos2 has also been linked to the development of IR (Perreault and Marette, 2001; Sugita et al., 2005; Yasukawa et al., 2005). Additionally, there are other immune cells residing in the adipose tissue that further contribute to inflammation and the development of IR, such as neutrophils, dendritic cells, mast cells, T cells and B cells (Liu et al., 2009; Winer et al., 2009; Stefanovic-Racic et al., 2012; Talukdar et al., 2012; DeFuria et al., 2013).

Furthermore, central obesity is also strongly associated with respiratory disease, with increased susceptibility to infections and higher hospitalisation rates in obese individuals compared to healthy weight controls (Petrova et al., 2020). Although the mechanism is not yet clearly understood, obesity has a multi-factorial impact on lung function (Salome, King and Berend, 2010). In people with obesity, mechanical changes observed are causing asthma-like symptoms such as wheezing and hyperventilation, primarily due to fat accumulation in the peritoneal and mediastinal cavity, leading to reduced lung volume (expiratory, resting, as well as tidal volume) (Dixon and Peters, 2018). Obesity-related inflammatory dysregulation has also been suggested to affect the severity and outcome of respiratory disease (Salome, King and Berend, 2010). This suggestion is supported by the increased expression of pro-inflammatory adipokine with a key role in the ventilatory drive regulation, namely leptin, and reduced expression of anti-inflammatory adipokine, adiponectin, in the adipose

tissue in the state of obesity compared to healthy status (Salome, King and Berend, 2010; Dixon and Peters, 2018). The detrimental effects of obesity on the severity and outcomes of respiratory disease were evident in the most recent pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), named COVID-19 with Public Health England (PHE, 2020) reported possible increased risk of death by 40% in patients with BMI of 35 to 40, while with a BMI of over 40, the increase may be as high as 90%. Consequently, there is an extreme burden placed not just on the affected individuals and their families. Still, there is also an extreme financial burden experienced by society due to obesity-related demands on the health care system. In the United Kingdom (UK), the National Health Service (NHS) spent £6.1 billion on overweight and obesity-related illnesses between 2014 and 2015. This figure is estimated to reach £9.7 billion by 2050 (PHE, 2017). Therefore, the causes, management and prevention, as well as the possible reduction of obesity, have been extensively studied in the last few years (Goran, 2000; Hui et al., 2002; Wynne et al., 2005; Jarolimova, Tagoni and Stern, 2013; Addison et al., 2014).

1.2. Appetite, satiety and energy homeostasis

Appetite and satiety are two interrelated physiological and psychological processes that regulate food intake and energy balance. Appetite refers to the desire to eat, influenced by hormonal signals such as ghrelin, environmental cues, emotional states, and sensory stimuli like the sight and smell of food (Blundell et al., 2010). In contrast, satiety is the sensation of fullness and satisfaction that follows eating, helping to suppress further intake until energy is again needed. This process is governed by complex interactions between the gastrointestinal tract, adipose tissue, and the brain, involving hormones such as leptin and cholecystokinin (Feinle-Bisset and Horowitz, 2021). The balance between appetite and satiety is essential for maintaining energy homeostasis.

Energy homeostasis is a state where energy consumption and expenditure are balanced (Keesey and Powley, 2008). The disruption of this balance by energy intake in excess of what is required for the body to maintain its energy homeostasis is said to cause obesity (Goran, 2000).

1.2.1. Appetite regulation along the gut-brain axis

Energy homeostasis, appetite and satiety are predominantly regulated by the hypothalamus, brain stem, and the brain's reward centres by conveying long and short-term peripheral signals of energy status (Wynne, Stanley and Bloom, 2004; Druce and Bloom, 2006). Peripherally secreted gut hormones such as peptide tyrosine tyrosine (PYY), glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK), the white adipose tissue secreted leptin, and the pancreas-derived insulin are key regulatory signals affecting the central appetite regulation within the brain directly or via the vagal nerve plexus (Makaronidis and Batterham, 2018). Mechanisms of appetite regulation, central or peripheral, are also influenced by hedonic cues such as the palatability or perception of food (Wynne, Stanley and Bloom, 2004; Ueno and Nakazato, 2016; Miller, 2019) (**Figure *1***).

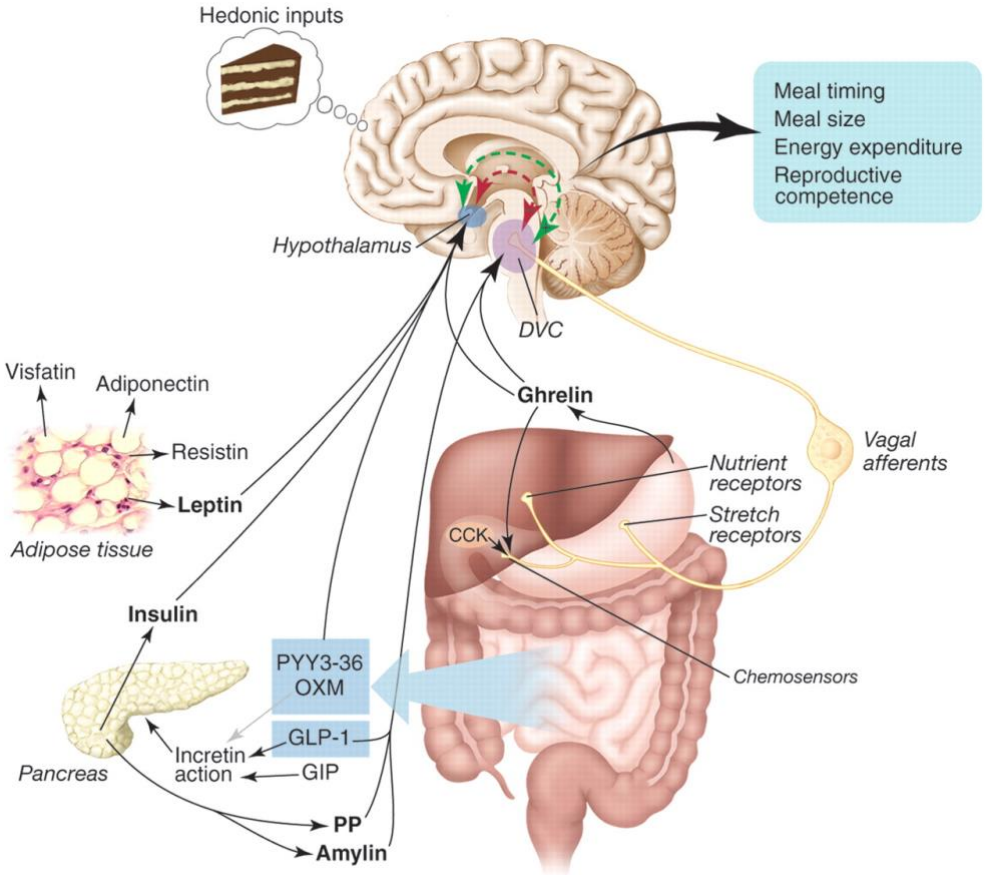


Figure 1. Energy balance regulation along the gut-brain axis (Adapted from Badman and Flier, 2005). The brain integrates long-term and short-term signals to regulate energy balance. Long-term signals are produced by adipose tissue (leptin) and the pancreas (insulin). After ingestion, short-term signals, such as CCK, GIP, GLP-1 and PYY, are released from the gut. These hormones can act locally to regulate gut motility or act in the brain to regulate food intake through two mechanisms: via the vagus nerve to stimulate ascending pathways from the gut to the brain, or by the circulation to the brain. The main regions in the brain involved are the hypothalamus, in particular the arcuate nucleus, and the dorsal vagal complex in the brain stem.

1.2.2. Central mechanisms of appetite regulation

The brain, namely the hypothalamus, plays a key role in maintaining energy balance within the body by integrating information received via the afferent and efferent pathways from and to the brainstem and peripheral organs (Nogueiras, 2021; Münzberg, Berthoud and Neuhuber, 2023). The brainstem, namely the nucleus of the solitary tract (NST), receives signals from the peripheral organs through circulating factors, e.g., gut hormones or neural inputs via the afferent synapses of the vagus nerve. In turn, the NST conveys the received signals to the arcuate nucleus in the hypothalamus (Vargas et al., 2024). Additionally, the circulating hormones and nutrients are detected by the area postrema within the brainstem and consequently relayed signals via the efferent and afferent pathways to the arcuate nucleus to further contribute to the moderation of appetite and satiety based on nutritional needs (Miller, 2019; Iovino et al., 2022). The control of appetite and satiety within the arcuate nucleus is orchestrated by two types of neurons via the integration of short-term hunger and satiety as well as long-term nutrient and energy availability signals (Gouveia, de Oliveira Beleza and Steculorum, 2021). The two types of neurons responsible for the integration of various long- and short-term signals are the agouti-related protein (AgRP) neurons activated by hunger and fasting and the proopiomelanocortin (POMC) neurons activated by energy intake (Chen and Knight, 2016).

The POMC neurons are catabolic, expressing POMC, which is a precursor for peptides such as a-melanocyte stimulating hormone (a-MSH) (Aoyama et al., 2018). The a-MSH binds primarily to the melanocortin 4 receptor (MC4R), which is widely spread in the hypothalamus (Siljee et al., 2013). The binding of a-MSH as well as agonists to MC4R leads to hyperphagia, thermogenesis and consequently weight loss in mice, while with the application of antagonists or with the distortion of the MC4R, opposing results were observed (Ji, Hong and Tao, 2022; Wu et al., 2023). Furthermore, in humans, the most common monogenic form of obesity is due to a mutation in MC4R, with a prevalence of 2% to 6% among the paediatric population affected by obesity (Concepción-Zavaleta et al., 2024).

The AgRP anabolic neurons express AgRP as well as Neuropeptide Y (NYP) (Yeo and Heisler, 2012). The binding of NPY to Y receptors and the AgRP antagonist to MC4R receptors causes a short-term increase in food consumption, leading to a potential increase in body weight (Gan, Cerbone and Dattani, 2024) (**Figure 2**).

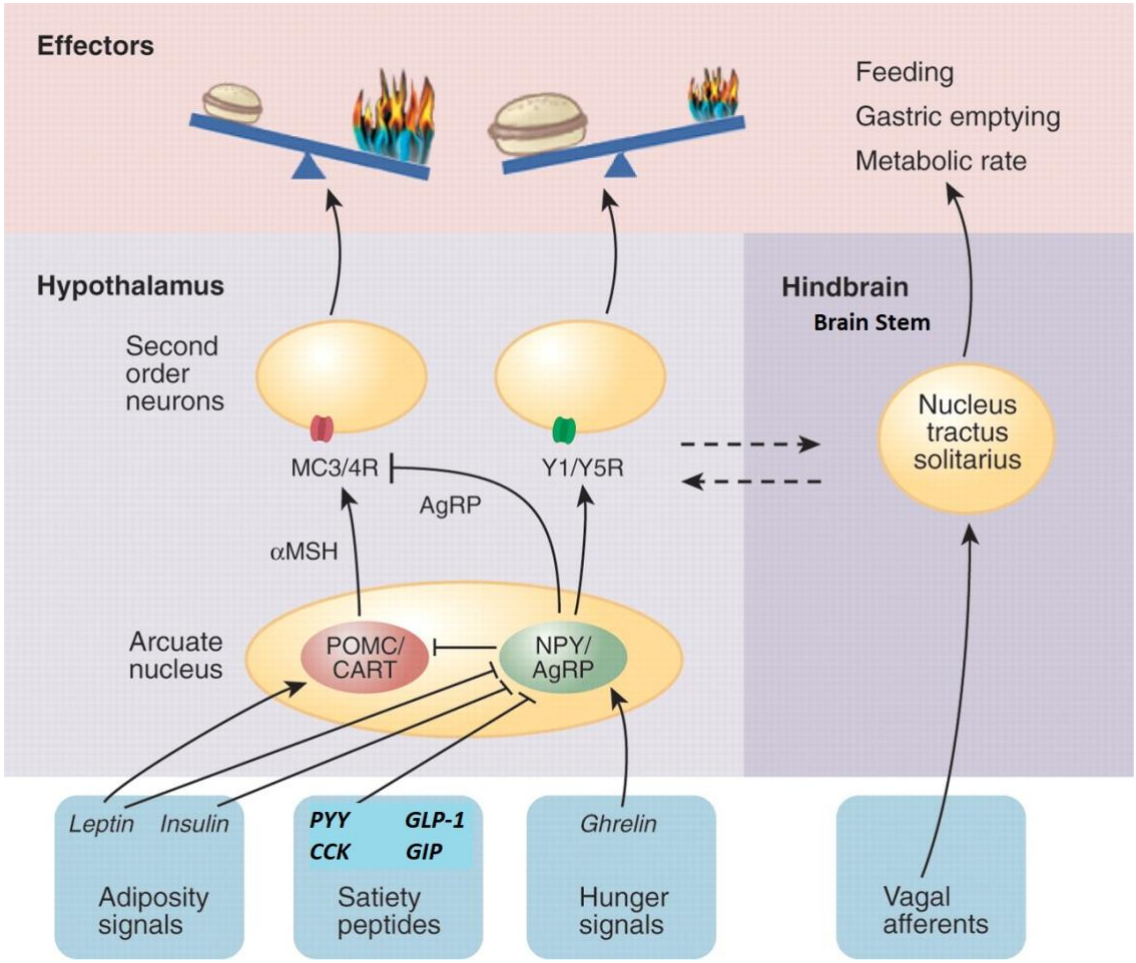


Figure 2. The action of gut peptides on the brain (Adapted from Badman and Flier, 2005). The arcuate nucleus integrates long-term or short-term signals from the periphery and the brain stem. The arcuate nucleus contains two distinct subsets of neurons controlling food intake. One acts as a stimulus to feeding; these neurons contain neuropeptide Y (NPY) and agouti-related peptide (AgRP). The second subset of neurons acts as an inhibitor to feeding; these contain α-MSH, and cocaine and amphetamine-regulated transcript (CART). Integration of peripheral signals within the brain involves the interplay between the hypothalamus and brain stem (hindbrain) structures, including the NTS (Nucleus tractus solitarius), which receives vagal afferent inputs. Inputs from the brain stem and hypothalamus are integrated, acting on meal size, frequency, and energy expenditure.

Although the brain is protected by the blood-brain barrier (BBB) from any potentially harmful presence circulating in the blood, such as pathogens, viruses, or solutes, this tightly selective regulation allows the entry of peripheral metabolic signals into the interstitial fluid of the brain, allowing the interaction with the areas

responsible for energy homeostasis (Aburto and Cryan, 2024). Furthermore, the BBB is not complete around the hypothalamus as it is presented with fenestrated capillaries, allowing increased permeability of peripheral signals such as hormones and nutrients to the brain (Rodríguez, Blázquez and Guerra, 2010; Aburto and Cryan, 2024).

1.2.3. Long-term peripheral appetite regulatory signals

The effects of the peptide hormones leptin and insulin are expressed peripherally as well as within the central nervous system (CNS), with a significant influence on the metabolism (Erichsen, Fadel and Reagan, 2022). Leptin and insulin play a key role in appetite regulation and energy homeostasis. There is also a bidirectional feedback loop between these two hormones, whereby while leptin inhibits insulin secretion and production via receptors found in pancreatic beta cells, insulin promotes leptin release from adipocytes. The "adipo-insular axis," which regulates body metabolism, is a link between the pancreas and adipocytes (Tsai et al., 2012) (**Figure *3***). Moreover, leptin and insulin are characterised as adiposity signals due to their proportionate secretion to stored adipose tissue (Martínez-Sánchez, 2020; Obradovic et al., 2021).

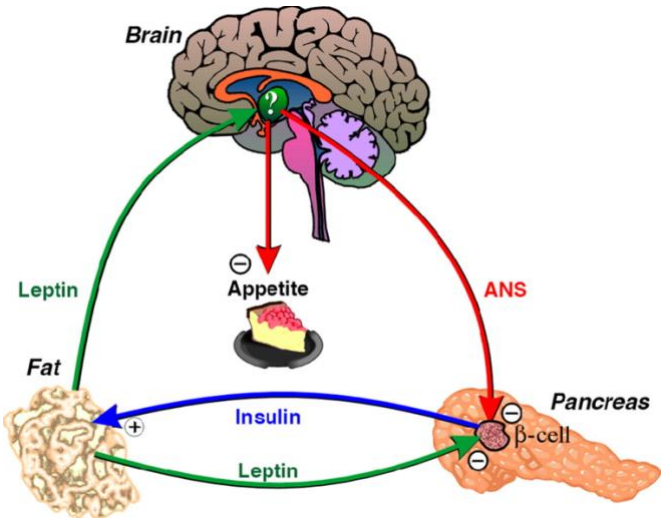


Figure 3. The adipo-insular axis (Adapted from Kieffer and Habener, 2000)**.** Leptin reduces appetite via its inhibitory effects expressed within the hypothalamus. A bidirectional feedback loop, or the adipo-insular axis, is present between adipose tissue and pancreatic B-cells via leptin and insulin hormones. Insulin is adipogenic and increases the production of leptin by adipose tissue. Leptin feeds back to reduce both insulin secretion and insulin gene expression. The suppressive effect of leptin on insulin production is mediated both by the autonomic nervous system (ANS) and by direct actions via leptin receptors on B-cells.

1.2.3.1. Leptin

Leptin is a 16 kDa-sized peptide hormone comprising 167 AAs, secreted primarily from subcutaneous white adipose tissue and secreted into the circulation (Obradovic et al., 2021). The circulating leptin levels are in proportion to body fat stores, with higher levels of plasma leptin observed in individuals with higher body fat (Izquierdo et al., 2019; Obradovic et al., 2021). Additionally, proportionate reduction in both BMI and plasma leptin levels with positive correlations was observed in morbidly obese Roux-en-Y gastric bypass patients with *(r = .78)* and *(r = .79)* at baseline and 6 months post-surgery, respectively (Miller, Nicklas and Fernandez, 2011). However, a 7-day long energy restriction led to a mean reduction of 66% *(p < 0.001)* in leptin levels in 21 obese women, indicating that leptin's secretion is tightly coupled to energy status and may have a superior influence on leptin regulation compared to fat mass (Wisse et al., 1999).

Circulatory leptin exists in two forms: bound to soluble leptin receptors or the free, unbound form (Obradovic et al., 2021). Binding to the soluble leptin receptors may affect the bioavailability and inhibit leptin signalling (Picó et al., 2022). The unbound, biologically active leptin is transported to the hypothalamus through the BBB via a saturable transporter (Banks, 2006; Pan and Myers, 2018; Miller, 2019) and exerts its effects upon binding to leptin receptors (LEP-R). Binding to LEP-R activates the downstream Janus Activated Kinases and the transcription factor signal transducer and activators of transcription (JAK/STAT) signalling cascade with a key role in energy balance (Boucsein, Kamstra and Tups, 2021). Leptin receptors are expressed on POMC and AgRP neurons, which, upon leptin binding, are activated and inhibited, respectively (Schwartz et al., 1996; Williams, Scott and Elmquist, 2011). The activation of POMC neurons via leptin binding leads to increased production of a-MSH and its binding to MC4R, initiating a reduction in food consumption (Rossi et al., 2011; Williams, Scott and Elmquist, 2011). The effects of leptin on appetite regulation are further supported by the observation of MCR4 antagonists and the deletion of LEP-R in the hypothalamus, eliminating the feeding inhibitory effects of leptin (Martínez-Sánchez, 2020).

1.2.3.2. Insulin

Insulin is secreted by the beta cells of the pancreas. It comprises 5.8 kDa molecules containing 51 AAs in a dimer configuration where the A and B chains are linked together by disulfide bonds (Westermeier et al., 2015). Additionally, to the well-described anabolic effects of insulin via glucose regulation at the periphery level, insulin also plays a key regulatory role in energy homeostasis within the central nervous system (Spanswick et al., 2000; Hill et al., 2010; Williams, Scott and Elmquist, 2011). Insulin secretion, similar to that of leptin, is influenced by energy intake proportionally to stored fat mass (Miller, 2019). However, in contrast with leptin, circulating insulin levels are highly correlated with visceral rather than subcutaneous fat (Lé and Wajchenberg, 2000).

Similar to leptin, insulin crosses the BBB via a saturable mechanism and binds to insulin receptors (InsR) abundantly found on the hypothalamic POMC and AgRP neurons (Banks, 2006; Belsham and Dalvi, 2021). Insulin also exerts its anorexigenic effects by activating POMC and inhibiting AgRP neuron signalling, which was shown to result in reduced energy intake and weight loss in nonhuman primates (Woods et al., 1979) as well as in lean, but not in obese rodents (Ikeda et al., 1986) with hypothalamic infusions. The anorexigenic effects of intranasally administered insulin in humans were also observed, where plasma insulin levels remained unaffected. However, the cerebrospinal fluid levels increased, leading to lower energy intake and reduced body fat (Hallschmid et al., 2004).

1.2.4. Short-term peripheral appetite regulatory signals

The gut hormones play a key role in the regulation of digestion, nutrient absorption, disposal of unutilised nutrients, as well as appetite, consequently having a crucial role in metabolic regulation (e.g., insulin secretion) (Gribble and Reimann, 2016). Gut hormones are characterised as acute regulators of appetite and food intake via signalling the brain directly as well as indirectly via the vagal nerve. The central appetite-regulatory effects of gut hormones are expressed in the hypothalamus and the brain stem (Makaronidis and Batterham, 2018).

1.2.4.1. Ghrelin

Ghrelin is a 28 AA peptide secreted primarily from the oxyntic cells of the stomach (Sakata et al., 2002). Ghrelin has an N-octanoyl group covalently linked to the hydroxyl group of its serine-3 residue, which is necessary for binding to its receptor (Kojima et al., 1999; Tschop, Smiley and Heiman, 2000), the growth hormone secretagogue receptor (GHS-R1) widely expressed in the hypothalamus (Gnanapavan et al., 2002). Ghrelin is an orexigenic peptide, also called the “hunger hormone”. As its title suggests, the levels of ghrelin peak during fasting and reduce after food consumption (Tschop, Smiley and Heiman, 2000; Kojima and Kangawa, 2005). Furthermore, it was observed that drinking water alone does not lead to a change in ghrelin levels, indicating that the mechanical expansion of the stomach alone is insufficient to cause a reduction in ghrelin (Tschop, Smiley and Heiman, 2000).

Ghrelin infusion peripherally or centrally leads to an increase in energy intake, a decrease in fat metabolism and consequently, weight gain in rats (Tschop, Smiley and Heiman, 2000). Similarly, intravenous administration of ghrelin in humans also led to a 28% (p <0.001) increase in food consumption as well as greater appetite when compared to saline infusion (Wren et al., 2001). It is suggested that peripheral ghrelin activates the hypothalamic regions via the vagal afferent neurons, which are also expressing GHS-R1, rather than by direct activation due to its slow rate of transport through the BBB (Date et al., 2002a; Rhea et al., 2018). Thus, ghrelin’s effects are expressed via activation of AgRP neurons and suppression of POMC neurons upon signals received from the vagal afferent pathways (Kojima et al., 1999; Nakazato et al., 2001; Date et al., 2006). Stimulation of appetite is also enhanced by the inhibition of gastric vagal afferent signals, as well as reversing the inhibition of AgRP neurons by leptin (Date et al., 2002). There is a competitive relationship between leptin and ghrelin within the hypothalamus, as leptin also directly inhibits AgRP neurons, blocking ghrelin-induced appetite stimulation (Sato et al., 2012).

1.2.4.2. CCK

CCK is predominantly released in the duodenum and jejunum (Larsson and Rehfeld, 1978). CCK is rapidly released into the circulation, and locally, proportional to fat and protein intake, and CCK levels remain elevated for up to 5 hours (Liddle et al., 1985). CCK contributes to appetite regulation by increasing pancreatin and bile release, therefore supporting fat and protein digestion (Liddle et al., 1985; Rehfeld et al., 2004). Additionally, CCK inhibits gastric emptying and increases intestinal motility (Liddle et al., 1985; Moran and Schwartz, 1994). In line with the above-described characteristics, CCK administration led to reduced food intake via smaller meal sizes and shorter durations in humans (Kissileff et al., 1981) as well as animals (Gibbs, Young and Smith, 1973). However, total energy intake and body mass seem to be unaffected in the long term due to increased frequency of meal consumption (West, Fey and Woods, 1984). Additionally, after the initial 24 hours of continuous infusion, the effects of CCK are diminished (Crawley and Beinfeld, 1983). Furthermore, rodents without CCK production maintain normal diets and body weights. Thus, CCK seems to be non-essential in appetite regulation (Woods, 2009).

CCK influences satiety via the vagal afferent nerve following binding to G-protein-coupled CCKA receptors (Wank et al., 1992). Vagal nerves project to the hindbrain, relaying information to the hypothalamus (Woods, 2009). CCK effects on appetite via the vagal nerves are supported by demonstrating the elimination of CCK-induced appetite suppression with vagal nerve lesions (Abelson, Nesse and Vinik, 1991). Moreover, it was suggested that the long-term energy regulatory effects of CCK may be affected by leptin as it enhances CCK binding to vagal afferent nerves (Peters, Ritter and Simasko, 2006).

1.2.4.3. PYY and GLP-1

Two of the most extensively studied hormones influencing energy intake are PYY and GLP-1 (Wynne et al., 2005). PYY and GLP-1 are both anorexigenic appetite hormones that enhance satiety and reduce hunger (De Silva and Bloom, 2012). These peptides are co-localised within the enteroendocrine L cells found in the innermost layer of the gut (the mucosa). L cells were detected along the entire intestinal tract, present with a low cell density in the upper small intestine, which

increases down the length of the intestine to the rectum (Bryant and Bloom, 1979; Hansen et al., 2013; Guedes et al., 2015). However, PYY and GLP-1 secretory characteristics are mostly dependent on the localisation of the L-cells. GLP-1 secretion is observed from the upper as well as lower parts of the intestine, while PYY secretion is detected only in L cells found in the distal parts of the ileum and colon (Rune E Kuhre et al., 2021). PYY and GLP-1 are secreted into systemic circulation in response to nutrients (e.g., glucose, AAs, lipids and short-chain fatty acids) binding to cell surface G-protein coupled receptors (GPCR) (Chaudhri, Small and Bloom, 2006). Furthermore, the released level of these hormones was found to be proportionate to calorie intake (Wynne, Stanley and Bloom, 2004). PYY and GLP-1 are bioactive peripheral signalling molecules with a direct effect on central appetite regulation, as the N-terminal within their structure allows them to readily cross the blood-brain BBB (Kastin, Akerstrom and Pan, 2002; Nonaka et al., 2003). PYY and GLP-1 have been observed to reduce food transit time within the gut and thereby slow gastric emptying, the process by which the contents of the stomach are moved into the duodenum; this mechanism is termed the ileal brake. This braking slows intestinal motility to allow more efficient digestion and nutrient absorption. Furthermore, it is also believed to induce satiety and reduce food intake (Van Citters and Lin, 2006).

PYY is a 36 AA length peptide secreted from the L-cells as early as 15 minutes after food intake, thus before the nutrients reach the distal part of the intestine where the PYY secretory L-cells are predominantly localised. This indicates that the early PYY secretion might be mediated via the vagal reflex (Onaga, Zabielski and Kato, 2002). PYY levels increase for up to 2h post-prandial and can remain elevated in the circulation for up to 6h (Adrian et al., 1985). Secreted levels of PYY seem to be influenced by macronutrients, with fat intake causing higher secretion compared to carbohydrates or protein (Lin and Chey, 2003). Additionally, other signals such as CCK, gastric acid and bile salt within the lumen increase while GLP-1 decreases PYY levels. However, PYY levels are unaffected by stomach distention (Näslund et al., 1999; Pedersen-Bjergaard et al., 2009).

Following secretion, PYY is cleaved by the enzyme dipeptidyl peptidase IV (DPP-IV), creating a truncated peptide, PYY3-36. PYY3-36 represents 40% of the total

circulating PYY. PYY3-36 binds specifically to GPCR Y2 found abundantly in the hypothalamus, resulting in appetite reduction and enhanced satiety via suppressing NPY and activating POMC neurons (Ballantyne, 2006). In mice, administering PYY3-36 peripherally led to reduced NPY mRNA and increased POMC mRNA expression, which was present up to 6 and 24 hours post-administration, respectively (Challis et al., 2003). Consequently, reduced food intake, lower weight gain (Batterham et al., 2002; Challis et al., 2003) and improved glucose regulation were observed in obese rodents (Pittner et al., 2004). In line with the animal studies, intravenous administration of PYY3-36 to obese humans also reduced food consumption by 30% (p <0.001), resulting in significantly reduced cumulative energy intake over 24 hours (Batterham et al., 2003). Additionally, Batterham et al. (2003) also showed that administration of PYY3-36 led to a decrease in ghrelin levels, which may potentially contribute to its appetite-suppressing effects (**Figure *4***).

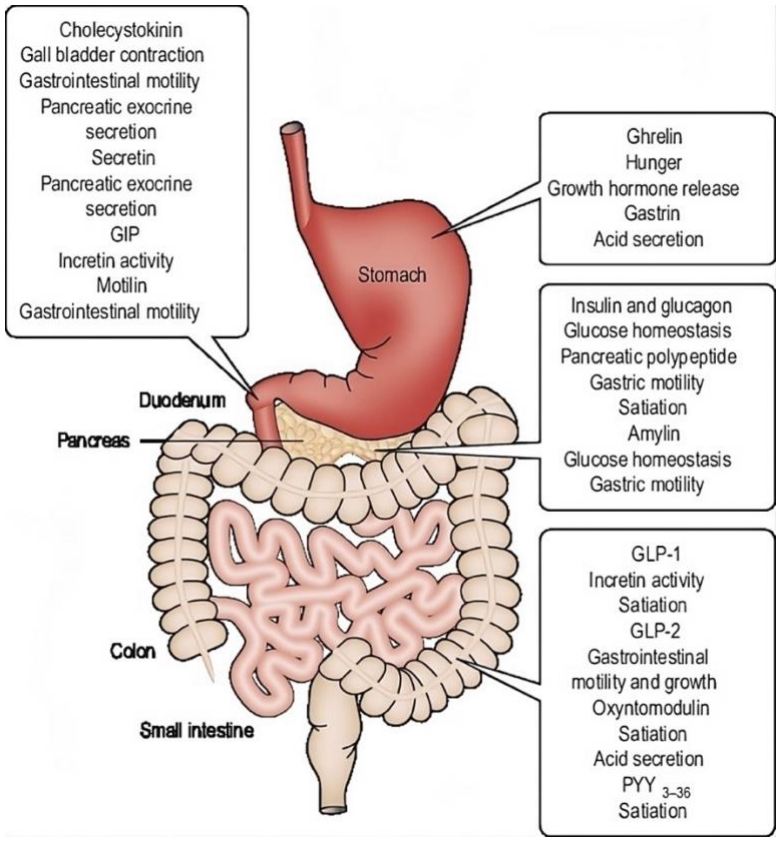


Figure 4. Gut peptides secreted from the gastrointestinal tract (Adapted from *Kevin G. Murphy & Stephen R. Bloom, 2006)*. The gastrointestinal tract releases a variety of hormones, including ghrelin and gastrin from the stomach, insulin, glucagon, pancreatic polypeptide and amylin from the pancreas, cholecystokinin, secretin, GIP and motilin from the small intestine, and GLP-1, GLP-2, oxyntomodulin and PYY 3-36 from the large intestine. These hormones signal to the periphery and to the central nervous system to regulate numerous biological processes.

GLP-1 is a short 30 AA peptide secreted from the L-cells rapidly after food consumption into the systemic circulation (Wynne, Stanley and Bloom, 2004). It exists in two major active forms in humans: GLP-1 (7-36) amide, representing 80% of circulating GLP-1, and GLP-1 (7-37) (Gupta, 2013). GLP-1 secretion is biphasic. The initial increase in GLP-1 levels is due to neural reflexes and circulating factors observed within minutes and lasts about 30 minutes post-prandially, while the second phase occurs an hour or so later due to the presence of nutrients in the distal gut (Elliott et al., 1993; Herrmann et al., 1995). The increase of GLP-1 is proportionate to energy intake and levels of 90 minutes post-prandial (TODD et al., 1998; Batterham et al., 2003). Similarly, insulin is secreted in two phases in response to GLP-1 levels due to its incretin effects. GLP-1 enhances glucose-stimulated insulin secretion upon binding to GLP-1 receptors (GLP-1R) on the beta cells of the pancreas (MacDonald et al., 2002; Vilsbøll and Holst, 2004). As previously mentioned, GLP-1 also slows gastric emptying, which also contributes to insulin secretion. By slower introduction of glucose into the circulation, the level of required insulin is reduced (Nauck et al., 1997). In addition, GLP-1's glucose-lowering effects are further amplified by concurrent inhibition of glucagon secretion (Godoy-Matos, 2014).

The satiety effect of GLP-1 is expressed upon its binding to G protein-coupled GLP-1R, found in the brainstem (Merchenthaler, Lane and Shughrue, 1999) as well as in the hypothalamus (Troke, Tan and Bloom, 2014). Peripherally administered GLP-1 prior to each meal led to 15% lower energy intake and 0.5 kg weight loss over 5 days in obese subjects. Additionally, it was shown to reduce body mass by 2 kg in patients with T2D after 6 weeks of administration (Zander et al., 2002). However, GLP-1 is exposed to cleavage by DPP-IV almost immediately after secretion, causing a very short half-life of less than 2 minutes, rendering its application as a weight loss agent impractical (Gupta, 2013). Nevertheless, the development of GLP-1 receptor agonists (GLP-1Ras) seems to provide a solution to this challenge (Holst and Madsbad, 2017). There are two types of GLP-1Ras, short- and long-acting, with a half-life of about 2 hours for the former and between 15 to 150 hours for the latter (Trujillo, Nuffer and Smith, 2021). GLP-1Ras have been demonstrated to be effective treatments for T2D, with additional popularity as it was observed not to cause hypoglycaemia and

presented appetite inhibitory characteristics (Meier, 2012; Østergaard, Frandsen and Madsbad, 2016).

1.2.4.4. Gastric inhibitory peptide (GIP)

GIP, also known as glucose-dependent insulinotropic polypeptide, is a 42 AA-long peptide secreted mainly within the duodenum and proximal jejunum from the K cells (Inagaki et al., 1989). GIP has many characteristics in common with the above-described GLP-1. Its release is stimulated by the ingestion of nutrients, mostly carbohydrates and lipids. After secretion, it is rapidly inactivated by DPP-IV, resulting in a short half-life of 4 to 7 minutes. GIP is also an incretin hormone, simulating glucose-dependent insulin secretion (Baggio and Drucker, 2004). However, unlike GLP-1, GIP has dual functions: increasing glucagon secretion in the normoglycaemic and hypoglycaemic states and reducing glucagon secretion in the hyperglycaemic state (Seino, Fukushima and Yabe, 2010).

Although the effect of GIP on appetite and body weight regulation is not yet well understood, mice with GIP receptor knockout demonstrated resistance to diet-induced obesity, unlike control mice, indicating a preference for fat as an energy source (Zhou et al., 2005; Naitoh et al., 2008). In transgenic mice, chronic elevation of GIP levels led to a reduction in diet-induced obesity and improved insulin sensitivity, glucose tolerance and beta cell function (Zhang et al., 1994; Yamada et al., 2006). Another potential mechanism of the body weight regulatory effects of GIP may be due to increasing glucagon levels, which were shown to cause a reduction in appetite and adipose tissue fat storage (Rix et al., 2015).

1.3. The gut microbiome and appetite

The described regulation of appetite-regulatory hormone secretion was also found to be majorly influenced by gut microbial metabolites as a result of dietary fibres (Cummings et al., 2001) as well as undigested proteins and AAs (Barker, 1981), fermentation by the gut in the distal gut. The gut microbiota has been found to have a key role in immunomodulation, maintenance of the gut mucosal barrier, protection against pathogens and metabolism of drugs, xenobiotics and nutrients (Jandhyala et al., 2015). Furthermore, the diversity of the gut microbiota has also been linked to obesity, with reduced diversity associated with increased fat

accumulation, low-grade inflammation and impaired glucose homeostasis (Le Chatelier et al., 2013). The human gut microbiota is a collection of a complex microbial ecosystem consisting of bacteria, yeast, fungi, viruses (Scarpellini et al., 2015), protozoa and archaea (Koskinen et al., 2017). About 90% of the human adult gut microbiota consists of Bactoidetes and Firmicutes phyla (Eckburg et al., 2005), with faecalibacterium prausnitzii (F. prausnitzii), a species within the genus Faecalibacterium, being one of the most abundant, representing around 5–15% of the healthy human gut microbiome. F. prausnitzii is one of the most important indicators of gut health as well as the main butyrate-producing bacteria found in the intestine (Lopez-Siles et al., 2017). However, the diversity of the bacteria within the microbiota is affected by the environment, genetics, antibiotic use, and diet (Zhu et al., 2015). Acute dietary alteration within 24 h of initiation was related to inducing large microbial shifts (David et al., 2014). Many species of Clostridium, Fusobacterium, Bacteroides, Actinomyces, Propionibacterium, as well as the Peptostreptococci genera have been identified to be involved in proteolytic fermentation (Pugin et al., 2017). Proteolytic fermentation results in secondary metabolite production, which could positively or negatively affect the host (Macfarlane et al., 1988). To date, the related metabolites have not been identified with certainty due to the complexity of the gut content (Sridharan et al., 2014). However, comparable to fibre fermentation, the metabolites produced are short-chain fatty acids (SCFA). SCFAs are also accompanied by branched-chain fatty acids (BCFA), ammonia, amines, hydrogen sulphide, indoles and phenols (Macfarlane, Gibson and Cummings, 1992). SCFAs, which are butyrate, acetate and propionate (S. Macfarlane & Macfarlane, 2003), have been observed to mediate appetite hormone secretion. Tolhurst et al. (2012) demonstrated that SCFAs induce GLP-1 secretion mediated by the GPCR, free fatty acid receptor 2 (FFA2), which is present in the murine enteroendocrine L-cells *in vitro*. Furthermore, in 2015, it was demonstrated by Psichas et al. (2015) that propionate treatment stimulated the release of GLP-1 as well as PYY from primary murine cells via FFA2. Furthermore, a recent study suggested that plant-derived proteins may have a more favourable effect on the gut microbiome and, consequently, on the metabolic health of the host in comparison to meat-derived proteins (Bel Lassen et al., 2021).

The gut microbiome is also highly capable of biotransformation, consequently producing a wide variety of physiologically active compounds that may affect the host (Thursby and Juge, 2017). These metabolites, such as the above-described SCFAs, are key in signalling between the GI tract and the CNS and may regulate physiological and pathological processes (Carabotti et al., 2015). Levels of SCFAs have been linked to mental health states, with patients experiencing major depressive disorder having reduced SCFA levels in their stool, urine, and plasma compared to nondepressed controls (Caspani et al., 2019; Averina et al., 2020). Also, increased levels of SCFAs following prebiotic consumption have been linked to a reduction in depressive and anxious behaviours in mice (Alagiakrishnan and Halverson, 2021). Reduced levels of SCFAs have also been indicated in neurodegenerative and cerebrovascular illnesses, such as Huntington's disease, Alzheimer's disease, Parkinson's disease, and stroke (Block et al., 2010; Ho et al., 2018; Sadler et al., 2020; Metzdorf, 2021). There is bidirectional communication between the gut and the CNS, which can be mediated by the gut microbiome metabolites binding to receptors in the host brain, stimulating the vagus nerve in the gut, modifying central neurotransmission or influencing neuroinflammation (Kenji, 2022). As previously mentioned, SCFAs express their effects on the host by binding to FFA2 receptors. Further metabolites produced by the gut microbiome are neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin and dopamine (Stephenson and Rowatt, 1947; Yano et al., 2015; Yunes et al., 2020). It has been suggested that gut neurotransmitters could potentially influence brain functionality by influencing the immune system, with serotonin activating immune cells and GABA reducing intestinal inflammation (Chen, Xu and Chen, 2021).

1.4. Obesity-related changes in appetite regulation

Metabolic hormone levels are negatively affected by obesity, which may present additional challenges when attempting weight loss or maintaining a lower body weight. Adiposity signalling hormone leptin presents stable levels in the circulation in the short term. However, it was shown that after just 1 week of a moderate energy deficit, leptin decreased by 54% and remained low after 6 and 12 weeks, and it was associated with increased hunger, appetite and consequently food intake in individuals (Keim, Stern and Havel, 1998).

Furthermore, although there are lower levels of ghrelin observed in obese patients compared to lean individuals (Shiiya et al., 2002), following weight loss, circulatory ghrelin levels increase, potentially contributing to regaining the lost weight (Avid et al., 2002). Additionally, ghrelin levels are suppressed by food intake in obese individuals,' possibly delaying the feeling of satiation and leading to overeating (English et al., 2002).

Moreover, PYY and GLP-1 were both found to have reduced rates of post-prandial secretion in obese patients (Batterham et al., 2003). However, the affected individuals retained sensitivity to the externally administered peptides (Wynne et al., 2005). Obese patients treated by gastric bypass have elevated PYY levels, possibly contributing to their appetite loss (Näslund et al., 1997; Bartolomé et al., 2002). GLP-1 levels also seem to increase and reach levels observed in healthy individuals after weight loss (Verdich et al., 2001).

1.5. Treatment approaches to obesity management

Obesity management strategies are based on the concept of net deficit in energy leading to weight loss. There are numerous approaches in practice with the same aim to generate a net energy deficit over a sufficient period, resulting in weight loss (Hall et al., 2011).

1.5.1. Altering macronutrient composition of diet

Fat, carbohydrates, and protein are the essential macronutrients in the human diet with varying energy content. Fat is the most energy-dense macronutrient, with 9 kcal per gram, while carbohydrates and proteins provide 4 kcal per gram each (Ruban et al., 2019).

Due to fat being the most energy-dense nutrient, it has become the principal target of weight loss strategies, leading to the creation of low-fat diets. Although low-fat diets tend to lead to weight loss when followed over a sufficient time, they were found to be not more effective than any other dietary weight loss intervention (Tobias et al., 2015).

Another dietary intervention is a low-carbohydrate diet, which is further categorised into low and very low-carbohydrate diets depending on the amount of carbohydrate consumed daily. Low-carbohydrate diets seem to be beneficial for weight loss, possibly resulting in a mean 12% weight reduction after one year of compliance (Hallberg et al., 2018). A very low-carbohydrate diet, also termed a ketogenic diet, was also demonstrated to be beneficial for the management of T2D with increased chances of partial or potentially complete remission compared to low-fat diets (Merrill et al., 2020). However, low-carbohydrate diets are a source of concern when assessing the daily intake of vitamins and fibres due to the reduced fruit and vegetable inclusion (Merrill et al., 2020). Additionally, adherence to this diet seems to be challenging due to its restrictive nature (Kumar et al., 2022).

Lastly, protein, the macronutrient observed to have the highest satiety effect, is consumed in increased amounts in high-protein diets. The rationale of high protein diets is to reduce the consumption of other, more energy-dense or less satiating fatty or sugary food items by inhibiting appetite due to the appetite regulatory characteristics of protein (Ruban et al., 2019). A systematic review and meta-analysis studying the effects of higher versus lower-protein diets on health outcomes suggested that higher-protein diets were more favourable compared to lower-protein diets. The meta-analysis at 3 months found a greater weight loss of 1.21kg (CI 1.88 to 0.57), a greater decrease in BMI (0.51kg/m^2, CI 0.77 to 0.26) and a greater loss in waist circumference (1.66cm, CI 2.66 to 0.62) in higher compared with lower-protein diets (Santesso et al., 2012).

1.5.2. Energy restricted diet

Energy-restricted diets aim to directly limit kilocalorie (kcal/energy units) consumption. With low and very low-calorie diets (LCD and VLCD), daily energy intake is restricted to 800 kcal to 1600 kcal and less than 800 kcal, respectively (Ruban et al., 2019). A meta-analysis of six randomised trials found that, although VLCD induces significantly greater weight loss short-term compared to LCD (16.1 ± 1.6% vs 9.7 ± 2.4% of initial weight; p = 0.0001), there was no significant difference in weight loss long-term after weight regain (6.3 ± 3.2% vs 5.0 ± 4.0%; p > 0.2) (Tsai and Wadden, 2006). Therefore, the current National Institute for

Health and Care Excellence (NICE) guidelines recommendation is to follow a low-calorie diet approach for sustainable weight loss with a 600 kcal/day deficit (NICE, 2021).

1.5.3. Pharmacotherapy

As previously described, lifestyle changes can reliably lead to the loss and maintenance of only a modest 5% to 12% of the initial weight, requiring the use of other methods to manage obesity, such as the application of pharmacotherapy. Pharmacological targeting of body weight regulation has already been pursued in the early 19th century. The first antiobesity treatments used, such as thyroid hormones, mitochondrial uncoupler dinitrophenol or monoaminergic systems (e.g. fenfluramine and sibutramine), targeted energy expenditure. However, numerous drugs in these classes failed due to the side effects observed, such as detrimental psychotropic or cardiovascular effects, skin rashes, peripheral neuritis, and hyperthermia leading to sudden deaths (Christoffersen et al., 2022). Currently, only three pharmacological interventions are available via the NHS: Orlistat, Liraglutide and Semaglutide (NHS, 2023).

Orlistat, also known as tetrahydrolipstatin, is an orally administered irreversible gastric and pancreatic lipase inhibitor. Orlistat forms a covalent bond with the lipases at their active serine site, preventing dietary fat hydrolysation into absorbable free fatty acids and monoglycerols (Heck, Yanovski and Calis, 2000). Therefore, orlistat acts locally within the gut, inhibiting dietary fat breakdown and reducing fatty acid absorption by approximately 30% (Bansal and Khalili, 2022). Although the reduced fat absorption leads to a decrease in available energy, the increased remaining levels of fat within the intestine cause steatorrhea, faecal spotting, abdominal pain, and diarrhoea. Additionally, there is an increased risk of fat-soluble vitamin (A, D, E, K) deficiency development as their absorption is also inhibited by Orlistat. Nevertheless, the weight loss achieved after 6 months of therapy is about 6 kg (Bansal and Khalili, 2022).

Most recently, appetite hormones PYY and GLP-1 have become a key research focus in the pharmacological treatment of obesity. Although, there are some challenges present due to these peptides' vulnerability to digestion by gastric acid (Troke, Tan and Bloom, 2014) and their short half-life in systemic circulation (Hui

et al., 2002; Addison et al., 2011) as well as negative side effects observed after administration (Todd et al., 2003; Gantz et al., 2007), the recently developed pharmacological agents (Liraglutide, Semaglutide and Tirzepatide) may deliver 15% - 20% from baseline after 12 to 18 months of treatment (Jastreboff et al. 2022; Bergmann et al., 2023).

Liraglutide is a once-a-day, subcutaneously administered GLP-1Ras, acting by mimicking the effects of GLP-1 in increasing satiety and reducing appetite (Ruban et al., 2019). Liraglutide may lead to weight loss between 5% and 10% of initial body weight and has been demonstrated to be more effective than orlistat (Mehta, Marso and Neeland, 2017). The majority of the side effects are gastric-related, such as nausea, diarrhoea, and vomiting (Mehta, Marso and Neeland, 2017).

Another novel pharmacological weight loss agent approved for use within the NHS is Semaglutide (Department of Health and Social Care (DHSC), 2023). Like Liraglutide, it is a potent GLP-1 analogue. However, due to its longer half-life of 7 days, it only requires once-weekly subcutaneous administration (Wilding et al., 2021). Semaglutide is available for individuals diagnosed with obesity or being overweight with weight-related comorbidities (Wilding et al., 2021; Bergmann et al., 2023). It was observed that Semaglutide reduces hunger and energy intake and increases satiety and fullness. Semaglutide also seems to be more effective than Liraglutide, with mean weight losses of 14.9%-17.4% in participants without diabetes (Bergmann et al., 2023). Semaglutide was also observed to improve cardiometabolic risk factors, physical function, and quality of life (Bergmann et al., 2023).

Although the above-described pharmacological agents are available on prescription from the NHS, Orlistat is only available to individuals with BMI ≥ 30 or BMI ≥ 28 with obesity-related conditions such as T2D, while Liraglutide and Semaglutide are accessible only for patients with a BMI ≥ 35 with non-diabetic hyperglycaemia and with increased risk of developing cardiovascular diseases (NHS, 2019). Additionally, weight loss support by these medications is considered only after a low-calorie diet combined with a regular exercise regimen has been unsuccessful in achieving a significant weight reduction (NHS, 2019).

Although the use of the above-described pharmacological agents may result in significant weight loss, continuous long-term therapeutic use has been shown to be necessary for sustained weight loss, with a 6.9% increase in mean weight reported once Semaglutide was swapped for placebo following a 1-year intervention (Bergmann et al., 2023).

The most recent and most effective pharmacological treatment to date approved by the Food and Drug Administration (FDA) for obesity is Tirzepatide, a peptide developed from the native GIP sequence that acts as an agonist of both the GIP and GLP-1 receptors (Coskun et al., 2018). Animal studies demonstrated that Trizepatide's binding capacity to GIP receptors was comparable to that of native GIP; however, it is five times weaker than that of native GLP-1 to GLP-1 receptors (Coskun et al., 2018). Furthermore, preclinical and clinical data suggest a presence of synergistic effects on appetite regulation by the dual activation of GIP and GLP-1 receptors, leading to significantly greater weight loss compared to the use of GLP-1 receptor agonist only (Coskun et al., 2018; Frías et al., 2021). Jastreboff et al. (2022) showed clinically significant weight loss following the use of Tirzepatide for 72 weeks leading to mean percentage change of −15.0% (95% confidence interval [CI], −15.9 to −14.2), −19.5% (95% CI, −20.4 to −18.5), −20.9% (95% CI, −21.8 to −19.9) −3.1% and (95% CI, −4.3 to −1.9) with 5-, 10-, 15-mg weekly doses of Tirzepatide and placebo respectively with statistically significant differences (p <0.001) present for all comparisons with placebo in 2539 obese or overweight adults. Furthermore, significant improvements were observed in systolic and diastolic blood pressure, fasting insulin level, and lipid levels in participants receiving Tirzepatide (Jastreboff et al. 2022). Treatment with Tirzepatide, comparable to selective GLP-1 receptor agonists, was related to gastrointestinal side effects such as nausea, vomiting and diarrhoea (Frías et al., 2021). Tirzepatide was approved in the UK for the treatment of obesity by the Medicines and Healthcare products Regulatory Agency (MHRA) in November 2023 for adults with a BMI ≥ 30 or a BMI ≥ 27 with obesity-related conditions such as high blood pressure. As of today, Trizepatide is not available via the NHS (MHRA, 2023).

1.5.4. Surgery

The most effective treatment for obesity to date is bariatric surgery; however, its application is considered only as a last option when other weight loss attempts have failed (Colquitt et al., 2014). The requirement to access the surgery via the NHS is a BMI ≥ 40, or a BMI between 35 and 40, with obesity-related comorbidities such as T2D present (NHS, 2020). Bariatric surgeries are superior in the weight loss achieved, which is approximately 20 % to 30 % of pre-surgery weight, as well as in the management of obesity-related conditions, such as T2D, with remission observed in 72% of the patients 2 years post-surgery (Sjöström et al., 2007). The success of bariatric surgery, namely Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG), is foremost dedicated to the increased postprandial circulatory levels of PYY and GLP-1, leading to increased satiety (Ruban et al., 2019). RYGB involves the creation of a small gastric pouch (~30 mL) that is connected to the proximal jejunum (Olbers et al., 2003). Therefore, nutrients bypass most of the stomach, the entire duodenum, and the proximal jejunum, leading to rapid delivery to the jejunum and ileum, where the highest number of enteroendocrine (primarily GLP-1 secreting) L-cells are located, triggering the enhanced secretion of anorexigenic gut hormones (Brethauer, Hammel and Schauer, 2009). With VSG, which involves the creation of a gastric sleeve reducing the stomach volume by about 75%, similar mechanistic effects are observed due to the rapid emptying of the high-pressure gastric remnant (Akalestou et al., 2022). Although successful, this treatment is expensive, invasive and carries several side effects (Livingston, 2005; Malinowski, 2006). Patients post-surgery are required to take daily vitamin and mineral supplements to prevent malnutrition as their absorption is reduced from the gut (Schweitzer and Posthuma, 2008). The development of gallstones, which are small stone-like formations usually made of cholesterol in the gallbladder, is also common within the first year after the operation, possibly requiring surgery for removal (Brandão De Oliveira, Chaim and Da Silva, 2003). In addition, scaring and reduced blood flow to the affected area can cause narrowing or blockage of the gut, leading to difficulty swallowing, vomiting, abdominal pain and constipation (Kalarchian et al., 2014). Post-operative reviews also highlighted that there is an increased risk of depression, and suicidal thoughts and attempts in patients undergoing bariatric surgery (Müller et al., 2019).

1.5.5. Dietary replacements and supplements to aid weight loss

A dietary approach for appetite regulation and weight reduction seems more preferable due to costs, inaccessibility, and the several side effects that may occur with surgery or pharmacological management of obesity. However, as previously presented, achieving a net energy deficit that results in weight loss and the maintenance of the new body weight is challenging, partially due to changes in appetite regulatory hormone levels related to obesity or following weight loss. Therefore, a large number of supplements and meal replacements have been developed and are marketed for weight loss and weight maintenance. These dietary supplements usually contain a single or a combination of nutrients (Dwyer, Allison and Coates, 2005). Although the scientific evidence supporting the efficacy of these supplements is sparse at present (Dwyer et al., 2005), meal replacement therapies seem to be successful in supporting weight loss, with 2.1% greater weight reduction compared to a conventional calorie control diet at 40 weeks (Ruban et al., 2019). Additionally, the increase in protein (Luhovyy et al., 2007) and fibre in the daily diet also showed the potential to contribute to the management and prevention of obesity (Slavin and Green, 2007).

The increase in fibre intake within the diet was suggested to have satiety-enhancing effects (Slavin and Green, 2007). Fibre is a group of plant-derived carbohydrates resistant to digestion and absorption in the intestine. They are polymers of three or more monomeric units (Stephen et al., 2017). Fibres may be categorised based on their characteristics, such as solubility, viscosity and fermentation. Based on the fibres' soluble characteristics (dissolution in water), they can be subtyped into soluble and insoluble fibres. Insoluble fibres (e.g., cellulose or lignin) stimulate increased secretion from the gut mucosa and peristalsis, leading to increased gut transit time. Soluble fibres can be further divided into viscous (psyllium, β-glucan, pectin, gum) and non-viscous (wheat dextrin, inulin) fibres. Viscosity represents the fibre's capability to form a gel with water. Viscous soluble fibres were shown to slow digestion and absorption of nutrients, resulting in increased levels of nutrients reaching the distal part of the gut, leading to enhanced GLP-1 secretion, consequently reduced appetite and increased satiety (McRorie, 2015).

Other common supplements are proteins, which are irreplaceable components of the human diet as they are macronutrients essential for the development and sustainment of life. Proteins are also referred to as the building blocks and the molecular machinery of the human body, responsible for most biological structures and functions (Chou, Affolter and Kussmann, 2012). Additionally, proteins are utilised for the production of hormones, enzymes and haemoglobin. Dietary proteins consist of AAs, and there are twenty AAs identified that are required for the maintenance of a healthy state. Nine of these AAs are termed essential, meaning that they cannot be synthesised by our body and need to be consumed in the diet. Inadequate levels of any of the essential amino acids (EAAs) may compromise tissue growth and maintenance (Weijzen et al., 2021). Therefore, it is important to consume a sufficient amount of high-quality proteins, around 0.8g per kg of body weight for adults per day, to preserve health (Department of Health, 1991).

High protein meals were demonstrated to increase PYY 3-36 levels significantly (Batterham et al., 2006) as well as GLP-1 expression compared to carbohydrate or fat (Van Der Klaauw et al., 2013). It is believed that different proteins have unique characteristics depending on the source of protein, AA content and absorption kinetics. Thus, proteins from different sources might have different effects on satiety and appetite regulation (Bendtsen et al., 2013). Dairy products that are a complete source of protein (containing all EAAs) and must be obtained from the diet were found to have promising effects on body weight and composition (Gilbert et al., 2011). Dairy proteins, particularly whey and casein, have been shown to reduce appetite and increase satiety (Pal et al., 2014) via the ileal brake effect (W L Hall et al., 2003) as well as via the involvement of enteroendocrine hormones (PYY, GLP-1 and CCK) secretion (Fromentin et al., 2012). Whey contributes 20%, while casein contributes 80% to the total protein content of dairy protein. Casein and whey proteins were observed to have different physiological effects that were related to their AA constitution (Faghih et al., 2011; Pennings et al., 2011). Whey protein is high in branched-chain amino acids (BCAAs) and contains bioactive components such as glycomacropeptide (GMP) and α-lactalbumin (αlac). The αlac fraction makes up 20% of whey protein, and it has been hypothesised to have a major contribution to satiety inductions

as it has a high content of essential AA such as leucine, lysine and tryptophan (Chatterton et al., 2006). Tryptophan has a key role in satiety signalling in the brain as it is a precursor of the anorexigenic signalling molecule serotonin. Furthermore, leucine and lysine are ketogenic AAs, which have been observed to be involved in reduced appetite under ketogenic conditions (Gloaguen et al., 2012). Although casein is also high in tryptophan, Westerterp-Plantenga et al. (2009) indicated that it may not play an important role in appetite regulation on its own but rather in combination with other AAs. Some novel plant-based proteins also show potential in relation to satiety and appetite regulation. Zhu, Lasrado and Hu (2017) presented in 44 healthy women in a double-blind placebo-controlled crossover trial that the consumption of 15 mg or 30 mg potato protease inhibitor II (PI2) one hour prior to a standard breakfast meal resulted in significantly lower postprandial hunger and significantly higher postprandial fullness compared to placebo. Potato protease inhibitors are fractions of potato proteins, making up 50% of the total protein content (Waglay, Karboune and Alli, 2014). Additionally, a recent study showed in 9 healthy male participants in a single-blind, triple-crossover design that administration of brown rice protein isolate led to a significant increase in GLP-1 levels at 120 min in comparison to circulating levels observed at 30 min (p = 0.024) and at 60 min (p = 0.04) post-consumption. Although differences in GLP-1 levels were observed between whey vs rice proteins at 60 min after consumption (with significantly higher circulatory levels in the whey condition, p = 0.001), this difference was not evident in subjective appetite perception (Tiekou Lorinczova et al., 2021). Additionally, recent studies suggest that the choice of plants as a source of protein may be a more viable option for individuals with an increased risk of developing insulin resistance or diabetes due to lower insulinemic response as well as improved glucose maintenance observed after consumption compared to animal-derived proteins (He et al., 2013; Tiekou Lorinczova et al., 2021).

Furthermore, it has been observed that under most conditions, protein is more satiating than carbohydrate or fat (Westman et al., 2002; Astrup, 2005). This suggests that an increase in protein intake may promote satiety and, therefore, aid weight loss through reduced energy intake (Dit El Khoury et al., 2006). Increased satiety from protein has been observed in a single meal (Bloom et al.,

2006) and over 24 h (Leidy, Mattes and Campbell, 2007). Moreover, the AA composition of protein has also been suggested to affect the satiety effects of proteins (Bendtsen et al., 2013).

1.5.5.1. Protein Isolates

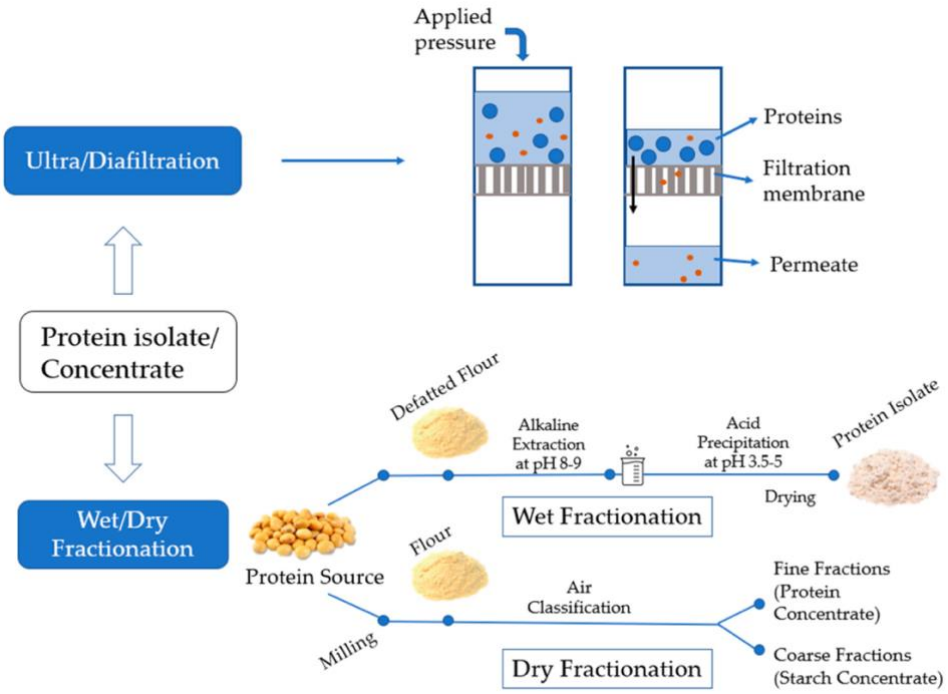


Figure 5. A schematic representation of protein fractionation from total to concentrates to isolates (Adapted from Nourmohammadi, Austin and Chen, 2023). Animal proteins, especially dairy proteins, are concentrated by ultrafiltration or diafiltration, followed by optional evaporation of the retentate prior to spray drying. For plant proteins, wet and dry fractionation has been widely used. Wet methods include mainly alkaline-isoelectric point precipitation, or water, salt, or acid extraction. By using acid or alkaline conditions, the protein recovery efficiency is high due to the increased solubility of proteins at pH values far away from their isoelectric point. Alkaline-isoelectric precipitation is more commonly used than other methods in food industries to extract proteins, followed by spray drying to produce a protein isolate.

Protein isolates, used as dietary supplements or ingredients in meal replacements, are highly concentrated protein fractions produced from whole food items by various processes (Sandberg, 2011). The protein extraction is based on the protein's solubilisation, followed by ultra- or microfiltration or isoelectric precipitation to recover the protein from the solution (Johnson et al., 2017). Protein isolates can also be produced by 'hydrophobic-out' or 'salting-out' processes (Owusu-Ansah and McCurdy, 1991). However, careful selection of the extraction process is crucial for maintaining the functionality of the protein

isolates, as although acidic precipitation and coagulation yield higher quantities of isolates, the functionality and the quality of the end product are often low (Cheng, Chen and Xiong, 2014). Protein isolates are often a by-product of mainstream manufacturing, such as whey protein and potato protein isolates, which are isolated from liquid whey, the waste product of cheese production (Pal et al., 2010), and potato fruit juice (PFJ), the waste product of starch production respectively (Pęksa and Miedzianka, 2021).

Whey protein is often used in meal replacement products or as a dietary supplement; however, in the past decade, the interest in plant-based protein products has increased substantially as more people follow vegan or vegetarian diets due to environmental or health awareness (Medawar et al., 2019). Various non-animal-derived protein sources are available for consumers on the market, such as soy protein, rice protein, pea protein or one that is relatively novel, potato protein (Tonsor, Lusk and Schroeder, 2022). However, while whey protein has been extensively researched (Patel, 2015), the differential appetite regulatory effects of plant-based proteins are not yet well understood. As it is evident from previous studies, proteins from different sources may have varying appetite regulatory effects (W. L. Hall et al., 2003; Pal and Ellis, 2010; Acheson et al., 2011). Thus, their metabolic effects should be fully elucidated to determine the most appropriate application of plant-based proteins.

1.5.5.1.1. Whey protein (WP)

As stated previously, WP is a by-product of cheese production, mostly from bovine milk. WP is extracted from the liquid whey that represents 20% of the milk (Faghih et al., 2011). Bovine whey production was estimated to be 168 million tons a year in 2020, of which about 50% is treated as waste, used for animal feed or as fertiliser, or disposed of without treatment (Pires et al., 2021). The other half is being processed to produce whey powder, WP concentrates or WP isolates (Kotoulas et al., 2019; Pires et al., 2021). WP isolation may be accomplished using several methods, such as microfiltration, ultrafiltration, cross-flow membrane filtration, ion exchange chromatography (IEC), followed by spray drying, in some cases in combination, depending on the desired concentration and quality of the WP (Tsakali et al., 2010).

Microfiltration and ultrafiltration are carried out at temperatures of around 55°C; however, while microfiltration applies membranes with a molecular weight cut-off (MWCO) of 200kDa, ultrafiltration membranes have a MWCO of 10 to 30kDa (Saxena et al., 2009). Microfiltration in the process of protein isolation is applied for the removal of phospholipoproteins, which were shown to hinder protein isolation due to their strong absorption on membrane materials (Saxena et al., 2009). Nevertheless, the ultrafiltration of the remaining permeate leads to close to complete elimination of fat and carbohydrates, namely lactose and minerals, resulting in an isolate with a protein concentration of possibly more than 90% after spray drying (Tunick, 2008). Additionally, IEC can be applied, following the ultrafiltration methods. This method is based on the ionic properties of WP. The major fractions of WP, which are β-lactoglobulin (β-lg), bovine serum albumin (BSA) and αlac, have a negative charge at the pH of liquid whey (pH 6.2-6.4), while the other protein fractions are positively charged at the same pH (Gerberding and Byers, 1998). Therefore, with the use of a negatively charged resin during IEC, the positively charged fractions, as well as some minerals, are selectively bound, resulting in protein isolated with protein content of up to 96% (Goodall et al., 2008). However, this fractionation of the WP, although leading to maximal protein yield, may affect its bioactivity due to the loss of fractions such as GMP, which is believed to have beneficial bioactive properties, such as antibacterial, prebiotic, remineralising, anti-tumoral, immune supportive, and digestion and metabolism modulatory activities (Córdova-Dávalos, Jiménez and Salinas, 2019). GMP was observed to increase CCK secretion (Beucher et al., 1994; Pedersen et al., 2000) in rats and was shown to reduce plasma ghrelin levels in mice (Sawin et al., 2016) as well as in individuals with phenylketonuria (MacLeod et al., 2010). Additionally, its supplementation on a high-fat diet was linked to lower body fat accumulation in rats (Xu et al., 2013).

Cross-flow membrane filtration is another microfiltration process carried out at a low temperature of 20°C in steel turbines containing ceramic filter membranes with pore sizes of 20 kDa diameter (Saxena et al., 2009; Barukčić, Božanić and Kulozik, 2015). The liquid whey passes over the porous membrane at varying pressures several times, leading to the eventual exclusion of fat and carbohydrates, yielding an isolate with a potential protein content of above 90%.

By the application of this method, the ratios of WP fractions present in the WP are close to those found in liquid whey (**Table 1**) due to no protein fraction exclusion and low temperature used during the process (Barukčić, Božanić and Kulozik, 2015).

1.5.5.1.2. Potato (*Solanum tubersosum*) protein (PP)

Potatoes are the world's fourth most important food crop after rice, wheat, and corn, with over 359 million metric tons of potatoes produced worldwide in 2020, according to the Food and Agriculture Organisation Statistics (FAOSTAT, 2020). As stated previously, a major portion of the harvested crop is used for starch production, with a by-product of PFJ. PFJ has been used as animal feed, fertiliser or for anaerobic biogas digestion (Pęksa and Miedzianka, 2021).

However, PFJ contains 1% - 2% (w/w) protein, rich in the essential AA lysine, methionine, threonine, and tryptophan and its well-balanced AA composition is comparable to WP (OECD, 2002) (**Table 2**). Additionally, PP has a high digestibility. Therefore, it has a superior nutritional value (Waglay and Karboune, 2016). PP fractions can be categorised into 3 main groups, which are patatin, protease inhibitors and high molecular weight fractions, all possessing specific bioactive functionalities such as antioxidant, angiotensin I-converting enzyme (ACE)-inhibitory, renin-inhibitory, lipid acyl hydrolase and anticancer activities (**Table 1**) (Pęksa and Miedzianka, 2021).

1.5.5.1.3. Rice (*Oryza sativa L.*) protein (RP)

Rice is a major staple food grain globally, with an estimated global annual production of 480 million metric tons (USDA, 2015). Milled rice consists of about 6-7% protein, while rice bran contains a high 11–15% protein (Juliano, 1985). RPs are divided into four main fractions: albumin, globulin, glutelin, and prolamin (**Table 1**) (Shih, 2003). Prolamin represents the major endosperm storage protein (Shewry and Halford, 2002), while it is a minor protein in all rice grain milling fractions. The dominant protein in rice is glutelin, which may represent more than 80% of the total protein in rice and is characterised by being completely insoluble in water but soluble under alkali conditions (0.1 M NaOH) (Luca et al., 2017).

Consumption of RP in comparison to WP as supplementation in resistance training practising individuals indicated no significant differences in post-exercise recovery and changes in body composition (Joy et al., 2013).

1.5.5.1.4. Pea (*Pisum sativum L.*) protein (PeP)

Pea is one of the most important legume crops produced worldwide, contributing 36% to total pulse production (Dahl, Foster, and Tyler, 2012). PeP isolate is a relatively new source of plant-based proteins used globally as a functional ingredient in the food industry (Boye, Zare, and Pletch, 2010). On average, peas contain 20–25% protein, with PePs (Dahl, Foster and Tyler, 2012) classified into four major classes: globulin, albumin, prolamin, and glutelin (Adebiyi and Aluko, 2011). Globulin and albumin are characterised as major storage proteins in pea seeds. Globulin can be subdivided into legumin, vicilin, and convicilin (**Table 1**), and it is soluble in salt solutions (Tzitzikasetal, 2006). The water-soluble albumin has metabolic and enzymatic characteristics with cytosolic functions and represents 18–25% of total protein content and is present in two types: PA1 (~10 kDa) and PA2 (~50 kDa) (**Table 2**) (McCarthy et al, 2016). PeP has a well-balanced AA profile with a high level of lysine (**Table 2**) (Schneider and Lacampagne, 2000; Nunes, Raymundo and Sousa, 2006).

PeP has also been used as a nutritional sports supplement due to its high BCAA content (Shimomura et al. 2004). A 12-week resistance training trial found that the supplementation with PeP promoted a greater increase in muscle thickness compared to placebo, and there was no significant difference observed between PeP and WP supplementation (Babault et al.,2015).

Table 1. Protein fractions of whey, potato, rice and pea protein isolates.

Source	Name	Molecular weight (kDa)	Function	Amount (% of total protein)
Whey	b-Lactoglobulin (b-lg)	~18	Source of essential and branched amino acids, enhanced retinol intake	50-55
	Immunoglobulins	150 - 1000	"Passive immunity"	10-15
	α-Lactalbumin (α-lac)	~14	Major protein in human milk, source of essential and branched amino acids, Ca+ binding protein, insulinotropic activities	20-25
	Bovine serum albumin (BSA)	69	Source of essential amino acids	5-10
	Glycomacropeptide (GMP) in sweet whey	7	Source of branched-chain amino acids Antibacterial, prebiotic, remineralising, anti-tumoral, immune supportive, and digestion and metabolism modulatory activities (Córdova-Dávalos, Jiménez and Salinas, 2019)	10-15
	Lactoferrin	78	Bactericidal, iron binding, tumour inhibiting activity	1-2
	Lactoperoxidase	89	Antimicrobial activity	0.5
	Lysozyme	15	Antimicrobial activity	0.002
Potato (*Solanum tubersosum*)	Patatin	40-45	Antioxidant, ACE-inhibitory, anti-inflammatory, and anti-cancer activities (Fu, Liu and Soladoye, 2020)	40
	Protease inhibitors	7-21	Digestion and metabolism regulatory activities (Zhu, Lasrado and Hu, 2017)	50
	High molecular weight proteins	>87	Unknown	10
Rice (*Oryza sativa L.*)	Albumin	16	Glucose absorption inhibitory (Ina et al., 2016) and Antioxidant activities (Wei et al., 2007)	5
	Globulin	46-52	Storage	12
	Glutelin	53-56	Storage	50-80
	Prolamin	10-16	Storage	5
Pea (*Pisum sativum L.*)	Legumin	330-400	Storage	6-24.5
	Vicilin	150-170	Storage	21-55
	Convicilin	~210	Storage	3.9-8.3
	PA1	~10	Storage	5-9
	PA2	~50	Unknown	10-20
	Lectins	~50	Protection against endogenous protease	2.5
	Lipoxygenases	n/a	Oxidation of fatty acids	<1
	Serine/trypsin protease inhibitor	10-16	Antitrypsic activity	<2

Table 2. Typical AA profile expressed as g per 100 g of product.

Amino Acids (AA)	Whey Protein Isolate	Potato Protein Isolate	Rice Protein Isolate	Pea Protein Concentrate
Histidine *	1.7	1.7	2.09	1.99
Isoleucine *, +	6.4	4.3	4.40	4.05
Leucine *, +	10.6	9.3	8.21	6.80
Lysine *	9.6	6.6	3.21	6.11
Methionine *	2.2	2.4	2.66	0.85
Phenylalanine *	3.0	5.5	5.38	3.86
Threonine *	6.7	6.2	3.57	3.05
Tryptophan *	1.4	1.0	1.12	0.79
Valine *, +	5.9	4.7	6.18	4.28
Arginine	2.1	3.7	7.64	6.84
Cysteine	2.2	0.7	2.09	0.85
Glutamic Acid/Glutamine	18.1	10.7	17.22	13.32
Glycine	1.4	3.9	4.33	3.28
Proline	5.5	4.0	4.49	3.55
Tyrosine	2.6	5.3	4.84	3.86
Alanine	5.0	5.2	5.67	3.44
Aspartic Acid/Asparagine	11.0	10.5	9.33	9.42
Serine	4.6	4.8	4.75	3.88
Total EAA	**47.5**	**41.7**	**38.82**	**31.78**
Total BCAA	**22.9**	**18.3**	**18.79**	**15.13**

1.6. Protein digestion and peptide sensing in the gut

1.6.1. Oral phase

Protein ingestion starts with mastication and salivation in the oral cavity (Liu et al., 2017). This mechanism changes the structure of food considerably, breaking it down into smaller, ideally <2mm particles while mixing with saliva, wetting and lubricating the food, creating a bolus ready to be swallowed (Helkimo, Carlsson

and Helkimo, 1978; Peyron, Mishellany and Woda, 2004). Saliva is a complex watery fluid produced by the parotid, the submandibular and the sublingual glands that contains mainly electrolytes (e.g. sodium, potassium, calcium, phosphate and bicarbonate) and proteins (e.g. immunoglobulin A, α-amylase, lysozyme, lactoferrin and mucosal glycoproteins) (Humphrey and Williamson, 2001; Aken, Vingerhoeds and Hoog, 2007; Almeida et al., 2008). Although salivary α-amylase plays a crucial role in carbohydrate digestion, contributing as much as 50% of starch digestion within 20 to 30 seconds, it does not significantly affect protein breakdown (Vingerhoeds and Hoog, 2007; Almeida et al., 2008; Bornhorst, Hivert and Singh 2014).

1.6.2. Gastric phase

The enzymatic digestion of protein starts in the stomach with pepsin, the only proteolytic enzyme in the human stomach, secreted from the gastric chief cells (Minekus et al., 2014). Pepsin is an endopeptidase that facilitates the cleavage of protein bonds in the centre of the peptide chains, producing oligopeptides (Smith and Morton, 2010). This proteolytic breakdown occurs in the lower part of the stomach, the antrum, where the optimal pH of 2 to 4 required for pepsin activity is achieved by hydrochloric acid secretion from the parietal cells (O'Connor and O'Moráin, 2014). The digestion within the antrum is also facilitated by the gastric mixing termed antral movements (Tyssandier et al., 2003; O'Connor and O'Moráin, 2014). Gastric digestion is responsible for the breakdown of around 15% of dietary proteins (Smith and Morton, 2010).

Furthermore, a crucial role of the stomach in the process of digestion is the gradual release of the gastric content, the chyme, into the duodenum, the most proximal part of the intestine (Lin et al., 2005; O'Connor and O'Moráin, 2014; Brodkorb et al., 2019). The transit time of the food from the stomach to the intestine, referred to as gastric emptying, may be affected by various factors, such as food consistency or nutrient content, varying between 30 min with liquids containing low levels of protein (Lin et al., 2005) and up to 4h with a typical western type solid meal (Dressman et al., 1990; Gardner, Ciociola and Robinson, 2002; Tyssandier et al., 2003). High protein content liquid was shown to have a gastric emptying time at around 120 min and 50% gastric emptying time of

40 ± 7 min and 50 ± 8 min assessed in younger (27 ± 2 years) and older (72 ± 1 years) obese (BMI > 33 kg/m^2) men respectively, with no significant difference between the groups (Oberoi et al., 2020).

1.6.3. Intestinal phase

The chyme in the duodenum is neutralised with bicarbonate, with the pH gradually increasing to around 6.5 and mixed with pancreatic juice and bile salts (Dressman et al., 1990). The pH rises along the length of the intestine, reaching around 7.5 at the distal end of the ileum (Dressman et al., 1990; Brodkorb et al., 2019). The increase in pH deactivates pepsin and enables peptide cleavage by the proteolytic enzymes and endopeptidases present in the pancreatic juice: trypsin, chymotrypsin and elastase (Li et al., 2020; McQuilken, 2024). The pancreatic juice also contains two carboxypeptidases (A and B) (Smith and Morton, 2010). Almost all (about 90%) of ingested protein is digested and absorbed in the small intestine (Rodríguez-Romero et al., 2022; McQuilken, 2024), and the pancreatic enzymes facilitate the degradation of at least 50% of the ingested protein in the duodenum (McQuilken, 2024). Proteolytic digestion is additionally facilitated by brush border enzymes, such as leucine aminopeptidase, tetrapeptides, dipeptidyl aminopeptidase, resulting in the formation of tetrapeptides, tripeptides and dipeptides, and some AAs (Fairweather et al., 2012; Ozorio et al., 2020). The proteolytic digestion was additionally demonstrated to be affected by the presence of bile salts by accelerating hydrolysis by pancreatic proteases (Gass et al., 2007).

The formed tripeptides, dipeptides and AAs are transported into the epithelial cells, enterocytes, by specific transporters, where the small peptides are cleaved to AAs by tri- and dipeptidases residing in the cytosol of the cells (Smith and Morton, 2010; Rodríguez-Romero et al., 2022; McQuilken, 2024). Small peptides were shown to be taken up by the proton-coupled Peptide transporter 1 (PEPT1). The transport of peptides via PEPT1 may lead to cell membrane depolarisation, resulting in Ca^{2+} influx from voltage-gated Ca^{2+} channels (Lu, Gribble and Reimann, 2021; Santos-Hernández, Reimann and Gribble, 2024). Comparably, the uptake of free AAs via the sodium-dependent neutral AA transporters (B(0)AT-1 and B(0)AT-2) was also suggested to lead to the depolarisation of the

cell membrane, resulting in extracellular Ca^{2+} influx (Santos-Hernández et al., 2024).

Additionally, AAs are sensed by a number of GPCRs found on the basolateral side of enteroendocrine cells (EECs). The proposed GPCRs involved in AA sensing are the calcium-sensing receptor (CASR), umami taste receptors (TAS1R1/TAS1R2), GPR142, GPR92 and GPRC6A (Fraga, Serrão and Soares-da-Silva, 2002; Fraga, Pinho and Soares-Da-Silva, 2005; Acar et al., 2018; Santos-Hernández, Reimann and Gribble, 2024). Although there are controversies on the importance of the specific GPCRs in protein sensing within the literature, it is suggested that the binding of AAs to these receptors activates protein kinase C, initiating intracellular Ca^{2+} release from the endoplasmic reticulum via the phosphatidylinositol pathway (Cho et al., 2014; Sánchez-Fernández et al., 2014; Acar et al., 2018; Lu, Gribble and Reimann, 2021; Rune E. Kuhre et al., 2021). The increase in intracellular Ca^{2+} from extracellular and intracellular sources was suggested to lead to GI hormone vesicle exocytosis (Acar et al., 2018; Lu, Gribble and Reimann, 2021; Watkins, Koumanov and Gonzalez, 2021).

1.7. Current in vitro intestinal models used to study nutrient sensing and gut hormone secretion

The current understanding of the mechanism and dynamics related to digestion, absorption, and gut hormone secretion within the intestine is greatly attributed to *in vitro* studies (Yamada and Cukierman, 2007; Li et al., 2020). *In vitro* studies mainly use primary cell cultures isolated from human or animal intestinal tissue or continuous cell lines derived from immortalised cells (Arango et al., 2013; Segeritz & Vallier, 2017). Immortalised cell lines have the advantage of lower cost, less complicated utilisation with existing previous knowledge of their required growth factors, as well as providing consistent and reproducible study results (Arango et al, 2013; Kapałczyńska et al., 2018). Moreover, the distinct characteristics of these cell lines enable their extensive utilisation for specific research applications such as pharmacological, medical, biological or nutritional (Mattei et al., 2014; Ponce de León-Rodríguez et al., 2019). Cell lines commonly

used in research related to nutrient sensing, gut hormone secretion and expression, and intestinal physiology are STC-1, GLUTag, NCI-H716, and Caco-2 (Simon-Assmann et al., 2007; Verhoeckx et al., 2015).

1.7.1. STC-1 cell line

The intestinal secretin tumour cell line (STC-1) is derived from a genetically induced murine enteroendocrine tumour within the duodenum (Rindi et al. 1990). This cell line possesses the characteristics of numerous enteroendocrine cells, therefore expressing as well as secreting many gut hormones (e.g. CCK, GIP, PYY, GLP-1&2 and oxyntomodulin) (Rindi et al. 1990; Hand et al. 2013).

STC-1 cells grow into a heterogeneous enteroendocrine cell line, forming a monolayer in a standard growth medium with a relatively long cell cycle of about 54 hours (Rindi et al. 1990). Gut hormone expression and secretion in this cell line can be induced by nutrients (e.g. monosaccharides and AAs), neuronal (e.g. neuropeptides hypocretin and GABA), as well as hormonal cues (e.g. bile acid and leptin) (McCarthy et al., 2015). This further supports the comparability of the STC-1 cell line to the *in vivo* epithelium, as hormonal and neuronal stimuli also induce gut hormone expression and secretion in the human GI tract (Engelstoft et al., 2008).

It has been suggested that this cell line provides a reliable and reproducible enteroendocrine cell model. However, variations are observed between laboratories and experiments due to exhibiting significant heterogeneity in morphology (confocal microscopy revealed heterogenous distribution of α-synuclein and the Serine-129 phosphorylated form (pS129)), physiology (the sensitivity of STC-1 cells to the neurotransmitter GABA was shown to vary significantly from cell to cell, due to the heterogeneous density and expression of $GABA_A$ receptors in this cell line), and genetics (only about ~7% of STC-1 cells has been demonstrated to express GIP) (McCarthy et al., 2015; Kuhre et al., 2016; Gorecki et al., 2025).

Nonetheless, this cell line has been used for nutrient interaction (Mangel et al. 1994; Hand et al. 2010; Wang et al. 2011), cell differentiation (Ratineau et al.

1997), immunology (Palazzo et al. 2007) as well as cancer research (Bollard et al. 2013) studies.

1.7.2. GLUTag cell line

The murine GLUTag cell line is derived from a genetically induced colonic endocrine cancer tumour (Drucker et al. 1994). This cell line is a GLP-1 secretory enteroendocrine cell line very closely mimicking the enteroendocrine L-cells' characteristics. GLUTag cells in standard growth medium supplemented with glucose and foetal bovine serum (FBS) form a polarised monolayer in 3 to 4 days. For GLP-1 secretion assays, it is advised to plant the cells on Matrigel® to avoid detachment from the basal surface, following repeated washing steps (Gil-Lozano & Brubaker, 2015).

This cell line has been extensively used to study nutrient as well as other hormonal cues stimulated GLP-1 secretion (Gil-Lozano & Brubaker, 2015). GLUTag cells have been shown to be highly sensitive to glucose stimulation when grown in a low glucose concentration media (<1 mmol/L), resulting in high levels of GLP-1 secretion; however, growing these cells in growth media with a higher glucose concentration (>5 mmol/L) diminishes the sensitivity of these cells to further increase in glucose concentration (Reimann and Gribble 2002). This observation is unlike the mechanism present in *in vivo* L-cells, where GLP-1 secretion stimulated by glucose is dose-dependent (Reimann et al., 2008). Lipids and proteins mediate GLP-1 secretion within the GLUTag cells in a comparable manner to primary L cell cultures (Hirasawa et al., 2005; Gameiro et al., 2005; Iakoubov et al., 2007; Tolhurst et al., 2011). Furthermore, bile acid (Dong & Brubaker, 2012), the parasympathetic nervous system (using muscarinic agonists) and GIP (Brubaker, Schloos & Drucker; 1998), leptin (Anini & Brubaker, 2003) as well as insulin (Lim et al., 2009) have been shown to induce GLP-1 expression as well as secretion in these cells. These characteristics further support this cell line's relevance to *in vivo* L cells, as physiological and hormonal stimuli mediate the early GLP-1 secretion within the GI tract, as the nutrient-related secretion is much delayed due to the L cell's predominant position in the distal part of the intestine (Dong and Brubaker 2012).

GLUTag is the most widely utilised and accepted cell line for the study of GLP-1 regulation as their response to cues, and the expressed protein profiles are closely comparable to the *in vivo* L cells (Tolhurst et al. 2012). However, as they lack a sufficient amount of GPR43, they are not an acceptable model for studying SCFAs-induced GLP-1 secretion, which is mediated via GPR43 receptors (Tolhurst et al. 2012).

1.7.3. NCI-H716 cell line

NCI-H716 cells were isolated from fluid accumulated in the abdominal cavity due to colorectal adenocarcinoma (Park et al., 1987), and they express characteristics of endocrine as well as mucus-secreting cells (de Bruine et al., 1993). Their endocrine characteristics are most comparable to L cell-like physiology, with a significant expression and secretion of active GLP-1 and PYY (Reimer et al., 2001; Jang et al., 2007).

These cells have characteristic spherical shapes when grown in suspension culture, forming occasional clumps of undifferentiated cells (Gagnon & Brubaker, 2016). It has been suggested that mounting them on a layer of type IV collagen and heparan sulphate proteoglycan in extracellular media containing basic fibroblast-growth factor can enhance their endocrine phenotypic expression, therefore inducing differentiation (de Bruine et al., 1993; Reimer et al., 2001).

The NCI-H716 cell line has been extensively used to study the effects of different nutrients on GLP-1 expression and secretion, as currently, it is the only available, suitable human *in vitro* cell line model (Gagnon & Brubaker, 2016). However, this cell line might be heterogeneous as it is not single-cell cloned (Park et al., 1987).

1.7.4. Caco-2 cell line

Caco-2 cells are derived from human colorectal adenocarcinoma and function as undifferentiated large intestinal cells or can spontaneously differentiate to resemble a small intestine-like phenotype with enterocyte-like absorptive properties (**Figure *6***). These cells express intestinal-like brush border structure on their apical surface as well as several receptors, transporters, enzymes (Antunes et al., 2013) and appetite hormones (Kosicka, Renshaw and Zariwala, 2013),

making them an ideal candidate as a small intestinal model (Ponce de León-Rodríguez, Guyot and Laurent-Babot, 2019).

Caco-2 cells are grown as confluent polarised monolayers and have been extensively used to study absorptive and transport kinetics (Smetanová et al., 2011; Zhang et al., 2013), bacterial adhesion/invasion or the effects of prebiotic supplementation on intestinal microbiota (Pearce et al., 2018). In fact, this cell line is the most widely used and accepted standard predictor model of drug permeability in the human intestine by regulatory authorities and pharmaceutical companies (Hidalgo, Raub and Borchardt, 1989; Hilgers, Conradi and Burton, 1990).

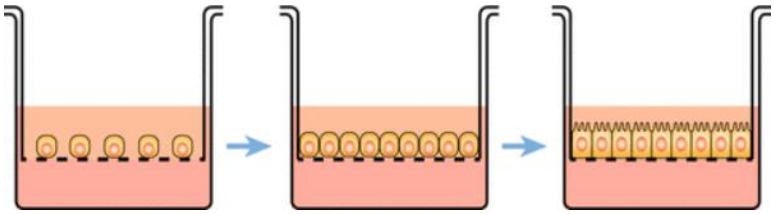


Figure 6. Differentiation of Caco-2 cells on a tissue culture insert (Adapted from Lea, 2015). Caco-2 cells differentiate spontaneously after reaching confluency (middle picture). Following a total incubation period of 21 days, microvilli develop on the apical surface of the cell monolayer, which is comparable to enterocytes.

The use of Caco-2 cells for appetite studies is currently scarce in the literature due to their novelty in this specific field. The expression of the appetite hormone PYY in the Caco-2 cell line was first demonstrated by Kosicka, Renshaw and Zariwala (2013), following 24 h treatment with L-Leucine, L-Arginine and glucose. Subsequently, Shobatake et al. (2019) showed that intermittent hypoxia increased PYY, GLP-1 and neurotensin (NTS) expression as well as secretion in Caco-2 following a 24 h exposure time. These observations indicate that, Caco-2 cells are a potential candidate as an enteroendocrine as well as an enterocyte model (Ponce de León-Rodríguez et al., 2019).

1.8. Scope of project

Recent research has focussed on gaining a better understanding of the dynamics of appetite regulation. Although the knowledge within this area is expanding, the mechanism of action of nutrient-induced satiety is not fully understood. Furthermore, while many studies indicate a positive association between increased protein consumption and maintenance of body weight as well as weight loss, the bioactive components responsible for increased satiety of these nutrients have not been identified with certainty.

The overall aim of this study is to seek to understand and explain the potential mechanisms underlying milk and plant-based proteins' specific effects on satiety and body-weight regulation to provide information for the generation of novel nutrient-based appetite suppressant approaches for diet modification.

To achieve the purpose of the study, the following objectives will be explored:

1. To establish the relative digestibility of various types of protein fractions (whey, potato, rice and pea protein isolate, and their blends in ratios of 50:50 and 30:70) following *in vitro* digestion
2. To establish the relative effect of various types of protein fractions (whey, potato, rice and pea protein isolate) on the dynamics of PYY and GLP-1 synthesis and secretion
3. To establish the relative effect of protein blends (whey, potato, rice and pea protein isolate) in varying ratios (50:50 and 30:70) on the dynamics of appetite regulation via PYY and GLP-1 synthesis and secretion
4. To investigate the mechanism of dietary protein-mediated appetite hormone signalling via the gut-brain axis

2. CHAPTER 2 – Materials and research methodology

2.1. Materials

The following protein isolates were used in this project: WP (Impact Whey Protein Isolate, Myprotein, Inc, Manchester, UK), PP (Solanic®200, ProteinmiXer.com®, Bonn, Germany), RP isolate (Organic Oryzatein® Silk 90, Axiom Foods/Growing Naturals, Inc., Los Angeles, CA, USA) and PeP (Puris™ Pea 870 H, Cargill, Inc., Minneapolis, MN, US).

Unless otherwise stated, all chemicals were analytical grade and cell culture grade where applicable. Caco-2 and STC-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Haemoglobin (from bovine blood, ref H2625), Pepsin (from porcine gastric mucosa, ref P7000), Trichloroacetic acid (TCA, ref T6399), Porcine pancreatin (ref P7545), p-Toluene-sulfonyl-L-arginine methyl ester (TAME, ref T4626), Trizma® hydrochloride (ref T3253), PEFABLOC(R) SC (ref 76307), $CaCl2(H_2O)_2$ (ref C7902), Potassium chloride (KCl, ref P9541), Potassium phosphate monobasic,*anhydrous (KH_2PO_4, ref P0662), Sodium chloride (NaCl, ref S7653), Magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$, ref M2670), Sodium hydroxide (NaOH, ref 30620), Ammonium carbonate ($(NH4)_2CO_3$, ref 207861-25G) were ordered from Merck Life Science UK Limited, Gillingham, Dorset, United Kingdom. Dulbecco's phosphate buffered saline (DPBS), Gibco™ Heat Inactivated Fetal Bovine Serum (HI-FBS, ref 11550356), Gibco® Antibiotic-Antimycotic (100X) (Anti-anti, ref 11570486), Gibco™ HEPES (1M) (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, ref 11560496), Gibco™ L-Glutamine (200 mM) (ref 11500626), Dulbecco's Modified Eagle Medium (DMEM) Glutamax® (ref 11584456), Gibco™ TrypLE Express Enzyme (1X) with Phenol Red (ref 10043382), Gibco™ Minimum Essential Medium (MEM with no glutamine or phenol red, ref 12057589), Gibco™ Hanks' Balanced Salt Solution (10X) (HBSS with no calcium, magnesium or phenol red, ref 15356878), Gibco™ Trypan Blue Solution, 0.4% (ref 15250061), Corning™ Dimethyl Sulfoxide (DMSO, ref 15303671), Halt protease inhibitor cocktail (100X) (ref 11834101), Hydrochloric acid solution 5M (HCl, ref 12963544), Tris base (ref 10376743), Thermo Scientific™ Thiazolyl Blue tetrazolium bromide, 98% (MTT, ref 11312727) were supplied by Fisher Scientific

UK Limited, Loughborough, Leicestershire. The Pierce BCA protein assay kit (ref 10678484) was also purchased from Fisher Scientific UK Limited, Loughborough, Leicestershire.

Clinical consumables, Caresite Single Extension (ref BN470108-01), Tegaderm IV Sterile Dressing 7cmx8.5cm (ref MM1633), 10mL IV Luer Lock Syringe (ref Y8IVL10), BD Vacutainer® Plastic K2EDTA tube with Lavender BD Hemogard™ Cap 16 x 100mm (10ml) (ref 367525) and Steroplast Spot Plasters 2.4cm Diameter (ref HCP249) were obtained from Health-Care Equipment & Supplies Ipswich, Suffolk, UK. Additionally, Tourniquet Single Use Adult Band (460mm x 25mm) (ref 8441/SINGLE), Medisave Professional 70% Alcohol IPA Pre-Injection Swabs (ref MS100), Vasofix Safety Pur 22g, 1 in, 0.9 x 25mm IV Catheter with Injection Port (ref 4269098S), 5ml Posiflush XS (ref 306571) and Gauze Swabs 7.5 x 7.5cm 8 Ply (ref 1655A) were ordered from Medisave UK Limited, Weymouth, Dorset.

The human PYY ELISA kit (ref EH0576) was purchased from FineTest, Wuhan Fine Biological Technology Co., Ltd., Wuhan, Hubei, China. The MILLIPLEX® Human Metabolic Hormone Expanded Panel v3 (GIP (total), GLP-1 (total), PYY (total), ref HMH3-34K-03) were purchased from Merck Life Science UK Limited, Gillingham, Dorset.

Experimental reagents were prepared using Milli-Q (MQ) water (water purified through a 0.22µm membrane filter with a resistivity of 18.2 MΩ) using the ELGA PURELAB Classic UFV water purifier system (ELGA LabWater Ltd., High Wycombe, UK).

Sterile syringe filters (Merck Millex™-HA Sterile Syringe Filter Unit, MCE, 0.45 µm, ref SLHAR33SB) were ordered from Merck Life Science UK Limited, Gillingham, Dorset. Cell culture flasks were from Nunc (Denmark), all culture plates and Eppendorf tubes were from Corning, UK, and all pipette tips and serological pipettes were from Starlabs UK Limited, Blakelands, Milton Keynes.

2.2. Cell culture

2.2.3. Cell line maintenance

Caco-2 and STC-1 cells were obtained at passage numbers 20 and 30, respectively and maintained in complete DMEM. Complete DMEM was prepared by supplementing 500 mL DMEM (with high glucose content) with 50 mL HI-FBS, 5 mL L-glutamine and 5 mL Anti-Anti, achieving a final concentration of 10%, 2mM and 100 U/mL of penicillin, 100 mg/mL streptomycin and 0.25 mg/mL of Gibco Amphotericin B from Anti-Anti, respectively. Cells were seeded in a 75 cm2 flask (T75) with a seeding density of 2-3x10^4 cells/cm2 in 15 mL complete DMEM pre-warmed to 37 °C, and the cell was grown under standard cell culture conditions in a humidified cell culture cabinet set at 37°C, supplemented with an air intake containing 5% CO^2. The medium was refreshed every 2 to 3 days, and cells were passaged routinely on a weekly basis. Caco-2 and STC-1 cells were used for experiments with passage numbers 36-45 and 30-40, respectively. Passage numbers between 20-50 are commonly used for Caco-2 cells. Furthermore, it is recommended to use passage numbers below 50 for Caco-2 cells as higher passage numbers can lead to changes in cell characteristics, potentially affecting their suitability as a model for intestinal permeability studies (Kus, Ibragimow and Piotrowska-Kempisty, 2023). Moreover, the recommended passage numbers for STC-1 cells are between 15 and 40, as the heterogeneity of the cells was observed to be reduced compared to earlier passages Heffernan et al., 2022).

2.2.4. Cell subculture

Caco-2 and STC-1 cells were obtained at passage numbers 20 and 30, respectively and maintained in complete DMEM. Complete DMEM was prepared by supplementing 500 mL DMEM (with high glucose content) with 50 mL HI-FBS, 5 mL L-glutamine and 5 mL Anti-Anti, achieving a final concentration of 10%, 2mM and 100 U/mL of penicillin, 100 mg/mL streptomycin and 0.25 mg/mL of Gibco Amphotericin B from Anti-Anti, respectively. Cells were seeded in a 75 cm2 flask (T75) with a seeding density of 2-3x10^4 cells/cm2 in 15 mL complete DMEM pre-warmed to 37 °C, and the cell was grown under standard cell culture conditions in a humidified cell culture cabinet set at 37°C, supplemented with an air intake containing 5% CO^2. The medium was refreshed every 2 to 3 days, and

cells were passaged routinely on a weekly basis. Caco-2 and STC-1 cells were used for experiments with passage numbers 36-45 and 30-40, respectively. Passage numbers between 20-50 are commonly used for Caco-2 cells. Furthermore, it is recommended to use passage numbers below 50 for Caco-2 cells as higher passage numbers can lead to changes in cell characteristics, potentially affecting their suitability as a model for intestinal permeability studies (Kus, Ibragimow and Piotrowska-Kempisty, 2023). Moreover, the recommended passage numbers for STC-1 cells are between 15 and 40, as the heterogeneity of the cells was observed to be reduced compared to earlier passages Heffernan et al., 2022).

2.2.5. Cell viability

Cell viability was assessed using trypan blue dye exclusion cell count. When counting viable cells, 50 μL of cell suspension was mixed with 50 μL of 0.4% (w/v) trypan blue. After mixing with a vortex (VX-200, Labnet International Inc., NJ, United States), the cell suspension was left to rest for 5 min at room temperature before proceeding with the cell count described in section 2.2.1. Trypan blue stains non-viable cells due to the incomplete cell membrane, differentiating from viable cells as the complete cell membrane of the live cells prevents the uptake of the dye. The total number of cells, as well as viable cells, were counted, and cell viability was calculated using the formula below.

$$Cell\ Viability = (Number\ of\ viable\ cells \div Total\ number\ of\ cells) \times 100$$

The cell viability assessment was performed as a precautionary measure when passaging cells to assure the healthy state of the cells, as well as when seeding cells into plates for experiments to obtain the required number of viable cells for the desired cell seeding density. Cell viability observed was at least 75% on all occasions for each cell line.

2.3. *In vitro* digestion

To stimulate *in vivo* conditions, *in vitro* digestion of protein isolates was carried out following a static internationally harmonised digestion protocol INFOGEST (Brodkorb et al., 2019). This method uses constant ratios of meal to digestive

fluids (1:1) and a constant pH for each step of digestion, which are oral, gastric and intestinal phases, while parameters such as electrolytes, enzymes (pepsin and pancreatin), bile, dilution, pH and time of digestion are based on available physiological data (Minekus et al., 2014). The INFOGEST method has been adapted in-house exclusively for the digestion of fluids with high protein content. Adaptations have been made firstly in gastric pH, reducing it from pH 3, found in the generalised protocol, to pH 2, as it has been shown previously to be ideal for protein digestion (Minekus et al., 2014). Secondly, the gastric digestion time has been reduced from 120 min to 60 min due to the shorter gastric transit time of liquids containing protein compared to conventional diets (Lin et al., 2005; Minekus et al., 2014). Prior to commencing the digestion of protein isolates, the simulated digestive fluids were prepared as described in section 2.3.1. Furthermore, the enzyme activity of pepsin and pancreatin was also measured to be able to apply the desired quantity of 2000 U/mL and 100 U/mL in the final digestion fluid, respectively (section 2.3.2.).

2.3.1. Preparation of the simulated digestive fluids

Three types of simulated digestion fluids were prepared to mimic the digestive environment during *in vitro* digestion at each phase. The three distinct phases applied in the digestion were oral, gastric and intestinal. The simulated digestion fluids were prepared at a 1.25x concentration at a volume of 400 mL at a time and kept in appropriately labelled 50 mL centrifuge tubes at -20 ºC.

Firstly, the electrolyte stocks were dissolved into 1 M solutions or to the highest concentration required for the simulated digestive fluids, and the NaOH and HCl stock solutions used for the pH adjustment were prepared using MQ water, following **Table *3***. The required amount of stock was measured using a laboratory analytical balance (Sartorius Entris® 224-1S; Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany) or a glass measuring cylinder in case of liquids and was dissolved in the required amount of MQ water in glass beakers with the use of a magnetic stirrer (Stuart SM5 Mini, Barloworld Scientific Ltd., Stone, Staffordshire, UK).

Table 3. Preparation of primary stock solutions required for the simulated digestion fluids.

Stock	Molecular weight (MW)	Stock amount	MilliQ water volume	Final volume	Final concentration
$CaCl2(H_2O)_2$	147.01	0.147 g	1 mL	1 mL	1 M
KCl	74.55	3.73 g	50 mL	50 mL	1 M
KH_2PO_4	136.09	1.36 g	10 mL	10 mL	1 M
$MgCl_2(H_2O)_6$	203.30	1.02 g	5 mL	5 mL	1 M
$(NH_4)_2CO_3$	96.09	0.48 g	5 mL	5 mL	1 M
NaCl	58.44	7.0128 g	60 mL	60 mL	2 M
NaCl (2M)	-	5 mL	5 mL	10 mL	1 M
NaOH	40.00	4 g	100 mL	100 mL	1 M
HCl (5M)	-	20 mL	80 mL	100 mL	1 M

Subsequently, the primary stock solutions were further diluted to the concentration required to prepare the simulated digestive fluids, as shown in **Table *4***.

Table 4. Preparation of secondary stock solutions required for the simulated digestion fluids.

Stock	Stock amount	MilliQ water volume	Final volume	Final concentration
$CaCl2(H_2O)_2$ (1M)	0.15 mL	0.35 mL	0.5 mL	0.3 M
KCl (1M)	15 mL	15 mL	30 mL	0.5 M
KH_2PO_4 (1M)	3 mL	3 mL	6 mL	0.5 M
$MgCl_2(H_2O)_6$ (1M)	0.3 mL	1.7 mL	2 mL	0.15 M
$(NH_4)_2CO_3$ (1M)	1 mL	1 mL	2 mL	0.5 M

The simulated digestion fluids for the oral, gastric and intestinal digestion phases were prepared at a 1.25× concentration using the previously prepared electrolyte stock solutions and water, according to **Table *5***.

The simulated salivary fluid (SSF) was prepared by adding the stated volumes of specific stock concentrations to 300 mL of MQ water without the addition of $CaCl_2(H_2O)_2$. Following that, the pH of the SSF was adjusted to 7 using 1 M NaOH or 1 M HCl if necessary, and the final volume was adjusted to 400 mL using MQ water.

Similarly, the simulated gastric fluid (SGF) was prepared by adding the stated volumes of specific stock concentrations to 300 mL of MQ water without the

addition of $CaCl_2(H_2O)_2$. However, the pH was adjusted to 2 using 1 M NaOH or 1 M HCl before the final volume was adjusted to 400 mL using MQ water.

The simulated intestinal fluid (SIF) was prepared following the same process as with the preparation of SSF, using the stated volumes of specific stock concentrations in **Table *5*** in relation to SIF.

Table 5. Required volumes of electrolyte stock solutions of digestion fluids for a volume of 400 mL at a concentration of 1.25x.

			SSF (pH 7)		SGF (pH 2)		SIF (pH 7)	
Salt solution added	**Stock conc.**		**Stock added to prepare 0.4 L (1.25x)**	**Final salt conc. in SSF (1x)**	**Stock added to prepare 0.4 L (1.25x)**	**Final salt conc. in SGF (1x)**	**Stock added to prepare 0.4 L (1.25x)**	**Final salt conc. in SIF (1x)**
	(g/L)	**(M)**	**(mL)**	**(mM)**	**(mL)**	**(mM)**	**(mL)**	**(mM)**
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaCl	58.4	1	6.8	13.6	-	-	-	-
NaCl	117	2	-	-	18.05	72.2	30.85	123.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl		5	0.11	1.1	1.56	15.6	0.84	8.4
$CaCl_2(H_2O)_2$	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

The simulated digestion fluids, as well as the stock solutions, were stored in appropriately labelled centrifuge or Eppendorf tubes at −20°C. The 0.3M $CaCl_2(H_2O)_2$ solution was added to the simulated digestion fluids only immediately before use to avoid precipitation during storage. Also, the digestive fluids were mixed with the necessary quantities of enzymes based on their measured activities just before use. Subsequently, simulated digestion fluids were diluted to 1x concentration, accounting for the required volume of HCl or NaOH used for pH adjustment between the digestion phases required due to the buffering capacity of the proteins digested.

2.3.2. Assessment of the activity of digestive enzymes

The assessment of the standard units of activity of the enzyme used was a critical step for the determination of the enzyme concentration required during the digestion, with 2000 U/mL required for pepsin and 100 U/mL required for pancreatin in the final digestion fluid, respectively.

2.3.2.1. Pepsin

Pepsin activity was determined using the spectrophotometric stop reaction method, based on Anson and Mirsky's work (1932). The principle of this method is that pepsin digests the substrate, haemoglobin, into smaller peptides soluble in TCA, detectable at UV absorbance of 280 nm, as follows:

$$Haemoglobin + H_2O \xrightarrow{pepsin} TCA\ soluble\ tyrosine\text{-}containing\ peptides$$

Firstly, all required reagents were measured out with a laboratory analytical balance or in volumetric glass and dissolved in MQ water in a glass beaker, as shown in **Table *6***.

Table 6. Reagents prepared in MilliQ water required for the pepsin activity assay.

Stock	Molecular weight (MW)	Stock amount	MilliQ water volume	Final volume	Final concentration
NaCl	58.44	0.2922 g	5ml	5ml	1 M
TCA	163.39	1.5g	30ml	30ml	5% (w/v)
HCl (1M)	-	5 mL	5 mL	10 mL	0.5 M
HCl (1M)	-	3 mL	7 mL	10 mL	0.3 M
HCl (1M)	-	0.3 mL	29.7 mL	30 mL	0.01 M
NaCl (1M)	-	1.5 mL	8.5 mL	10 mL	0.15 M

Furthermore, 10 mM Tris buffer in 150 mM NaCl at pH 6.5 was prepared to solubilise pepsin by dissolving 0.0121g Tris (MW 121.14 g/mol) in 10 ml of 150 mM NaCl in a glass beaker. The pH was adjusted to 6.5 with 500 mM HCl using dropwise pipetting with a 1 mL Pasteur pipette.

Subsequently, the substrate was prepared by dissolving 0.5 g of haemoglobin in 20 mL MQ water in a glass beaker, and the pH was adjusted to 2 by dropwise addition of 300 mM HCl using a 1 mL Pasteur pipette. Once pH 2 was reached, MQ water was added to achieve a final volume of 25 mL, resulting in a haemoglobin concentration of 2% (w/v).

The pepsin enzyme stock solution was prepared by dissolving 5 mg of pepsin in 5 ml of the previously prepared 10 mM Tris buffer and was kept on ice until the preparation of 6 different dilutions was required just before the assay (**Table *7***).

Table 7. Constitution of different pepsin concentrations.

Pepsin concentration	Volume of stock solution	Volume of 10mM HCl	Total volume
10 µg/ml	10 µl	990 µl	1000 µl
15 µg/ml	15 µl	985 µl	1000 µl
20 µg/ml	20 µl	980 µl	1000 µl
25 µg/ml	25 µl	975 µl	1000 µl
30 µg/ml	30 µl	970 µl	1000 µl
35 µg/ml	35 µl	965 µl	1000 µl

The pepsin enzyme activity measurement was carried out by firstly adding 500 µl of haemoglobin solution into 24 x 2 mL Eppendorf tubes (4 x for each pepsin concentration; 2 for active measurements and 2 for blanks with every concentration), followed by incubation in a benchtop orbital shaker (Thermo Scientific MaxQ™ 4450, Thermofisher Scientific Inc., Waltham, MA, USA) at 37°C for 3-4 minutes to achieve temperature equilibration. After incubation of the Eppendorf tubes, 100 µl of the appropriate concentration of pepsin solutions was added into the appropriately labelled active Eppendorf tubes in duplicates. The tubes were incubated for a further 10 minutes, followed by the addition of 1 mL of TCA (5% w/v trichloroacetic acid) into each tube to stop the reaction, which also caused the precipitation of the haemoglobin. Finally, 100 µl of the different pepsin solutions was added into the appropriately labelled Eppendorf tubes in duplicate. The Eppendorf tubes were centrifuged at 6,000 g for 30 minutes to pellet the precipitate haemoglobin in order to get a clear soluble phase available for absorbance measurement. The soluble phase was placed into quartz cuvettes with a 1 cm light path length, and the absorbance was read at 280 nm using a Perkin Elmer UV/VIS Lambda 365 spectrophotometer (Perkin Elmer Italia, Milano, Italy).

The enzyme activity was calculated using the following equation:

$$\frac{Unit}{mg} = \frac{[A280\ Test\ -\ A280\ Blank] \times 1{,}000}{\Delta t\ \times\ X\ \times\ 0.001}$$

Where Δt stands for the duration of the reaction (10 minutes), X stands for the concentration of pepsin powder (µg) in 1mL of assay solution (10, 15, 20, 25, 30, 35 µg), 1,000 is the dilution factor to convert µg to mg and 0.001 equals to ΔA_{280}

per unit of pepsin produced per minute at pH 2 and 37°C, measured as TCA-soluble products.

2.3.2.2. Pancreatin

The amount of pancreatin was normalised to the trypsin activity based on Hummel's kinetic spectrophotometric rate determination method and following the recommendations from the Worthington laboratory (Hummel, 2011; Bergmeyer, 2012). Porcine trypsin activity is based on p-toluene-sulfonyl-L-arginine methyl ester (TAME): one unit hydrolyses 1 µmol of TAME per minute at 25 °C, pH 8.1.

$$TAME + H2O \xrightarrow{trypsin} p\text{-}Toluene\text{-}Sulfonyl\text{-}L\text{-}Arginine + Methanol$$

For the assessment of trypsin activity, pancreatin was dissolved in 1 mM HCl (pH 3) by mixing with a magnetic stirrer for 10 min in a beaker surrounded by ice to maintain a low temperature to preserve enzyme activity. Afterwards, pancreatin was diluted in 4 different concentrations ranging from 0.1 to 1 mg/mL (**Table *8***). Pancreatin solutions were mixed using a vortex before pipetting them into the enzyme reaction vessel.

Table 8. Constitution pancreatin enzyme solutions at different concentrations.

Pancreatin concentration	Volume of pancreatic stock solution (1 mg/mL)	Volume of 1 mM HCl	Total volume (µl)
1 mg/ml	150 µl	-	150 µl
0.7 mg/ml	105 µl	45 µl	150 µl
0.4 mg/ml	60 µl	90 µl	150 µl
0.1 mg/ml	15 µl	135 µl	150 µl

The assay solution, 46 mM Tris/HCl buffer, containing 11.5 mM $CaCl_2$ at pH at 8.1 and 25°C (30 mL) was prepared by dissolving 0.217 g of Trizma® hydrochloride (MW 157.60) and 0.0383 g $CaCl_2$ (MW 110.98) in 25 mL MQ water followed by the pH adjustment to 8.1 using dropwise addition of 500 mM NaOH with 1 ml Pasteur pipette. Finally, the total volume was adjusted to 30 mL with MQ water. The 10 mM TAME substrate was prepared by dissolving 0.0189 g TAME (MW 378.87 g/mol) in 5 mL MQ water.

The trypsin activity assay was carried out by mixing 2.6 mL of the previously prepared assay solution with 0.3 mL of 10 mM TAME in quartz cuvettes. Then, 100 µL of the pancreatin solution of the tested concentration was added, and the content of the cuvette was mixed with gentle inversion to ensure even dispersion. For the blank measurements, the pancreatin was replaced by the assay solution. The kinetic spectrophotometric measurements were obtained every 10 seconds for the duration of 10 minutes at 247 nm using a Perkin Elmer UV/VIS Lambda 365 spectrophotometer (Perkin Elmer Italia, Milano, Italy).

First, the slope from the initial linear portion of the curves (unit absorbance/minute) for both blank and test reactions was determined for the calculations. The calculation did not include the measurements with pancreatin concentrations that did not achieve linearity during the measurement. The Units/mg activity of trypsin was calculated following the equation below:

$$\frac{Units}{mg} = \frac{[\Delta A247\ Test\ -\ \Delta A247\ Blank] \times\ 1000\ \times 3}{540\ \times\ X}$$

Where ΔA_{247} stands for the slope of the initial linear portion of the curve (unit absorbance/minute) for the test and blank measurements, 540 is the molar extinction coefficient (L/(mol × cm) of TAME at 247 nm, 3 is the volume of reaction mix in millilitres and X is the quantity of trypsin in the final reaction mixture in mg.

2.3.3. Digestion of protein isolates

The protein isolated was digested using a three-phase, oral, gastric and intestinal phase method. Each phase was executed at a specific pH for a pre-defined duration, and samples were obtained in a timely manner at each phase. All samples obtained were kept on ice until further processing. At the end of digestion, the end-point digests were centrifuged at 5000g for 15 min at 4°C using a mid-bench centrifuge (Thermo Scientific Heraeus Multifuge X1R Centrifuge, Thermofisher Scientific Inc., Waltham, MA, USA). All samples obtained during digestion were aliquoted into 200 µl while the soluble phase of the end-point digesta was separated into 5 mL aliquots and kept at -20°C until required (**Figure 7**). The digestion of the protein isolates was carried out based on the protocol

produced by Brodkorb and colleagues (2019), with modifications in the pH and duration of the gastric phase, as detailed in section 2.3.

The protein isolates were prepared freshly on the day of digestion with two different initial protein concentrations, 100 mg/mL and 200 mg/mL, corresponding to 20 and 40 g total protein content of a 200 mL meal replacement, respectively (**Table *9***).

Table 9. Constituents of protein isolates solutions at different final protein concentrations.

Protein Isolates (PI)	Protein concentration in PI (%)	Weight of PI (mg)	Milli-Q water (mL)	Final volume (mL)	Final concentration (mg/mL)
Whey Protein	90	555	5	5	100
Whey Protein	90	1111	5	5	200
Potato Protein	90	555	5	5	100
Potato Protein	90	1111	5	5	200
Rice Protein	90	555	5	5	100
Rice Protein	90	1111	5	5	200
Pea Protein	80	625	5	5	100
Pea Protein	80	1250	5	5	200

The blends of WP and plant-based protein isolates were prepared with a ratio of 50:50 and 30:70, respectively. 30% contribution of the total protein by WP represented the smallest required amount to create complete protein source isolate blends based on individual essential AA composition (**Table 2**). The protein isolate blends were prepared freshly on the day of digestion with an initial protein concentration of 200 mg/mL, corresponding to 40 g of total protein content of a 200 mL meal replacement (**Table *10***).

Table 10. Constituents of protein isolate blend solutions at 200 mg/mL of final protein concentrations.

Protein Isolate Blends (whey:plant-based protein)	Weight of WP (mg)	Weight of plant-based protein (mg)	MilliQ water (mL)	Final volume (mL)	Final concentration (mg/mL)
WP/PP (50:50)	555	555	5	5	200
WP/PP (30:70)	333	777	5	5	200
WP/RP (50:50)	555	555	5	5	200
WP/RP (30:70)	333	777	5	5	200
WP/PeP (50:50)	555	625	5	5	200
WP/PeP (50:50)	333	875	5	5	200

Whey protein isolate (WP), Potato protein isolate (PP), Rice protein isolate (RP), Pea protein isolate (PeP)

2.3.3.1. Oral phase

For the oral phase of the digestion, 5 mL of the protein isolate solutions were diluted with prewarmed 37°C SSF (1x) pH 7 to achieve a final ratio of 50:50 (v/v) in duplicates, achieving a final volume of 10 mL in each tube. Previously, the necessary amount of $CaCl_2(H_2O)_2$ to achieve 1.5 mM in the final mixture and the required volume of water to dilute the stock solution of SSF (1.25x) were added. Human salivary amylase was omitted due to the lack of starch in the digested material. Additionally, a blank digestion tube (BD) was prepared where the protein isolate solution was replaced by water, but all required enzymes and bile salts were added during digestion.

The oral phase ran for 2 min at 37°C and 70 rpm using the benchtop orbital shaker (Thermo Scientific MaxQ^{TM} 4450, Thermofisher Scientific Inc., Waltham, MA, USA). 1 x 1ml of sample was obtained at the end of the oral phase from each digestion tube and transferred to ice immediately.

2.3.3.2. Gastric phase

Before commencing the gastric phase digestion, the SGF was prepared by adding $CaCl_2(H_2O)_2$ to achieve a final concentration of 0.15 mM in SGF (1x), porcine pepsin to achieve an activity of 2,000 U/mL in the final gastric digestion mixture. Subsequently, the volume of HCl required to achieve pH 2 during the gastric phase was added.

Due to varying pH buffering capacities, the volume needed varied based on the protein isolate digested. The buffering capacity of the specific protein isolates was assessed previously with a pH test adjustment experiment, where the protein isolates were exposed to oral, gastric and intestinal phase digestion without the addition of enzymes. During the pH test adjustment experiment, the pH of the digesta was adjusted to pH 2 and pH 7 at the beginning of the gastric and intestinal phases, respectively. The required amount of HCl or NaOH to achieve the desired pH levels was carefully recorded. Finally, the required volume of water was added to the SGF to achieve a final concentration of 1x.

After the SGF (1x) preparation, 10 mL was added to the oral bolus (final ratio 1:1), leading to a final volume of 20 mL of gastric mixture. The gastric mixture was incubated at 37 °C for 1 h at 70 rpm using the benchtop orbital shaker (Thermo Scientific MaxQ^{TM} 4450, Thermofisher Scientific Inc., Waltham, MA, USA). Samples (1 mL) were obtained every 30 min (15 min for digestion verification assessment) during gastric digestion. After 1 h, the pepsin activity was ceased by raising the pH to 7 by adding the required volume SIF (1x). Samples were stored on ice until further processing.

2.3.3.3. Intestinal phase

The SIF was prepared by adding $CaCl_2(H_2O)_2$ to achieve a final concentration of 0.6 mM in SIF (1x), porcine pancreatin to achieve a final trypsin activity of 100 U/mL, and bile to reach a final concentration of 10 mM in the final intestinal digestion mixture. Afterwards, the volume of NaOH required to achieve pH 7 during the intestinal phase was added. Finally, water was added in order to achieve a 1× concentration of the SIF. Of the prepared SIF (1x) electrolyte and enzyme solution, 20 mL was added to the gastric chyme to achieve a final ratio of 1:1 (vol/vol). The samples were incubated at 37 °C, using a shaking incubator to mix the digestive mixture sufficiently for 2 h, with the same parameters used during oral and gastric phase digestion. Samples in a volume of 1 mL were obtained every 30 min (15 min for digestion verification assessment), and 10 µl of 100 mM Pefabloc® was added to each 1 mL sample to block possible residual enzyme activity of the pancreatin (Egger et al., 2016). Samples were stored on ice until further processing. After adding Pefabloc® with a final 1 mM concentration, end-point samples were centrifuged at 5000g for 15 min at 4 °C to separate the soluble phase from the insoluble. The soluble phases of the samples were stored in 5 mL aliquots at -20 °C until required for analysis.

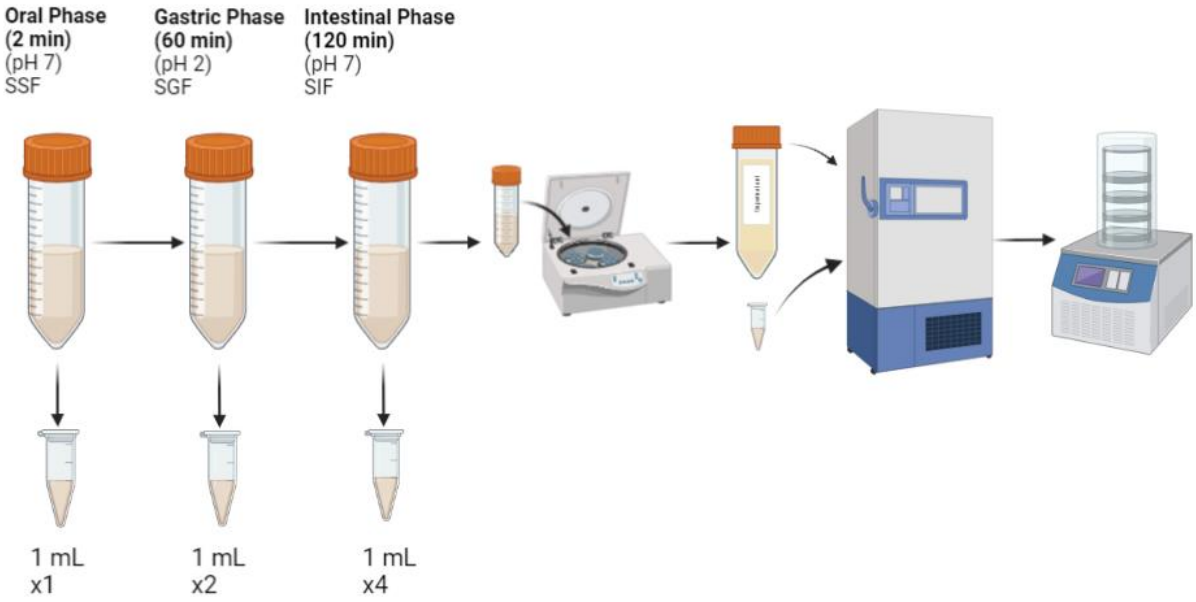


Figure 7. Schematic representation of the in-vitro digestion process (Created with BioRender.com).

2.4. Heat inactivation of enzymes

The liquid phase of the digested samples was placed in a Labnet AccuBlock™ Digital Dry Bath thermoblock (Labnet International, Inc., New Jersey, US) dry block heater at 100 °C for 5 min to denature enzymes to stop any potential remaining activity. After heating, the samples were centrifuged at 5000g for 15 min (Thermo Scientific Heraeus Multifuge X1R Centrifuge, Thermofisher Scientific Inc., Waltham, MA, USA) and filtered using a 0.45 μm syringe filter.

2.5. Characterisation of protein digests

2.5.1. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis

The protein degradation of protein isolates during *in vitro* digestion was determined by electrophoresis. The proteins of the digesta samples were separated using a polyacrylamide gel matrix after treatment with SDS, also known as sodium lauryl sulphate. During SDS-PAGE, the proteins are separated based on their polypeptide chain length. SDS is a detergent with a strong protein-denaturing effect and binds to the protein backbone at a constant molar ratio. In the presence of SDS and a reducing agent that cleaves disulfide bonds critical for proper folding, proteins unfold into linear chains with negative charge proportional to the polypeptide chain length. The polymerised acrylamide

(polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size, depending on the concentration of the acrylamide used (**Figure *8***).

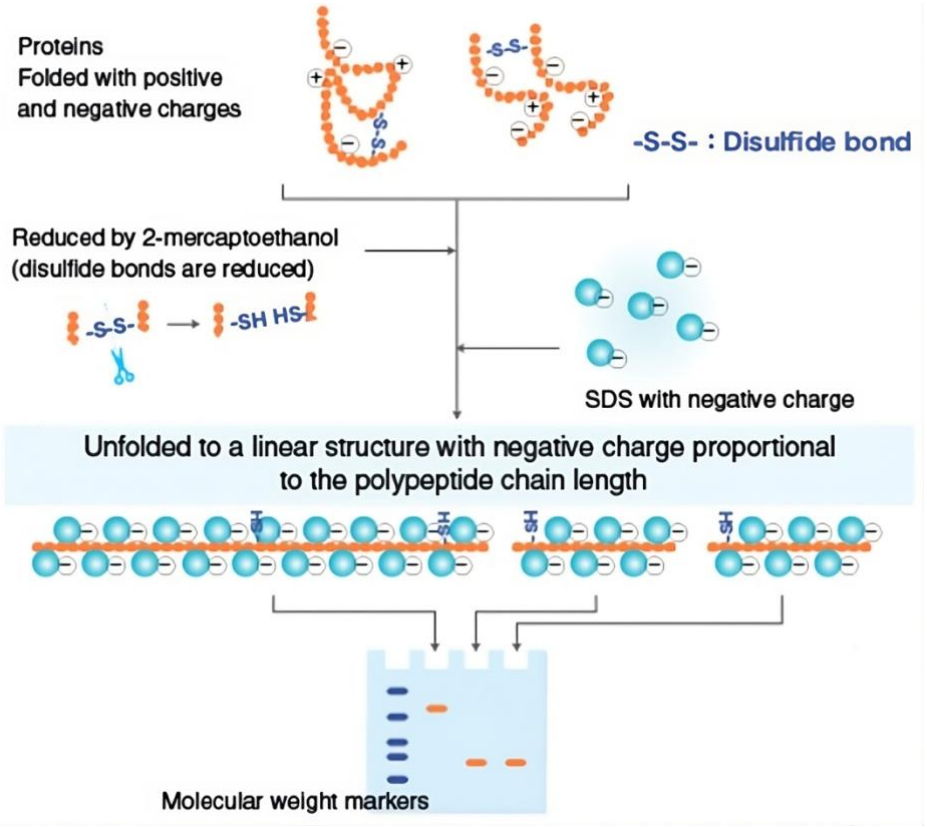


Figure 8. Principle of SDS-PAGE (Adapted from ruo.mbl.co.jp).

2.5.2. Separation of proteins on SDS-PAGE gel

The samples were brought to the same concentration based on their dilution during *in vitro* digestion using MQ water to obtain an equal loading of digested protein in each lane. The samples were diluted with sample buffer containing 62.5mM Tris (pH 6.8), 25% glycerol, 0.01% Coomassie Brilliant Blue R250, 2% SDS and 5% β-mercaptoethanol in a ratio of 1:1 to enable protein tracking through the gel during electrophoresis. After dilution, the samples were heated at 95 °C for 5 min, cooled to room temperature, and loaded (20 µL) onto the 4-16% acrylamide 1.0 mm gels. The gels were cast manually using the Bio-Rad mini-format handcast system using two-thirds 16% running gel solution and one-third 4% stacking gel solution (**Table 11**). The running gel was added between the glass plates and left to set for ~ 1 h until polymerised, before the stacking gel was added. A comb was placed into the stacking gel to form wells for sample loading.

Table 11. Reagents required for the preparation of running and stacking gel solutions.

Reagents	Running gel (16%)	Stacking gel (4%)
Distilled H_2O	2070µl	3000µl
Running gel solution (1.5 M Tris hydrochloride (Tris-HCl) pH 8.8)	2500µl	-
Stacking gel solution (0.5 M Tris-HCl pH 6.8)	-	1260µl
10% Sodium dodecyl sulphate (SDS)	100µl	50µl
30% acrylamide/bisacrylamide	5280µl	660µl
10% ammonium persulphate (APS)	50µl	25µl
N,N,N',N',Tetramethylethylenediamine (TEMED)	5µl	5µl

The electrophoresis was conducted in a Mini-PROTEAN Tetra System connected to a Bio-Rad Power Pac 1000 (Bio-Rad Laboratories, Hercules, CA, USA) using a running buffer containing 25 mM Tris-base, 192 mM glycine and 0.1% SDS at 200 V for 45 min. The gels were marked with Coomassie Brilliant Blue R250 staining solution consisting of acetic acid, methanol, and H_2O with a 1:4:5 ratio (v/v/v), and destained in an acetic acid:methanol:H_2O solution (1:4:5, v/v/v). A pre-stained molecular weight marker comprising a range of proteins with sizes that varied from 10 to 250 kDa was used (PageRuler™ Plus Prestained Protein Ladder, Fisher Scientific UK Limited, Loughborough, Leicestershire). Gels were documented using a UVIpro gel documentation system (UVItec, Cambridge, UK).

2.5.3. Determination of digested proteins

2.5.3.1. Preparation of the derivatisation reagent

Ophthaldialdehyde (OPA) is a highly effective derivatisation agent for AAs because it readily reacts with amino groups to form highly fluorescent products when in the presence of excess thiols. The AA derivatisation reagent was prepared fresh each day by dissolving 1.905 g of sodium tetraborate in 40 mL MQ water; this solution was brought to 50 °C and shaken in a shaking incubator at 200 rpm for 1 hour to aid solubilisation. After 1 hour of incubation, the sodium tetraborate buffer was cooled to room temperature. Subsequently, 40 mg of OPA was dissolved in 2 mL of ethanol, and 44 mg of dithiothreitol and 50 mg of SDS were added to the sodium tetraborate buffer. This derivatisation reagent was further diluted with MQ water, bringing it to a final volume of 100 mL to obtain the final working solution. Then, the solution was covered with aluminium foil to

prevent the development of colour that could influence the subsequent measurements.

2.5.3.2. Separation of free amino acids and peptides

Free AAs and peptides within the digested samples were separated from intact and undigested proteins by precipitation using TCA. In brief, 0.332 mL of 5% TCA was mixed with 0.2 mL of the digested sample, followed by centrifugation at 10,000 g for 30 min at room temperature. The supernatants were then filtered using a 0.45 µm syringe filter (25 mm diameter, 0.45 µm pore size PVDF membrane).

2.5.3.3. Determination of free amino acid groups

The concentration of free amino groups released after *in vitro* digestion was measured by the OPA spectrophotometric assay according to the method of Nielsen et al. (2001) with minor modifications. To determine the absorption value, 200 µL of the OPA reagent was added to 10 µL of each tested sample (blank, standard solutions and digested supernatants) in a 96-well plate. The reaction was allowed to occur over 15 minutes at room temperature before the measurement was taken at 340 nm. The free amino group concentration of *in vitro*-digested samples was determined with reference to a calibration curve constructed using L-leucine (0 - 10 mM) prepared freshly on the day of the assay.

2.6. Assessment of cell viability

The Caco-2 and STC-1 cells, after trypsinisation, were seeded in 96-well plates at a seeding density of 3 × 104 cells/cm^2 or 9,600 cells per well and 1.5 × 10^5 cells/cm^2 or 468,750 cells per well, respectively. Cells were grown till confluency before the treatment with the digests. The unheated as well as heat-inactivated digests of WP, PP, RP, PeP and BD and the heat-treated blended protein digests WP/PP (50:50), WP/RP (50:50), WP/PP (30:70), WP/RP (30:70), WP/PeP (50:50) and WP/PeP (30:70) with the initial protein concentration of 200mg/mL were prepared at dilutions ranging from 1x to 10x using MEM, as seen in **Table 12**.

Table 12. The different concentrations of treatments tested for cell viability.

Treatment dilution	Ratio (Digesta:MEM)	Digesta (µl)	MEM (µl)	Estimated digested protein concentration (mg/mL)
ND	1:0	1500	-	25 mg/mL
1x	1:1	750	750	12.5 mg/mL
2x	1:2	500	1000	8.3mg/mL
3x	1:3	375	1125	6.25mg/mL
4x	1:4	300	1200	5mg/mL
5x	1:5	250	1250	4.17mg/mL
6x	1:6	214	1286	3.57mg/mL
7x	1:7	188	1312	3.13mg/mL
8x	1:8	167	1333	2.78mg/mL
9x	1:9	150	1350	2.5mg/mL
10x	1:10	136	1364	2.27mg/mL

BD was used as a control treatment to indicate the potential cytotoxic effects of bile acids and enzymes within the digesta at various concentrations. Additionally, MEM, which was also used as a control, indicated normal cell viability and served as a reference when assessing cell viability with the different treatments.

The cells were treated with unheated or heat-inactivated digests at different concentrations for 24 or 4 hours, respectively, while maintaining the cell culture conditions required for Caco-2 and STC-1 cells in the tissue culture incubator. After the incubation period, 20 µL of MTT dissolved in DPBS was added to each well, and the cells were allowed to uptake MTT for 4 hours at 37 °C, 5% CO_2. The identification of live cells is based on the metabolically active cells reducing the original yellow colour of MTT to purple via mitochondrial dehydrogenases (**Figure *9***).

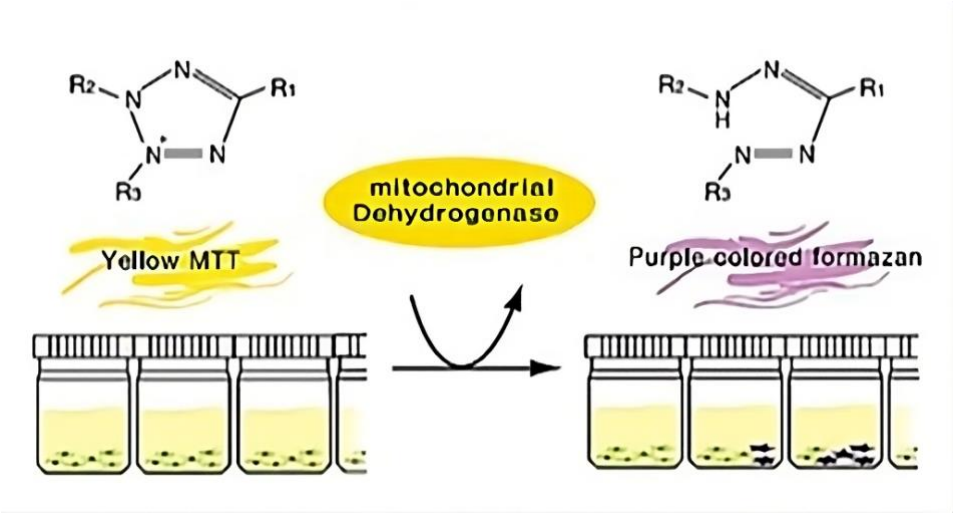


Figure 9. Principle of MTT assay (Adapted from Jethva, Bhatt and Zaveri, 2021).

Subsequently, the media was removed, and 150 µL of DMSO was added to each well and incubated for 1 hour at 37 °C and 170 rpm in the benchtop orbital shaker while protecting from the light. This incubation allowed the DMSO to solubilise the metabolic product of MTT, formazan. The optical density was read at 490 nm using a microplate reader (SPECTROstar® Nano, BMG Labtech GmbH, Ortenberg, Germany). The percentage cell viability was calculated using the following equation:

$$Cell\ viability\ (\%) = [Signal\ - Background] \div [Blank - Background] \times 100$$

2.7. Assessment of the effects of protein digests on appetite hormone regulation

2.7.1. Treatment of Caco-2 cells

Caco-2 cells were used to create an *in vitro* model of the small intestinal mucosa, and the STC-1 cells represented the *in vitro* model of intestinal L-cells. As described above, Caco-2 and STC-1 cells were grown in complete DMEM in a 5% CO2 environment at 37 °C. Caco-2 cells were thawed and grown in plastic T75 cell culture flasks until they reached 70% confluence. Adherent cells were then detached from the surface of the flasks via trypsinisation and seeded into 6-well plates at $4x10^3$ cells/cm^2 seeding density and grown to confluency over 2 weeks with Caco-2 cells. Seeding density and the time required to achieve a confluent monolayer with STC-1 cells were $1.5x10^5$ cells/cm^2 and 5 days,

respectively. Based on methods previously described by Zariwala et al (2013), Caco-2 cells were exposed to MEM 2 mL/well for 24 h before the treatment with digests. After the 24 h MEM treatment, the Caco-2 cells were treated with 2 mL non-heated or 1 mL heat-inactivated digesta at a concentration deemed suitable following the cell viability assay for 2 h and 6 h or 2 h and 4 h, respectively. While following the methodologies previously published by Kondrashina, Papkovsky and Giblin (2018), once STC-1 cells reached confluency, complete media was aspirated and cells were washed once with modified DPBS buffer (without bicarbonate but with 10 mM HEPES) and then pre-incubated in the same buffer for 1 h to acclimatise. Buffer was then aspirated and replaced with 1 mL/well protein digesta diluted in the modified DBPS buffer for a final concentration, which was identified as suitable based on viability assays in STC-1 cells. All non-sterile solutions were filter-sterilised (0.45 µm) prior to cell exposures for 4 h at 37 °C.

2.7.2. Lysis of Caco-2 and STC-1 cells

Following the treatment with digesta, the supernatant was collected from the wells and the cells were lysed at 4 °C as previously described by Zariwala et al. (2013), using 350 µl ice-cold lysis buffer (50 mM NaOH supplemented with 1 ug/ml protease inhibitor cocktail) whilst rocking gently for 60 min in ice trays on a plate shaker (8 rpm). Cell lysates were collected using sterile cell scrapers and passed through a 25G needle to reduce the viscosity of the suspension. Supernatants, as well as cell lysates, were aliquoted into microcentrifuge tubes and kept at -20 °C, ready for further analysis.

2.7.3. Assessment of cell lysates' total protein content

The total protein content of the Caco-2 cell lysates after treatments with protein digests was assessed using the ThermoFisher Scientific Pierce BCA Protein Assay Kit, following the manufacturer's protocol (as previously described by Kim et al., 2011), using the BSA stock (2 mg/ml) provided in the kit as the standard. The total protein concentration was quantified based on bicinchoninic acid (BCA) via colourimetric detection. This method combined the reduction of Cu^{+2} to Cu^{+1} by protein bonds in an alkaline medium (the biuret reaction) with the colourimetric detection of the cuprous cation (Cu^{+1}) using a bicinchoninic acid-

containing reagent. The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex is water-soluble and exhibits a strong absorbance at 562 nm (**Figure 10**).

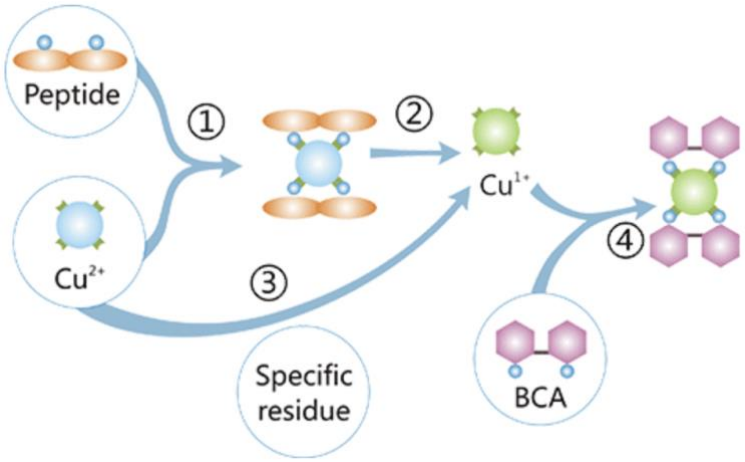


Figure 10. Principle of the BCA assay (Adapted from cusabio.com/c-21049.html)

The BSA standards were prepared in a concentration ranging from 0 to 2000 µg/mL using 50 mM NaOH, as presented in **Table *13***. Samples were also diluted using 50 mM NaOH in a ratio of 1:2 by adding 20 µl of sample to 40 µl of solvent. The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B), provided in the kit used.

The samples and standards were tested in duplicates with a 25 µL working volume. After the addition of 200 µL working solution into each well, the plates were incubated at 37 °C for 30 minutes. The absorbance was measured at 562 nm using a microplate reader (SPECTROstar® Nano, BMG Labtech GmbH, Ortenberg, Germany) after the plate had cooled down to room temperature.

The blank-corrected standard curve was plotted to determine the protein concentration of each unknown sample using a four-parameter (quadratic) equation on PRISM (Version 9, Graphpad Software Inc., San Diego, CA, USA).

Table 13. Reagents required for the preparation of BSA standard.

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 =Blank

2.7.4. Assessment of intracellular PYY levels following unheated digest treatments

To analyse the relative appetite regulatory effect of the four protein fractions (WP, PP, RP and PeP isolate), intracellular PYY levels were analysed using the Human PYY ELISA Kit (FineTest, Wuhan Fine Biological Technology Co., Ltd., Wuhan, Hubei, China). The kit's sensitivity and detection range were 18.75 pg/mL and 31.25–2000 pg/mL, respectively, with an intra-assay CV of 6.6% and an inter-assay CV of 9.4%. The assay procedures were performed according to the manufacturer's instructions, and plates were read using a microplate reader (SPECTROstar® Nano, BMG Labtech GmbH, Ortenberg, Germany).

Firstly, standards were prepared using the provided PYY standard and sample dilution buffer via serial dilution (**Figure *11***). Standards and samples were tested with a volume of 50 µL. A biotin-labelled antibody with a volume of 50 µL per well was used to label PYY by covalent attachment of biotin. After 45 min incubation at 37 ºC, 100 µL/well HRP (horseradish peroxidase) - Streptavidin Conjugate was added to bind the previously added biotin with streptavidin while the conjugated HRP provided enzyme activity for detection using TMB (3,3',5,5'-Tetramethylbenzidine) substrate (90 µL/well), yielding a blue colour, detectable at 450 nm.

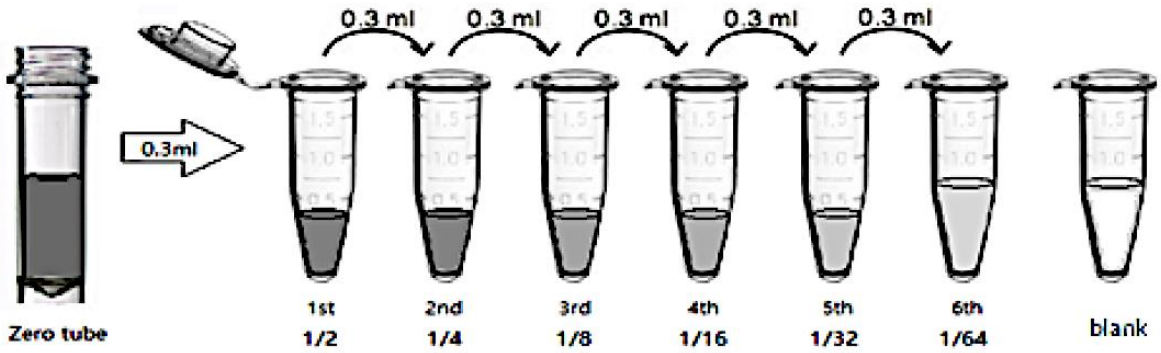


Figure 11. Serial dilution of PYY standard (Adapted from Human PYY ELISA Kit, FineTest, Wuhan Fine Biological Technology Co., Ltd., Wuhan, Hubei, China).

The detected intracellular PYY concentrations were normalised to total protein concentration, previously measured by BCA, for each sample prior to statistical analysis, as described below.

2.7.5. Assessment of intracellular and secreted GLP-1, PYY and GIP levels following heat-inactivated digest treatments

The total concentrations of GLP-1, PYY and GIP within cell lysates and supernatants following treatments with heat-inactivated digests were analysed using a customisable 3-plex Human Metabolic Panel Milliplex kit (Merck Millipore, Darmstadt, Germany). The assays were performed according to the manufacturer's instructions. The Luminex Magpix® xPONENT software and plate reader instrument (Luminex Technologies, Austin, TX, USA) were used to perform multianalyte profiling of circulating concentrations of metabolic markers. Standard curves were generated for each metabolic hormone, and the mean fluorescence intensity (MFI) of each metabolite in each well was correlated to determine concentrations. The detected intracellular and secreted GIP, GLP-1 and PYY concentrations were normalised to total protein concentration, previously measured by BCA, for each sample prior to statistical analysis, as described below.

2.8. Assessing the effects of whey and potato protein-enriched breakfasts on appetite regulation in healthy male participants

2.8.1. Participants

From the initially recruited twelve healthy male individuals, ten successfully completed the study and were included in the statistical data analysis (Appendix C).

Participants' suitability for the study was assessed using a Health Screening Questionnaire. The questionnaire was completed as part of the study sign-up process via Jisc (Jisc, Bristol, UK), a General Data Protection Regulation (GDPR) compliant online platform, to which access was provided via the recruitment posters (Appendix B). The health assessment included questions related to pre-existing medical conditions such as diabetes, high blood pressure or coronary heart disease. In addition, dietary and supplementation aspects were captured. Individuals consuming more than 14 units of alcohol per week, presenting allergic reactions to ingredients in the test meals provided, suffering from illnesses that affect taste or appetite, gastrointestinal disorders, eating disorders, depression and/or smokers were not suitable for participation.

Volunteers suitable to participate were provided with a detailed participant information sheet and were invited for their first visit. On the first attendance day, prior to participation, the volunteers were explained in detail about the study and were offered answers to any questions they may have had. An informed consent form was signed upon the volunteers' decision to participate. Baseline anthropometric measurements were also collected on the same day by trained research staff. Height was measured using a Seca Leicester Height Measure (Seca GmbH & Co. KG, Hamburg, Germany). Weight, body mass index (BMI) and body fat% were measured using the Seca 515 medical Body Composition Analyser (Seca GmbH & Co. KG, Hamburg, Germany). Ethical approval (ID: ETH2223-1377) was granted by the College Research and Knowledge Exchange Ethics Committees (CREC) – Liberal Arts and Sciences (LAS), University of Westminster, in accordance with the ethical standards of the Helsinki Declaration of 1975 (Appendix A).

2.8.2. Study Design

A single-blind (blinding of participants), randomised, triple cross-over study design was employed. Participants received three different oat-based breakfast meals in a random order, which was generated using the online service Randomization.com (Dallal, 2013). The porridge meals prepared using coconut milk were of equal mass and calories and contained the same amount of WP or PP, except for the Control porridge. The Control porridge contained no added protein (**Table *14***).

Table 14. Composition of breakfast meals

	Control porridge			Whey protein			Potato protein		
	Weight (g)	Energy (kcal)	Protein (g)	Weight (g)	Energy (kcal)	Protein (g)	Weight (g)	Energy (kcal)	Protein (g)
oat	81	300	9	54	200	6	54	200	6
coconut milk (water)	500	100	0.5	300 (150)	60	0.3	300 (150)	60	0.3
protein isolate	-	-	-	38	141.74	34.2	38	141.36	34.39
Total	**581**	**400**	**9.5**	**542**	**401.74**	**40.5**	**542**	**401.36**	**40.6**

The interventions were administered on different days after an overnight fast of 10 hours at minimum, with at least a two-day washout period between different intervention meals. Furthermore, participants were instructed to consume a carbohydrate-based meal the night before study attendance day; however, their dietary intake was not controlled in any other way (**Figure *12***).

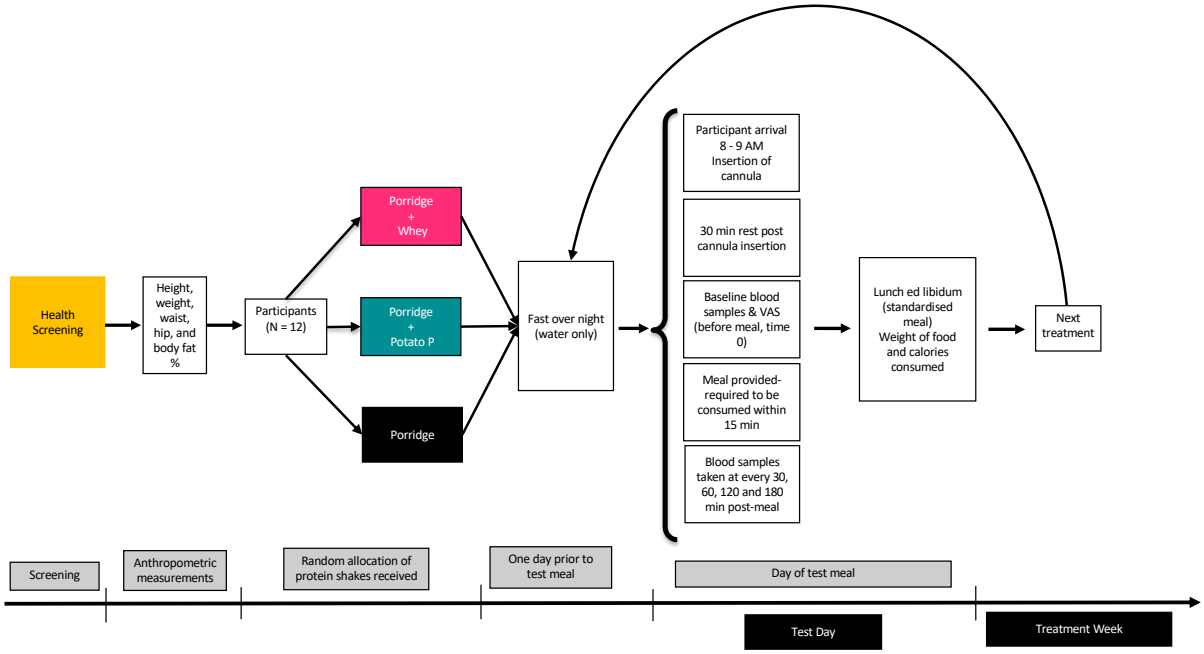


Figure 12. Overview of study design.

2.8.3. Study Protocol

Participants were requested to attend the research laboratory following a 10-hour overnight fast and avoid caffeine consumption. On arrival, a cannula was inserted in the antecubital fossa via an Introcan Safety® IV catheter (22G) (B. Braun, Sheffield, UK), and the participants were asked to rest for 30 min to avoid any interference due to stress potentially caused by the procedure. The baseline blood sample (Time 0; T0) was collected after the resting period. Finger-prick glucose measurements, using the blood glucose monitoring system SD CodeFree™ Blood Glucose Meter with SD Biosensor test strips (SD Biosensor, Inc., Suwon, Gyeonggi-do, Republic of Korea), were also obtained concurrently at the same time points. Following the baseline blood sample collection, participants were provided with one of the intervention breakfasts (**Table *14***) identified based on the randomised order allocated to them.

The full amount of breakfast had to be consumed within 15 min. Further blood samples were then drawn at timepoints T30, T60, T120 and T180 during each visit. Approximately 10 mL of blood was collected from each participant per timepoint using Becton Dickinson (BD) Vacutainer ® EDTA tubes (BD, Oxford, UK). Blood in the EDTA (ethylenediaminetetraacetic acid) tubes were kept on ice

and centrifuged (Hettich 340r, Hettich GmbH & Co. KG, Tuttlingen, Germany) within 30 min of collection, for 20 min at 3857 g at 4 °C. Plasma supernatants were aliquoted into 1.5 mL microcentrifuge tubes immediately post-centrifugation and stored at −80 °C. Furthermore, visual analogue scales (VAS) (Flint et al., 2000) were completed by all participants to measure subjective aspects such as palatability (measured during consumption of breakfast and lunch) and satiety (measured at T0, T30, T60, T120 and T180). To assess the potential delayed effects of increased protein intake at breakfast, a ready-made pasta-based meal (**Table *15***) (Tesco Three Cheese Pasta Bake 750G; Tesco plc, Welwyn Garden City, AL7 1GA, UK) was provided for lunch at libitum after the 3-hour intervention. Participants were given 20 minutes to eat as much as they liked from the provided meal. The pasta dish was measured before serving and after eating. The weight difference was used to calculate the consumed energy over lunch.

Table 15. Nutritional information of Tesco 3 cheese pasta bake/100g

Energy	548 kJ / 130 kcal
Fat of which saturates	3.1 g 1.7 g
Carbohydrate of which sugars	18.8 g 2.8 g
Fibre	1.7 g
Protein	5.9 g
Salt	0.25 g

2.9. Plasma Analysis

Plasma was analysed to determine the primary outcomes of this study, which was to assess the acute effects of WP and PP isolate enriched porridge in comparison to a general breakfast porridge on insulin, total GLP-1 (amount of GLP-1, 7–36 and 9–36 forms), PYY, GIP and ghrelin. For each biomarker, the assay kits were from the same Lot number.

Plasma insulin concentration was analysed using an insulin ELISA (enzyme-linked immunosorbent assay) kit (DRG Insulin Elisa kit, DRG Instruments GmbH, Marburg, Germany). The measurement involved a solid-phase enzyme-linked immunosorbent assay based on the sandwich principle. The analytical sensitivity

was 0.076 ng/mL, the assay range was 0.076–4.33 ng/mL, and an intra-assay coefficient of variation (CV) of 2.5% and an inter-assay CV of 4.8% were determined. Total plasma ghrelin was detected using a competitive Millipore Human Ghrelin (Total) ELISA kit (Sigma-Aldrich, Darmstadt, Germany). The kit's sensitivity was 9 pmol/L (20 µl sample size), with an intra-assay CV of 2.2% and inter-assay CV of 6.3%. All assay procedures were performed according to the manufacturer's instructions, and plates were read using a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany). Total plasma GLP-1, PYY and GIP were analysed using a customisable 3-plex Human Metabolic Panel Milliplex kit (Merck Millipore, Darmstadt, Germany). The assays were performed according to the manufacturer's instructions. The Luminex Magpix® xPONENT software and plate reader instrument (Luminex Technologies, Austin, TX, USA) were used to perform multianalyte profiling of circulating concentrations of metabolic markers. Standard curves were generated for each metabolic hormone, and the MFI of each metabolite in each well was correlated to determine concentrations.

2.10. Satiety and Palatability

The palatability of the breakfast meals provided was assessed using VAS (Flint et al., 2000), consisting of five characteristics related to visual appeal: smell, taste, aftertaste and palatability (where 0 = good and 100 = bad). The satiety effects of the porridge meals were evaluated using a VAS measurement tool (a psychometric response scale using a 100 mm line with anchor statements at either end of the line). The satiety VAS comprises eight characteristics of interest and includes levels of hunger (0 = not hungry at all, 100 = never been hungrier), satisfaction (0 = completely empty, 100 = cannot eat another bite), fullness (0 = not at all full, 100 = totally full) and prospective food intake (0 = nothing at all, 100 = a lot). The collected data was used to calculate an average appetite response across the intervention period; an equation adapted from Zafar et al. (2013):

$$Average\ appetite = \frac{[prospective\ food\ intake + hunger + (100 - fullness) + (100 - satisfaction)]}{4}$$

2.11. Statistical analysis

Data analysis of the digestibility of different proteins was carried out using one-way and two-way repeated measures analysis of variance (ANOVA) for between and within-group analysis, respectively. The post-hoc test, Tukey's, was applied, and data are expressed as mean and 95% confidence intervals of the mean (CI) for the between-group differences and mean ± standard deviation (SD) for the within-group differences.

The analysed data from cell viability and the intracellular PYY level assessment are expressed as mean ± SD. MTT data was analysed using one-way and PYY data with two-way repeated measures ANOVA. Post-hoc test, Tukey's, was applied to assess the differences between and within treatment conditions. The reported *p*-values were adjusted for multiple analyses. Data are expressed as mean and the 95% confidence interval of the mean for difference analysis. A significant difference was declared when the 95% confidence interval of the treatments compared did not contain zero, and the *p*-value of the overall F-test was less than 0.05.

The number of participants recruited for the human study was based on the power calculation utilising the results of our previous pilot study on gut peptides (Tiekou Lorinczova et al., 2021) using the mean differences and standard deviations detected between the area under the curve of GLP-1, glucose and insulin with WP vs PP considering a Type I error (α) = 0.05 (two-sided), and Type II error (β) = 0.20 (power of 80%). The power calculation was carried out using G*Power 3.1.9.2 software statistical analysis software (Kiel, Germany) (Faul et al., 2007; Erdfelder et al., 2009). The power calculation indicated the requirement for ten participants to complete the study. Twelve participants were recruited to account for potential dropouts.

The Shapiro–Wilk test was used to test the normality of the data collected in the human study part of this project. The test provided no evidence to reject the hypothesis that all data were normally distributed. A two-way (condition (WP vs. PP vs. Control) * time) repeated measures ANOVA was used to assess differences in glucose, insulin, GLP-1, GIP, PYY, ghrelin, palatability, and

average appetite. Where a significant main effect was found following the ANOVA, Bonferroni post hoc paired comparisons were performed. The level of significance was set at $p < 0.05$. All descriptive data are presented as mean ± standard deviation unless otherwise stated. Data was analysed using the GraphPad PRISM software package (Version 10, Graphpad Software Inc., San Diego, CA, USA).

3. CHAPTER 3 – Digestion of protein isolates and characterisation of digests

3.1. Introduction

Protein consumption has increased globally over the last few decades (Lumsden et al., 2024) due to the availability and accessibility of the nutrient (Henchion et al., 2017) as well as changing preferences towards higher protein intake (Hertzler et al., 2020). The desire to consume a diet with higher protein content may be related to its positive impact on health, such as aiding muscle mass increase (Houston et al., 2008) or appetite reduction (Kohanmoo, Faghih and Akhlaghi, 2020). The appetite-regulatory effects of proteins largely contributed to their effect on appetite-regulatory hormones, e.g. PYY, GLP-1 or CCK (De Graaf et al., 2004; Karhunen et al., 2008; Fromentin et al., 2012). Such appetite-regulatory effects of meat or diary-derived proteins such as WP are widely studied and presented in the literature, showing appetite-regulatory effects by reducing appetite, increasing satiety, and subsequently reducing calorie intake (Alfenas, Bressan and de Paiva, 2010; Astbury et al., 2010; Pal et al., 2014). In order to comprehend the mechanisms of the appetite regulatory effects of differing proteins, numerous studies assessed the specific characteristics (e.g. AA profile, digestibility, absorption rate) of proteins derived from differing sources (Pennings et al., 2012; Kalman, 2014; Purpura et al., 2014; Almeida et al., 2015; Melchior et al., 2023). Such studies highlighted the comprehensive AA profile (Chapter 1,**Table 2**) and a favourable absorption rate of WP (Pennings et al., 2012; Purpura et al., 2014; Moore et al., 2015). M et al., 2014; Pennings et al., 2012Thus, WP has been widely applied to nutritional supplements to assist muscle gain and weight loss (Patel, 2015b).

Meanwhile, the interest in plant-based eating patterns has also increased over the last decade, with a rising number of individuals following a vegan or vegetarian diet due to health or environmental interests (Medawar et al., 2019). It was suggested that the amount of plant material required as a feed to produce 1 kg of high-quality animal protein would contain approximately 6 kg of plant protein (Pimentel and Pimentel, 2003). Therefore, direct human consumption of

plant-derived proteins may be more environmentally sustainable than the consumption of animal-derived proteins (Tonsor, Lusk and Schroeder, 2022). Additionally, the global CO_2 equivalent greenhouse gas (CO_{2eq} GHG) emission per 100 grams of protein produced was shown to be different depending on the source, with milk, rice, potato and pea contributing 9.50, 6.27, 2.71 and 0.44 kg CO_{2eq} GHG in 2010, respectively (Poore and Nemecek, 2018). However, a few recent studies focusing on the appetite regulatory effects of various plant-based proteins, such as soy, RP, PeP or, most recently, PP, showed varying benefits as well as drawbacks between the varying sources or compared to dairy-based proteins (Gorissen et al., 2018; Tiekou Lorinczova et al., 2021). The observed differences in the appetite regulatory effects of the various proteins might be due to their AA profile, absorption rate, digestibility as well as bioactive characteristics of their digested fractions (Schaafsma, 2000; Newgard et al., 2009; Mathai, Liu and Stein, 2017; Rigamonti et al., 2020).

One of the well-established and accepted approaches for studying the digestibility of different nutrients, including proteins, is the harmonised INFOGEST *in vitro* digestion method (Santos-Hernández et al., 2020; Sousa et al., 2020; R. M. C. Ariëns et al., 2021; Brodkorb et al., 2019; Rasera et al., 2023; Zhou et al., 2023). This method has been developed and validated with the aim of harmonising *in vitro* protocols simulating human digestion while accounting for physiological conditions (Minekus et al., 2014; Egger et al., 2016).

Thus, in this study, the harmonised INFOGEST *in vitro* digestion method was applied to investigate and compare the digestibility of a novel plant-based PP isolate to the well-researched dairy-based WP as well as plant-based RP and PeP isolates, which are widely used for increasing protein content in meals and meal replacement products.

The protein isolates used in this study were of high purity, with a minimum protein content of 90% in WP, PP and RP and at least 80% in PeP isolate. These proteins are of high quality and contain all essential AAs (**Table *16***).WP and PP isolates also qualify as complete proteins based on the AA requirements. However, the RP and the PeP isolates do not qualify as complete proteins based

on the criteria defined by the Food and Nutrition Board (FNB) of the National Academy of Sciences (Meyers, Hellwig and Otten, 2006). RP and PeP isolates contain insufficient amounts of lysine and sulphur-containing AAs methionine and cysteine, respectively (**Table *16***).

Table 16. Essential amino acids profile of a typical complete protein as characterised by FNB of the National Academy of Sciences compared to whey, potato, rice and pea protein isolates used in the current study.

Essential AA [mg/g of protein]	Complete protein	Whey protein isolate	Potato protein isolate	Rice protein isolate	Pea protein isolate
Tryptophan	7	14	10	11	8
Threonine	27	67	62	36	31
Isoleucine	25	64	43	44	41
Leucine	55	106	93	82	68
Lysine	51	96	66	*32	61
Methionine + Cystine	25	44	31	48	*17
Phenylalanine + Tyrosine	47	56	108	102	77
Valine	32	59	47	62	43
Histidine	17	17	17	21	20

Amino acid composition as provided by the manufacturers. *Indicates amino acid levels lower than required to be characterised as a complete protein (Meyers, Hellwig and Otten, 2006).

Most plant-based proteins (such as pea, rice, and wheat) are insufficient to sustain human health due to their unbalanced AA profile. In addition, they may contain antinutritional components, such as protease inhibitors, that decrease protein digestibility, further reducing the pool of readily available AA from these sources (Amin et al., 2022). A widely applied approach to improve the nutritional quality of plant-based proteins is the use of protein blends. Blending plant-based proteins with WP, although not vegan, could provide a complete protein source with potentially increased appetite regulatory effect while reducing the production-related environmental impact (Alvarez-Jubete, Arendt and Gallagher, 2010; Sá, Moreno and Carciofi, 2020; Duque-Estrada et al., 2023).

Thus, this study aims to identify and compare the digestibility of plant-based, PP, RP and PeP to WP and their blends in ratios of 50:50 and 30:70 (WP to plant

protein) by observing changes in their fractions and analysing the free AA release during *in vitro* digestion.

3.2. Results

3.2.1 Enzyme activity

3.2.1.1 Pepsin

The activity of pepsin was identified as the key factor in gastric digestion via inter-laboratory studies. Variability in pepsin activity was shown to result in fluctuating protein hydrolysis during the gastric digestion phase (Minekus et al., 2014; Egger et al., 2016; Brodkorb et al., 2019). To achieve harmonisation between laboratories, the INFOGEST protocol suggests the use of pepsin with a final concentration of 2000 U/mL (Brodkorb et al., 2019). Thus, the porcine pepsin activity (U/mg) was assessed with a classic spectrophotometric enzymatic assay adapted from Anson involving 10 min of haemoglobin hydrolysis (Anson and Mirsky, 1932; Anson, 1938). The mean calculated activity of the porcine pepsin based on six different enzyme concentrations (10, 15, 20, 25, 30 and 35 µg/mL) was 448.7 (± 25.5) U/mg (**Table *17***).

Table 17. Parameters used for the estimation of pepsin activity.

Pepsin concentrations (µg/mL) = µg in assay	Test (A 280 nm)	Blank (A 280 nm)	Δ A Test (280 nm)	Activity (U/mg)
10	0.6097	0.567	0.043	425.5
15	0.62985		0.063	418.0
20	0.65815		0.091	455.0
25	0.677		0.110	439.4
30	0.70925		0.142	473.7
35	0.73535		0.168	480.6
			Average	**448.7**
			St dev	**25.5**

3.2.1.2 Pancreatin

As pancreatin comprises a mixture of digestive enzymes in differing amounts, the final concentration of pancreatin (100 U trypsin activity/mL of digesta) used during the intestinal digestion phase was based on the activity of trypsin, the

primary proteolytic enzyme in the lumen of the small intestine. The measurement of trypsin activity in porcine pancreatin observed with kinetic measurements over 10 min showed that the concentration of 0.4, 0.7 and 1 mg/mL reached a plateau due to cleaving all available substrates. However, a linear phase with 0.1 mg/mL concentration was not observed; therefore, this concentration was excluded from the following calculations to estimate trypsin activity expressed in U/mg (**Figure *13***).

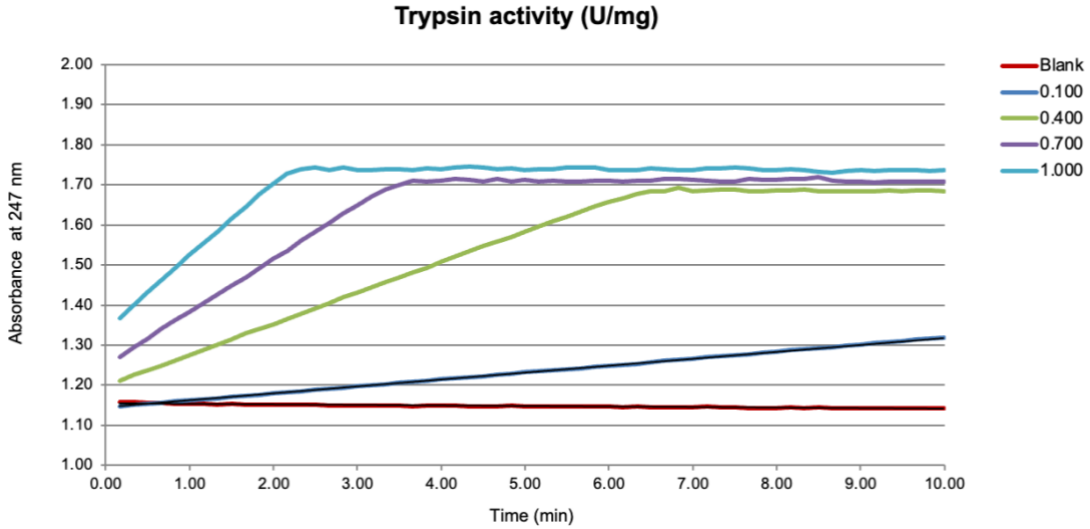


Figure 13. Trypsin activity observed over a duration of 10 min. Trypsin activity was measured by the proteolytic cleavage of its substrate TAME (p-toluene-sulfonyl-L-arginine methyl ester) at different concentrations of pancreatin used (Blank 0, 0.1, 0.4, 0.7 and 1 mg/mL). The release of p-Toluene-Sulfonyl-L-Arginine was calorimetrically detected (λ = 247nm).

The blank measurement slope and the slope of the initial linear phase of trypsin's kinetic activity with 0.4, 0.7 and 1 mg/mL of pancreatin concentration were established. Using the data presented in **Table *18***, the trypsin activity was calculated to be 10.5 (±0.2) U/mg.

Table 18. Parameters used for the estimation of trypsin activity within the pancreatin.

Volume of reaction mixture (mL)	Molar extinction coefficient TAME (247 nm)	Stock Solution (mg/mL)	Concentrations tested (mg/mL)	Volume of enzyme solution (mL)	Amount of trypsin (mg)	Blank Slope	Enzyme Slope	Trypsin activity (U/mg)
3	540	1.0	*0.100	0.100	0.010	-0.002	0.017	2.700
			0.400	0.100	0.040		0.075	10.740
			0.700	0.100	0.070		0.130	10.480
			1.000	0.100	0.100		0.182	10.250
							Average	10.500
							St dev	0.200

*Measurements of 0.1 mg/mL pancreatin concentration were excluded from the final calculation.

3.2.2. Digestion of protein isolates

The suitability of the in-house adjustments of the INFOGEST digestion protocol was analysed by assessing the protein hydrolysis of WP with an initial protein concentration of 100 mg/mL The protein hydrolysis was analysed with Coomassie-stained SDS PAGE after the 2-minute oral phase and after every 15 minutes during gastric (**Figure *14***) and intestinal digestion phases (**Figure *15***). An equal load of WP was added to each lane with WP digesta. A molecular weight marker, an undigested WP control, as well as blank digesta were included. Protein bands indicated by arrows are proteins that were previously identified as protein fractions of WP.

As evident in **Figure *14***, WP was unaffected during the oral phase of the digestion, with all bands present comparably to undigested WP. However, by the end of the gastric phase of digestion, only one band was predominantly present, namely β-lg, compared to undigested WP, which was unaffected without the presence of pepsin. Additionally, while α-lac is clearly present in the WP control, it was hydrolysed and not detected within the WP digesta by the end of the gastric phase.

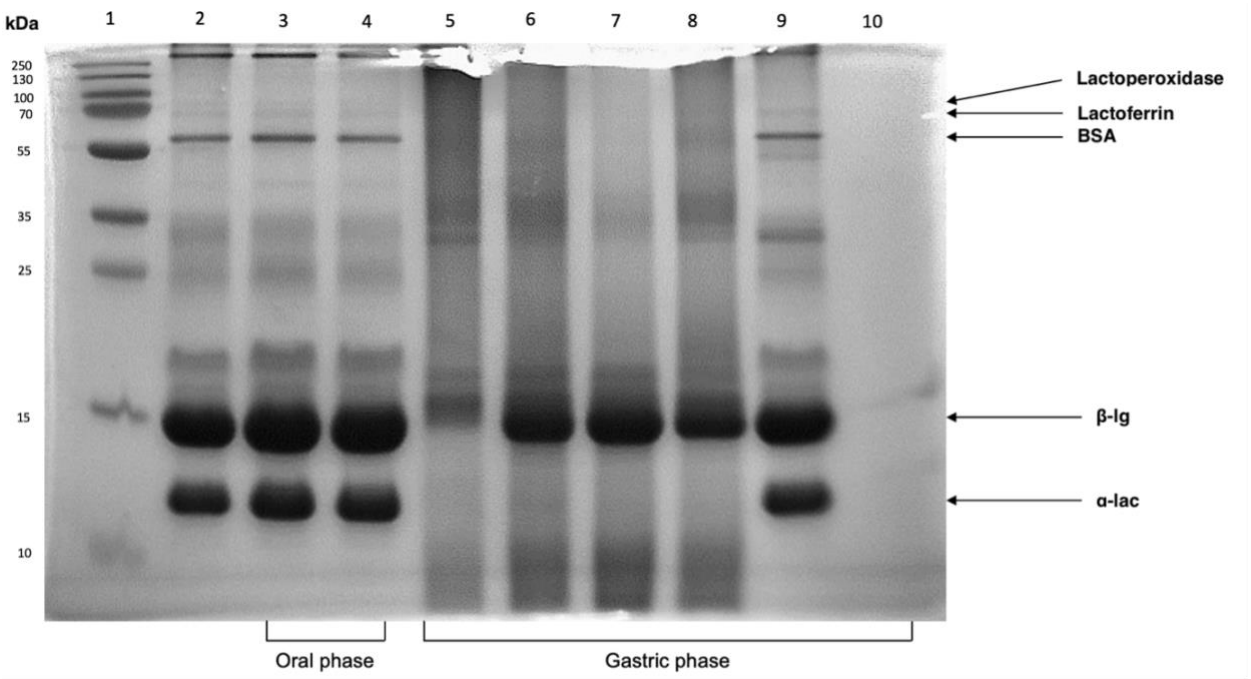


Figure 14. SDS-PAGE patterns of whey protein isolate (WP) following oral and gastric phase digestion. Lane 1, protein marker; **Lane 2**, WP (100 mg/mL); **Lane 3**, WP oral phase 2 min; **Lane 4**, food control (FC) oral phase 2min; **Lane 5**, WP gastric phase 15 min; **Lane 6**, WP gastric phase 30 min; **Lane 7**, WP gastric phase 45 min; **Lane 8**, WP gastric phase 60 min; **Lane 9**, FC gastric phase 60 min; **Lane 10**, Blank Digestion (BD). Samples loaded into each lane contained 10 µg of protein.

In contrast, by the end of the intestinal phase, β-lg was also digested, and there were no protein bands from the WP digest detected, while the WP control showed no presence of proteolysis without the addition of digestive enzymes (**Figure *15***). Additionally, there were no significant differences detected between the 15-minute timepoints within the same digestion phases (**Figure *14***, **Figure *15***). The visible bands on the SDS-PAGE gel, other than the molecular weight ladder and WP control, are digestive enzymes originating from pancreatin.

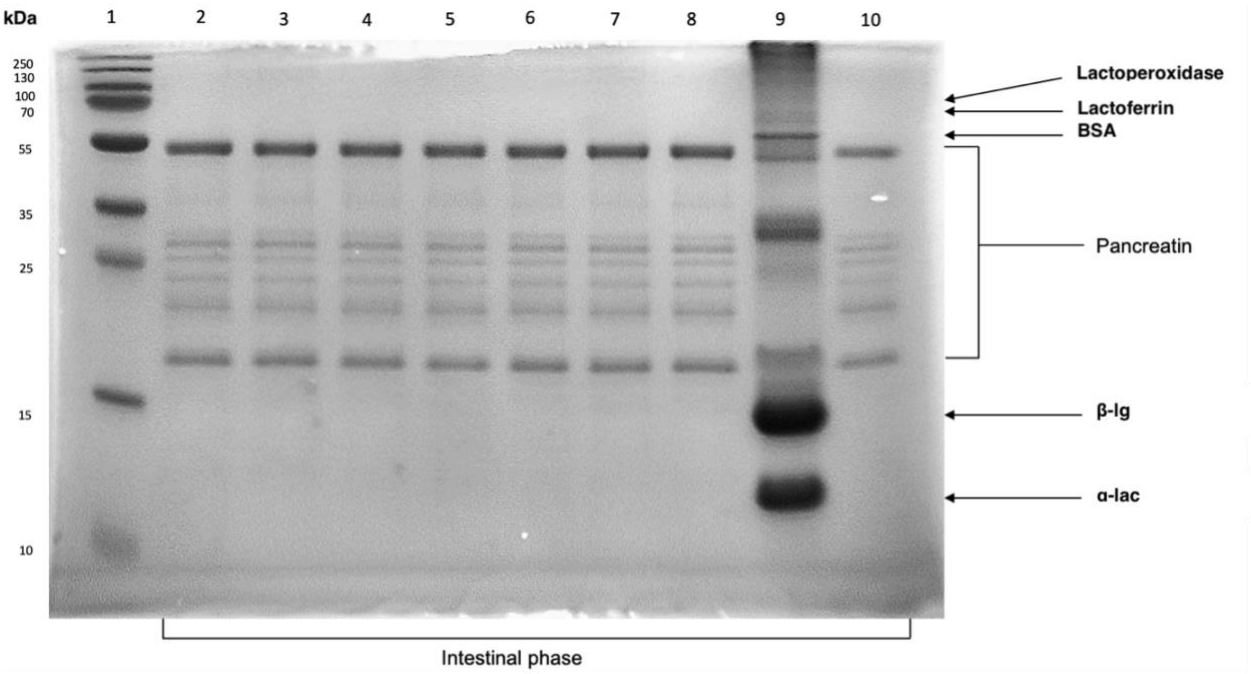


Figure 15. SDS-PAGE patterns of WP digests following the intestinal phase digestion. Lane 1, protein marker; **Lane 2**, WP intestinal phase 30 min; **Lane 3**, WP intestinal phase 45 min; **Lane 4**, WP intestinal phase 60 min; **Lane 5**, WP intestinal phase 75 min; **Lane 6**, WP intestinal phase 90min; **Lane 7**, WP intestinal phase 105min; **Lane 8**, WP intestinal phase 120 min; **Lane 9**, FC intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

3.2.3. SDS-PAGE gel electrophoresis

Similarly, the hydrolysis of protein isolates with both initial protein concentrations of 100 mg/mL and 200 mg/mL was analysed with Coomassie-stained SDS PAGE, with the aim of understanding the differences in the extent of protein breakdown between the four different protein isolates as well as two different concentrations. Samples used for the analysis were obtained every 30 minutes during the gastric and intestinal phases of the *in vitro* digestion. Each lane containing the digesta was loaded with an equal amount (10 µg) of protein. Additionally, a molecular weight marker, non-digested protein isolate solutions and blank digesta controls were also loaded onto the gel. Bands indicated by arrows are previously identified fractions of protein isolates.

The electrophoresis analysis of WP (100 mg/mL) showed by the end of the gastric phase, all major protein fractions were hydrolysed except for β-lg. However, β-lg was also undetectable during the intestinal phase, comparable to our previous WP (100 mg/mL) digestion. Additionally, the pancreatin-derived digestive enzymes were clearly identified (**Figure 16**).

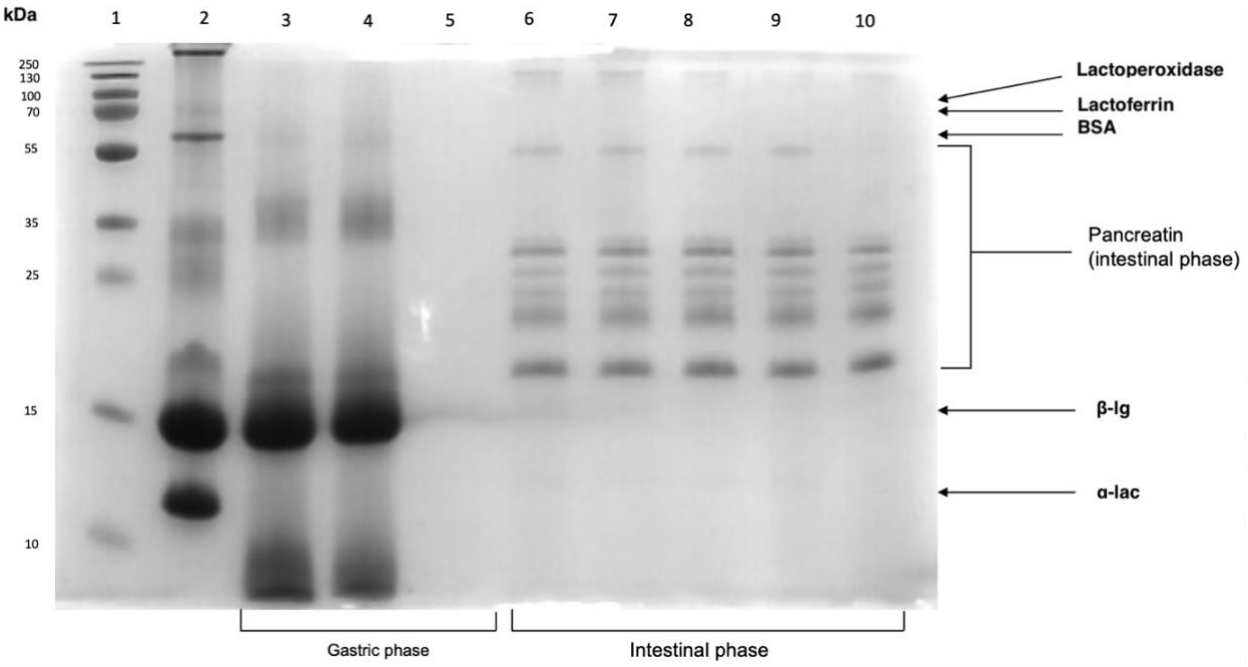


Figure 16. SDS-PAGE patterns of whey protein isolate (WP) digests following the gastric and intestinal phase digestions (initial protein concentration 100 mg/mL). Lane 1, protein marker; **Lane 2**, WP (100 mg/mL); **Lane 3**, WP gastric phase 30 min; **Lane 4**, WP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, WP intestinal phase 30 min; **Lane 7**, WP intestinal phase 60 min; **Lane 8**, WP intestinal phase 90 min; **Lane 9**, WP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples load*ed* into each lane contained 10 µg of protein.

The analysis of WP (200 mg/mL) digests indicated complete digestion with no protein fraction bands detectable at the end of the intestinal phase. However, there are fewer pancreatin-derived digestive enzymes detected compared to BD (**Figure *17***).

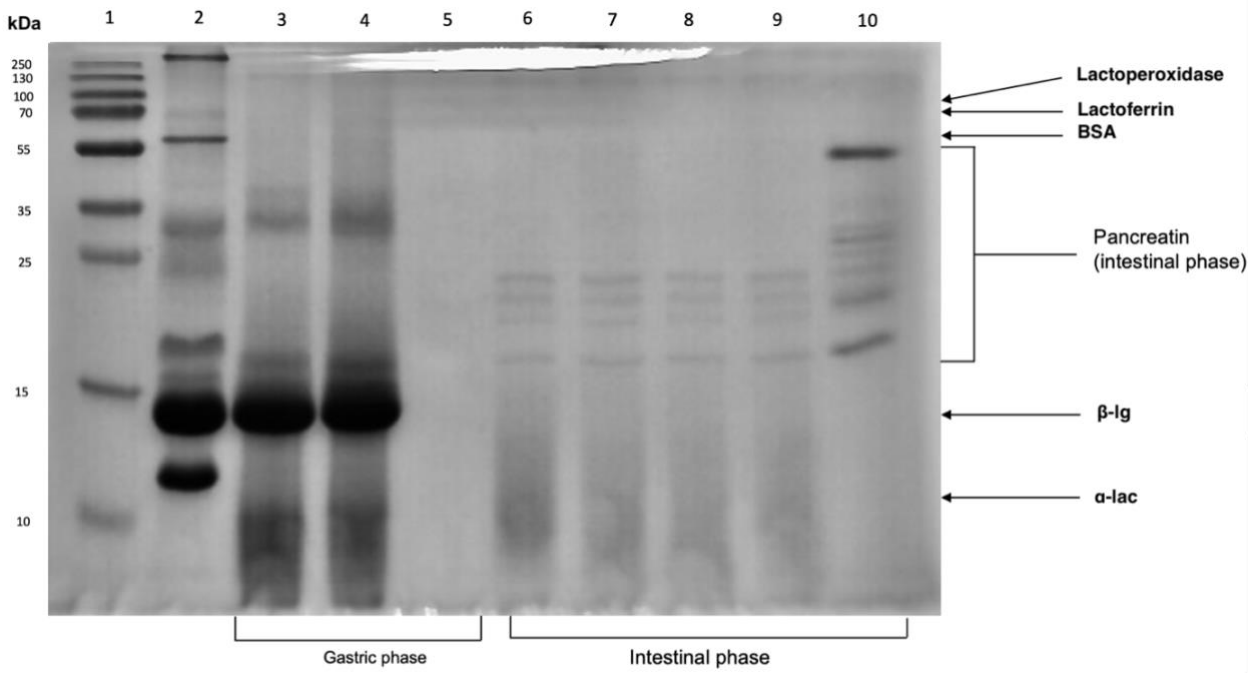


Figure 17. SDS-PAGE patterns of whey protein isolate (WP) digests following the gastric and intestinal phase digestions (initial protein concentration 200 mg/mL). Lane 1, protein marker; **Lane 2**, WP (200 mg/mL); **Lane 3**, WP gastric phase 30 min; **Lane 4**, WP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, WP intestinal phase 30 min; **Lane 7**, WP intestinal phase 60 min; **Lane 8**, WP intestinal phase 90 min; **Lane 9**, WP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

Electrophoresis analysis of the undigested sample revealed that PP was primarily comprised of patatin (migrating at about 43 kDa). There were also two main bands present representing high molecular weight proteins (migrating at about 100 and 180 kDa). Additionally, lower intensity bands were also noted as the protease inhibitors (ranging from 22 to 9 kDa). It is evident on the SPS-PAGE gel that the PP with the initial concentration of 100 mg/mL was partially hydrolysed at the end of the gastric phase, with only the patatin and protease inhibitor bands remaining detectable. By the end of the intestinal phase, the patatin band also diminished. However, protease inhibitors were still detectable, although at a lower rate. The pancreatin-derived digestive enzyme bands detected within the intestinal digesta samples were comparable to those observed in the BD (**Figure 18**).

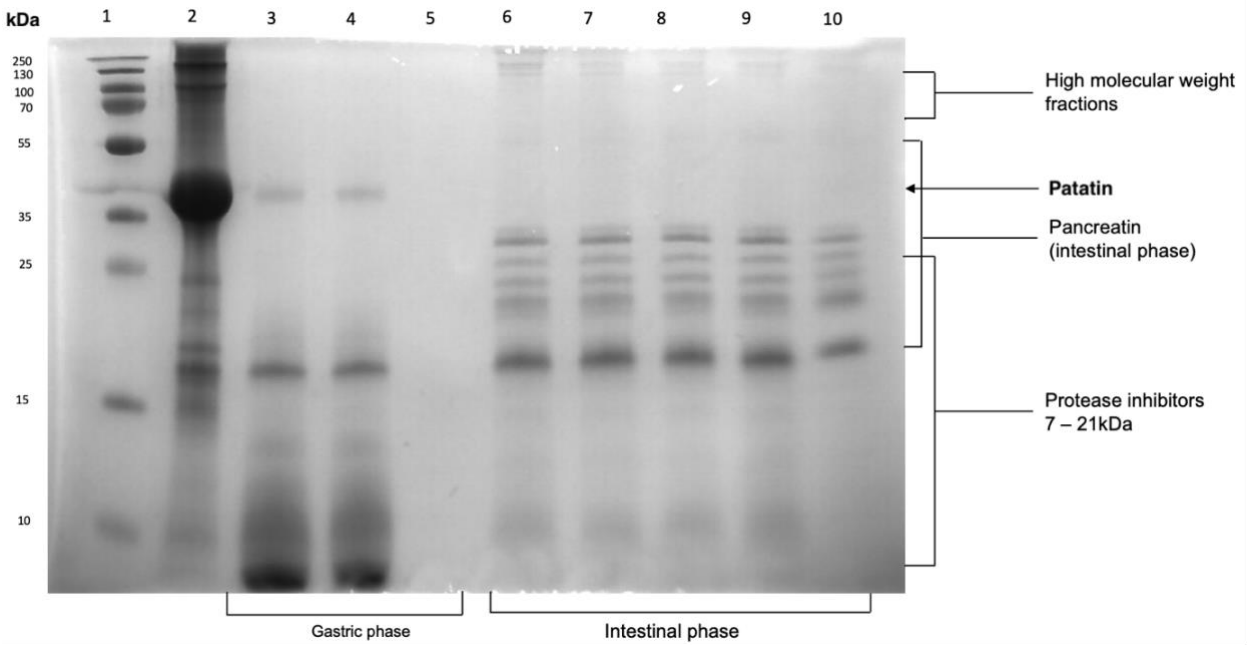


Figure 18. SDS-PAGE patterns of potato protein isolate (PP) digests following the gastric and intestinal phase digestions (initial protein concentration 100 mg/mL). Lane 1, protein marker; **Lane 2**, PP (100 mg/mL); **Lane 3**, PP gastric phase 30 min; **Lane 4**, PP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, PP intestinal phase 30 min; **Lane 7**, PP intestinal phase 60 min; **Lane 8**, PP intestinal phase 90 min; **Lane 9**, PP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

When analysing the hydrolysis of PP with 200 mg/mL initial concentration, the visualised band pattern seems to be comparable to the lower protein concentration digesta except for the detected protease inhibitors. Protease inhibitors seem to be detectable at a higher rate following the intestinal phase compared to the lower initial concentration. Additionally, there are fewer pancreatin-derived digestive enzymes detected compared to BD (**Figure 19**).

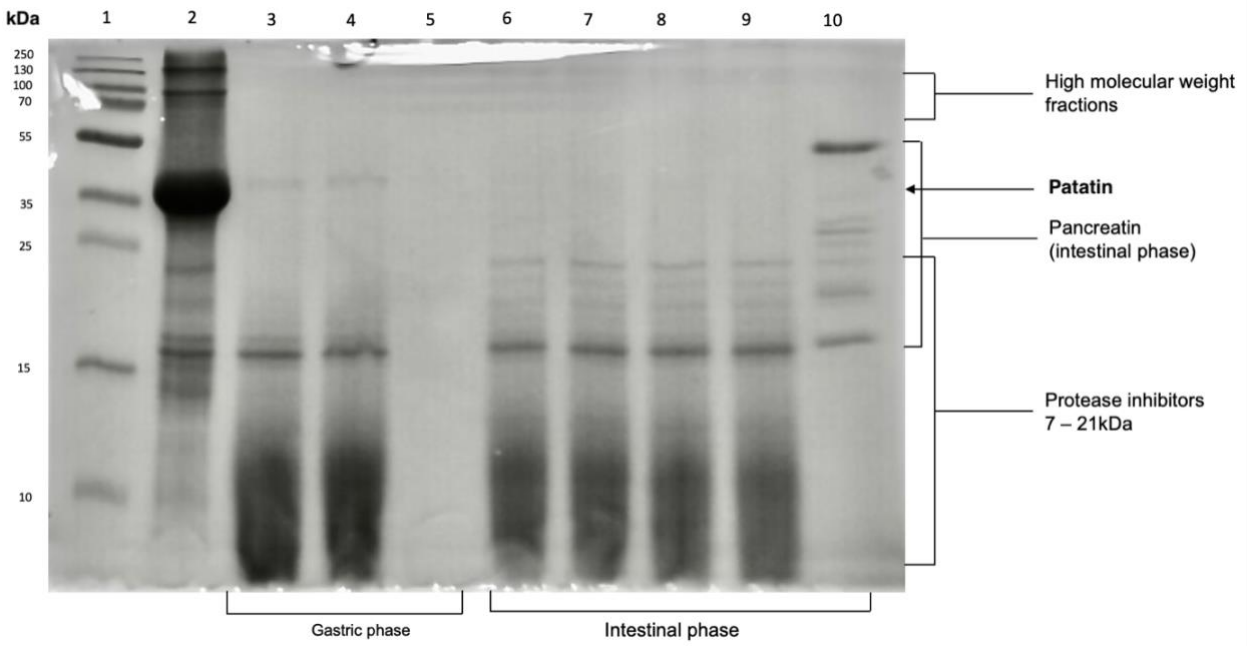


Figure 19. SDS-PAGE patterns of potato protein isolate (PP) digests following the gastric and intestinal phase digestions (initial protein concentration 200 mg/mL). Lane 1, protein marker; **Lane 2**, PP (200 mg/mL); **Lane 3**, PP gastric phase 30 min; **Lane 4**, PP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, PP intestinal phase 30 min; **Lane 7**, PP intestinal phase 60 min; **Lane 8**, PP intestinal phase 90 min; **Lane 9**, PP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

Observing **Figure 20**, it is evident that in the RP control with 100 mg/mL protein concentration, only prolamin (~ 13 kDa) was detected with SDS-PAGE, however in the gastric phase samples (30 and 60 min), the protein band corresponding to glutelin (was also visible). By the end of the intestinal phase, glutelin was undetectable. However, the prolamin remained present in the digesta samples. The pattern of pancreatin-derived digestive enzyme bands within the digesta samples was comparable to that observed in the BD.

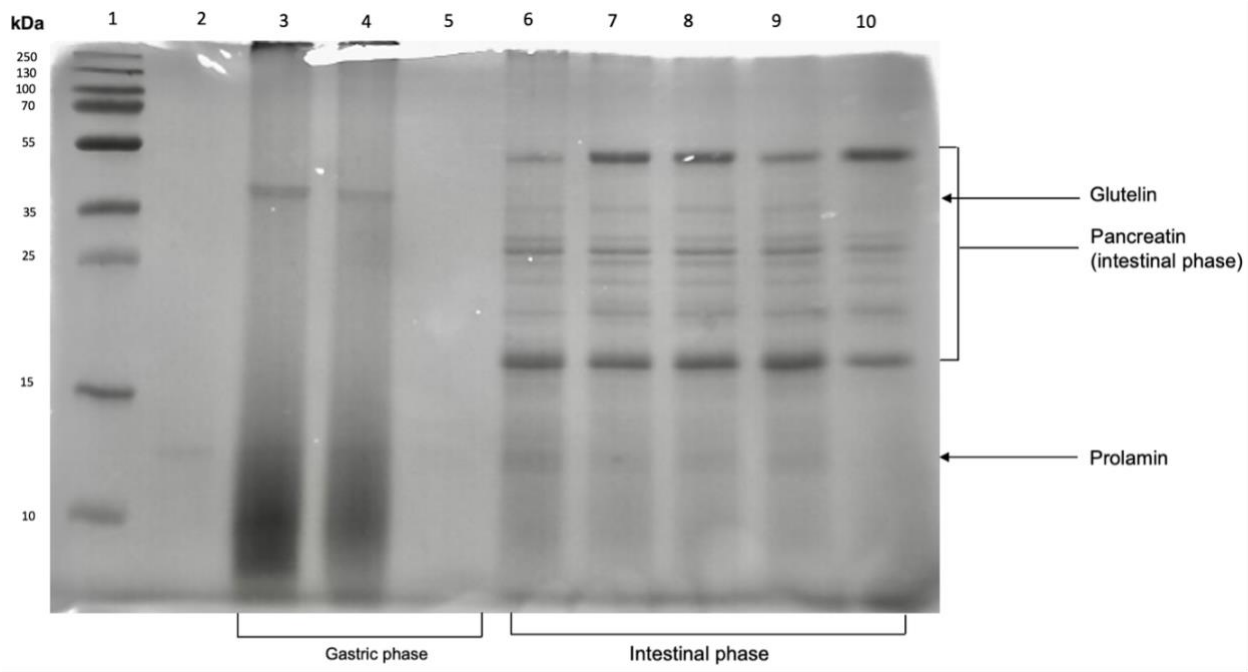


Figure 20. SDS-PAGE patterns of rice protein isolate (RP) digests following the gastric and intestinal phase digestions (initial protein concentration 100 mg/mL). Lane 1, protein marker; **Lane 2**, RP (100 mg/mL); **Lane 3**, RP gastric phase 30 min; **Lane 4**, RP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, RP intestinal phase 30 min; **Lane 7**, RP intestinal phase 60 min; **Lane 8**, RP intestinal phase 90 min; **Lane 9**, RP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

Similarly, the SDS-PAGE analysis of RP digesta with the initial concentration of 200 mg/mL showed that only the prolamin protein band was detectable in the undigested RP control. However, during the gastric digestion phase glutelin was also observable. Comparably to the RP digesta with lower protein concentration, the prolamin band was still visible by the end of the intestinal digestion phase. However, less of the residual pancreatin-derived enzyme bands were evident compared to BD (**Figure 21**).

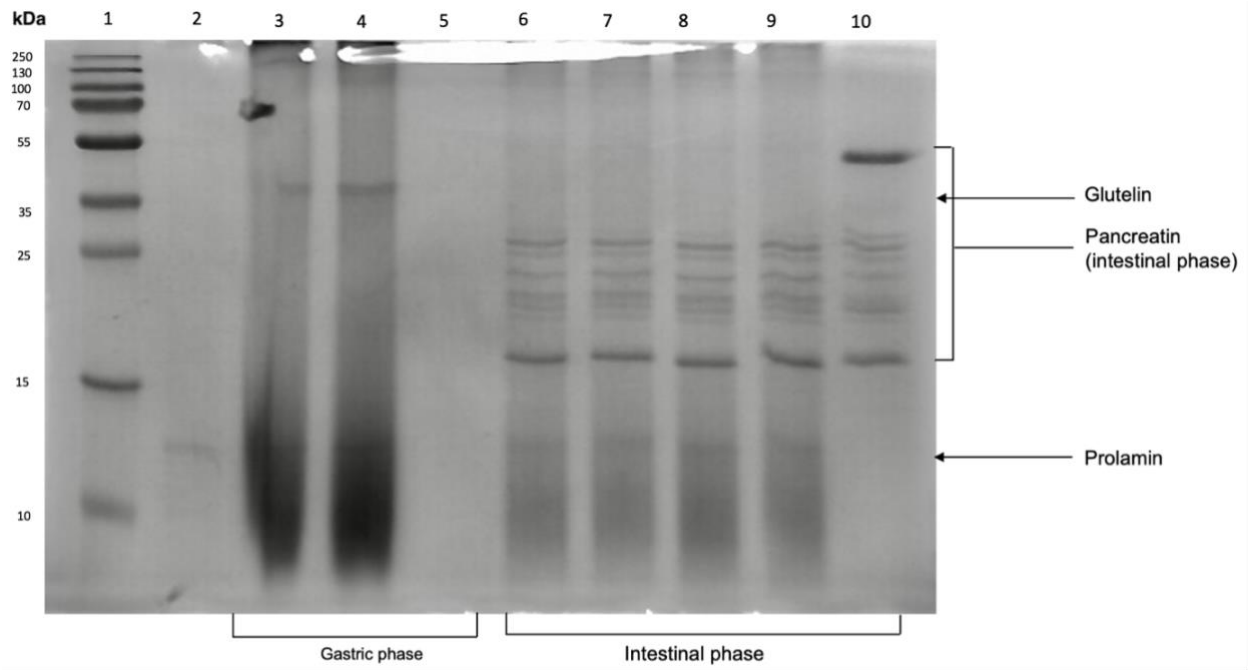


Figure 21. SDS-PAGE patterns of rice protein isolate (RP) digests following the gastric and intestinal phase digestions (initial protein concentration 200 mg/mL). Lane 1, protein marker; **Lane 2**, RP (200mg/mL); **Lane 3**, RP gastric phase 30 min; **Lane 4**, RP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, RP intestinal phase 30 min; **Lane 7**, RP intestinal phase 60 min; **Lane 8**, RP intestinal phase 90 min; **Lane 9**, RP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

The electrophoretic profiles of PeP digesta at different stages of gastric and intestinal digestion are shown in Figures 18 and 19. The different globulin fractions: legumin (23 and 20 kDa), vicilin (54–50, 37–34 and 15-13 kDa) and convicilin (80–75 kDa), as well as albumin fractions: PA1 (~14 kDa), PA2 (~20 kDa) of undigested PeP dispersion, are shown in lane 2 and where identified according to the previous literature (Lu et al., 2019; Shanthakumar et al., 2022). During the digestion, a new protein band with an approximate size of 40 kDa was also detected. Additionally, by the end of the gastric digestion phase, the remanence of legumin, PA1, and PA2 were still evident. During the intestinal phase, a protein band with an approximate size of 14 kDa was detectable within the digests as well as the control BD sample, while the residual pancreatin-derived enzyme bands were less evident compared to BD (**Figure 22**).

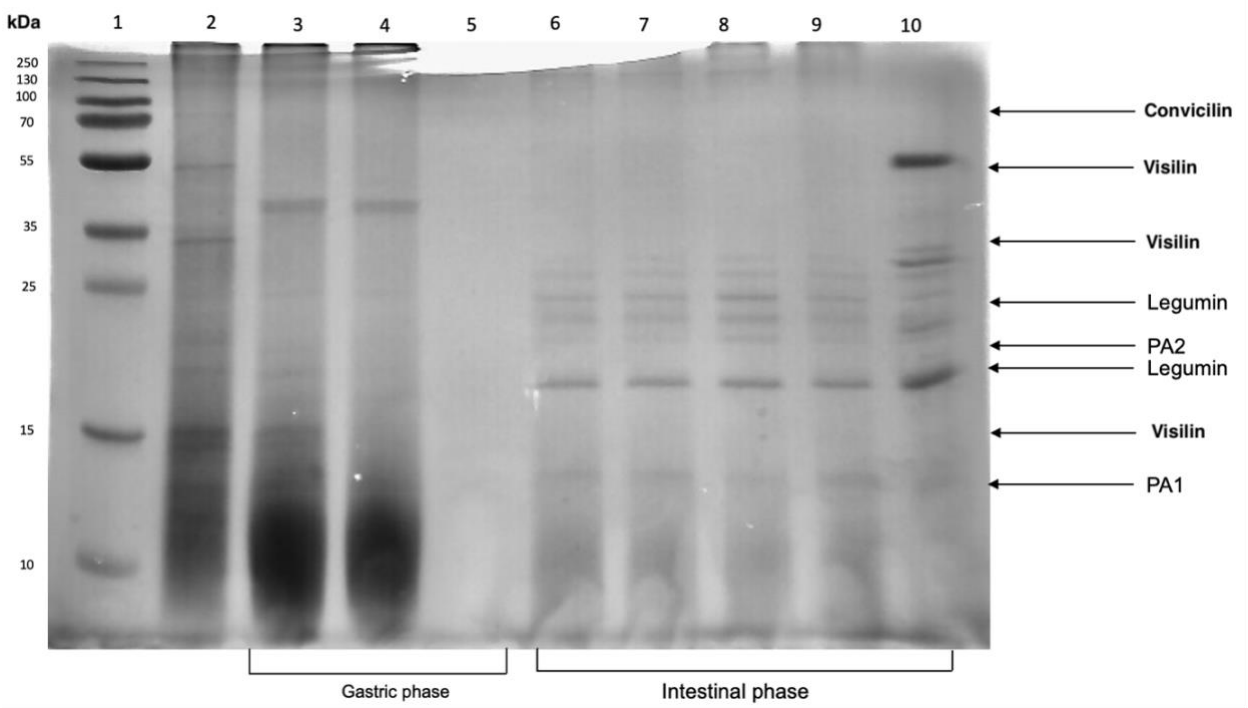


Figure 22. SDS-PAGE patterns of pea protein isolate (PeP) digests following the gastric and intestinal phase digestions (initial protein concentration of 100 mg/mL). Lane 1, protein marker; **Lane 2**, PeP (100 mg/mL); **Lane 3**, PeP gastric phase 30 min; **Lane 4**, PeP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, PeP intestinal phase 30 min; **Lane 7**, PeP intestinal phase 60 min; **Lane 8**, PeP intestinal phase 90 min; **Lane 9**, PeP intestinal phase 120 min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

The analysis of the PeP digesta with an initial protein concentration of 200 mg/mL was comparable to the one with an initial concentration of 100 mg/mL. By the end of the gastric digestion phase, the remanence of legumin, PA1, PA2, and visilin was still evident, and the new protein band, with an approximate size of 40 kDa, was also detectable. However, the ~14 kDa protein band previously observed with the lower protein concentration digesta, as well as the control BD, was not detected when analysing PeP digesta with 200 mg/mL initial concentration. Additionally, the residual pancreatin-derived enzyme bands were less evident compared to BD (**Figure 23**).

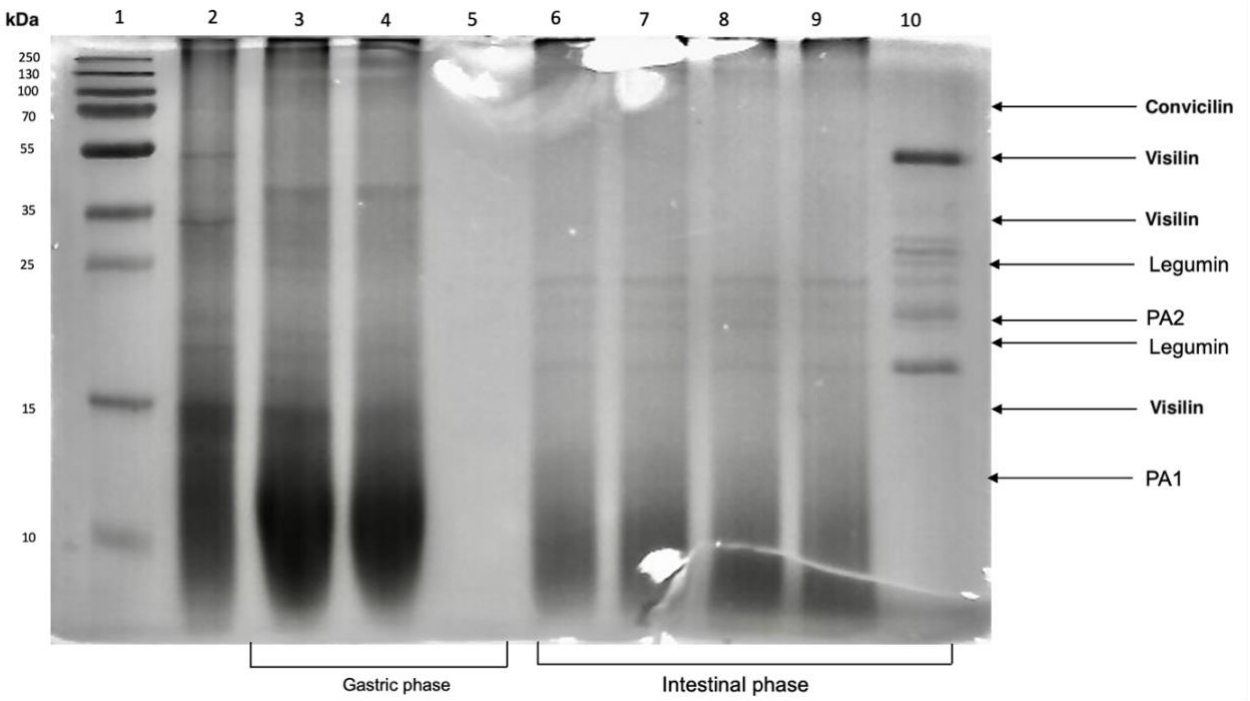


Figure 23. SDS-PAGE patterns of pea protein isolate (PeP) digests following the gastric and intestinal phase digestions (initial protein concentration 200mg/mL). Lane 1, protein marker; **Lane 2**, PeP (200mg/mL); **Lane 3**, PeP gastric phase 30 min; **Lane 4**, PeP gastric phase 60 min; **Lane 5**, BD gastric phase 60 min; **Lane 6**, PeP intestinal phase 30 min; **Lane 7**, PeP intestinal phase 60 min; **Lane 8**, PeP intestinal phase 90min; **Lane 9**, PeP intestinal phase 120min; **Lane 10**, BD intestinal phase 120 min. Samples loaded into each lane contained 10 µg of protein.

3.2.4 OPA

The OPA analysis was utilised to determine the percentage digestibility of the protein isolates and their blends used in this study based on the measured L-Leucine equivalent free AA concentration of gastric and intestinal phase end-point samples in triplicate.

The results of OPA spectrophotometric assay analysis of unblended and blended protein isolates following gastric and intestinal phase digestion are summarised in **Table *19***, where the mean results and standard deviations of sample triplicates are presented in mg/mL as well as % L-Leucine equivalent concentration.

Table 19. The concentration of free amino acids in the digested protein samples.

	Gastric phase		Intestinal phase	
	L-Leuc eq. concentration mean (±SD) mg/mL	Concentration mean (±SD) of initial 200mg/mL %	L-Leuc eq. concentration mean (±SD) mg/mL	Concentration mean (±SD) of initial 200mg/mL %
WP	3.72 (0.42)	1.86 (0.21)	8.08 (1.06)	4.04 (0.53)
PP	6.99 (0.34)	3.49 (0.17)	6.97 (1.11)	3.49 (0.55)
RP	2.42 (0.95)	1.21 (0.47)	7.06 (1.22)	3.53 (0.61)
Pep	6.57 (0.84)	3.29 (0.42)	6.75 (0.68)	3.38 (0.34)
WP/PP (50:50)	6.58 (0.57)	3.29 (0.29)	6.23 (1.21)	3.12 (0.60)
WP/RP (50:50)	3.80 (0.36)	1.90 (0.18)	8.47 (0.56)	4.24 (0.28)
WP/PeP (50:50)	5.94 (0.48)	2.97 (0.24)	6.54 (1.94)	3.27 (0.97)
WP/PP (30:70)	7.29 (0.29)	3.64 (0.14)	7.11 (0.94)	3.55 (0.47)
WP/RP (30:70)	2.98 (0.13)	1.49 (0.07)	8.96 (0.54)	4.48 (0.29)
WP/PeP (30:70)	4.86 (0.11)	2.43 (0.06)	7.87 (0.59)	3.93 (0.29)

The evaluation of digestibility of proteins using OPA spectrophotometric assay showed that the release of free AA or bioaccessible fractions after gastric phase digestion, when comparing unblended protein isolates, was significantly higher with PP compared to WP (1.63%, CI 2.38 to 0.88, p <0.0001) and RP (2.28%, CI 1.53 to 3.03, p <0.0001). Additionally, free AA concentration after gastric phase digestion was also significantly higher with PeP compared to WP (1.42%, CI 2.17

to 0.67, p <0.0001) and RP (2.07%, CI 2.82 to 1.32, p <0.0001) (**Figure *24***). No significant differences between the digests of different unblended proteins were observed in free AA concentration after the intestinal phase digestion.

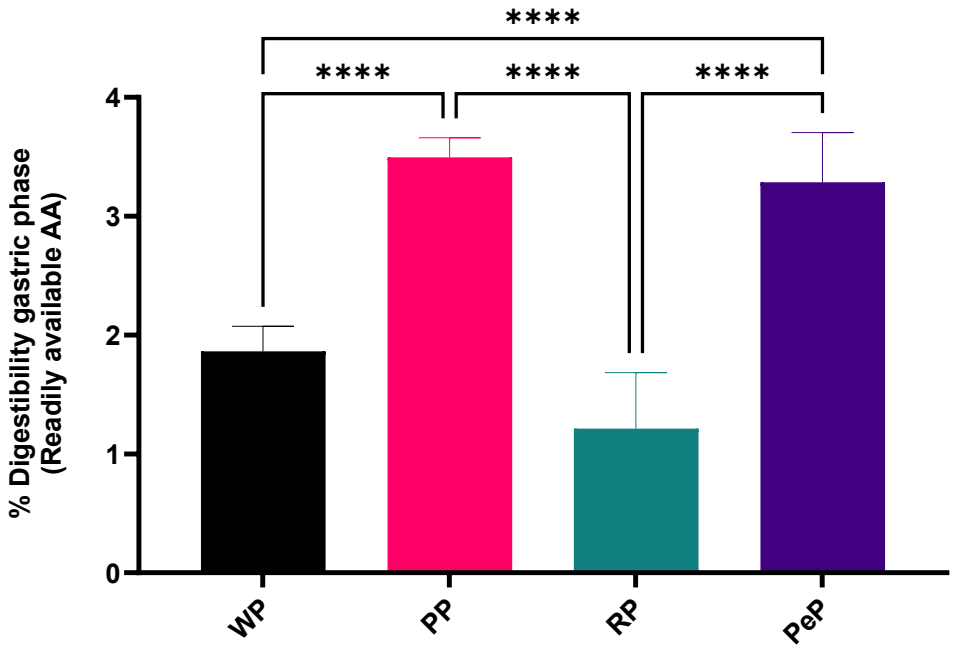


Figure 24. L-Leucine equivalent free amino-acid (AA) concentration (%) in unblended protein digests following gastric phase digestion. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly higher in potato protein (PP) vs whey (WP) and rice protein (RP) and in pea protein (PeP) vs WP and RP following gastric phase digestion (**** p <0.0001 in all cases).

The analysis of free AAs released from unblended protein isolates during digestion showed significantly higher concentrations at intestinal phase end-points compared to gastric phase end-points with WP and RP isolates, with mean differences of 2.18 ± 0.41% (p = 0.0029) and 2.32 ± 0.41% (p = 0.0019), respectively. No significant differences were observed over time with PP and PeP isolates (**Figure *25***).

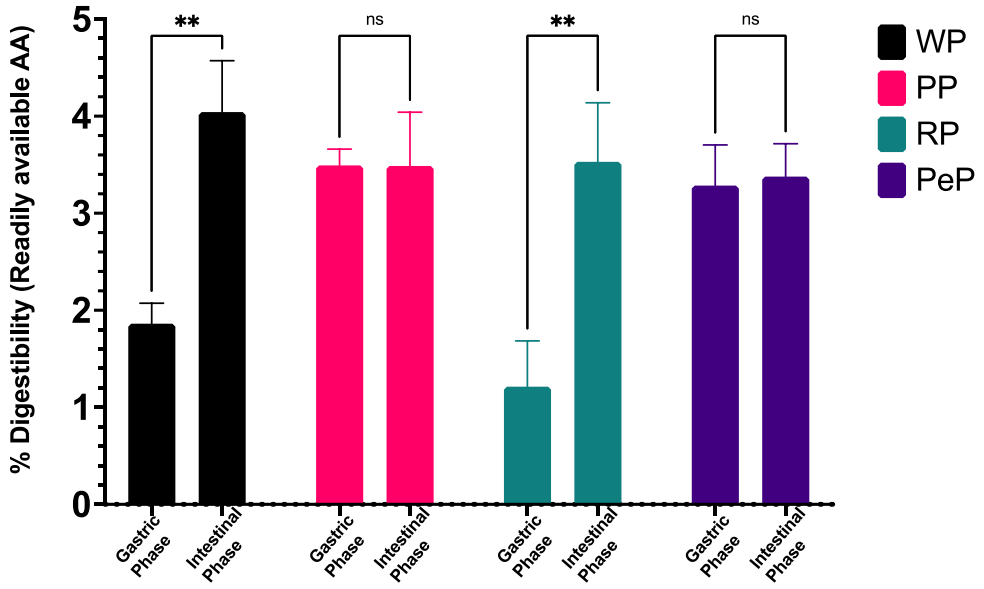


Figure 25. L-Leucine equivalent free amino-acid (AA) concentration (%) in unblended protein isolate digests following gastric and intestinal phase digestion (n=3 for each group). Results are presented as mean ± standard deviation. Data was analysed using a mixed effect model, Type III test, followed by Tukey's test for multiple comparison correction. Concentration of readily available AA was significantly higher in whey protein (WP) and rice protein (RP) following intestinal phase digestion vs gastric digestion (** $p < 0.01$, ^{ns} $p > 0.05$).

The analysis of bioaccessible fractions post gastric digestion in WP and PP isolate blends in a ratio of 50:50 and 30:70, respectively, showed that blending PP with WP resulted in significantly higher concentrations of free AA within the digests compared to unblended WP isolate digests with the same initial concentration. The observed significant differences were 1.43% (CI 2.18 to 0.68) and 1.78% (CI 2.53 to 1.03) between WP and the blended protein isolates at 50:50 and 30:70 ratios, respectively, with significance of p <0.0001. There were no significant differences observed between the unblended PP digests and the digests of WP and PP isolate blends or between the digests of blended proteins with differing ratios (**Figure *26***).

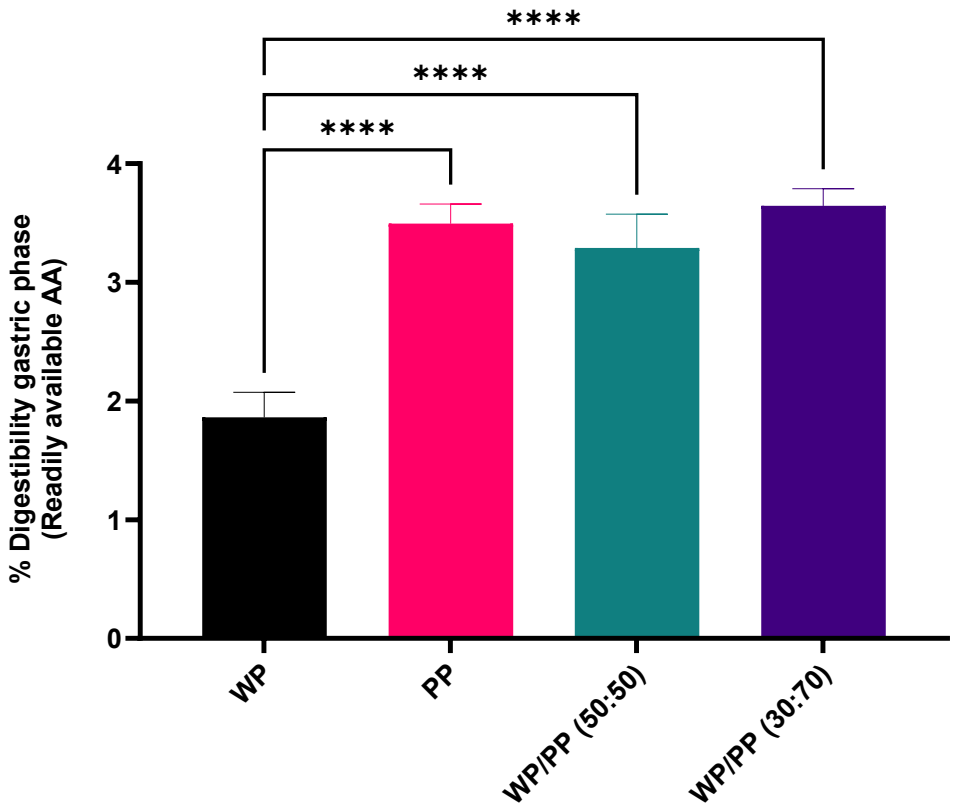


Figure 26. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of blended whey (WP) and potato protein (PP) isolates in a ratio of 50:50 and 30:70, respectively, following gastric phase digestion in comparison to unblended WP and PP isolates. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly lower in WP vs PP as well as WP and PP blends in both 50:50 and 30:70 ratios following gastric phase digestion (**** p <0.0001 in all cases).

The analysis of bioaccessible fractions in intestinal end-point samples of WP and PP isolate blends in a ratio of 50:50 showed that blending PP with WP resulted in significantly lower concentrations of free AA within the digests compared to unblended WP isolate digests with the same initial concentration. The observed significant difference was 1.60% (CI 0.21 to 2.99) with a significance of p = 0.0172. There were no significant differences observed between the intestinal digest blend of WP and PP isolates at the ratio of 30:70 and other relevant conditions of intestinal end-point digests presented in **Figure *27***.

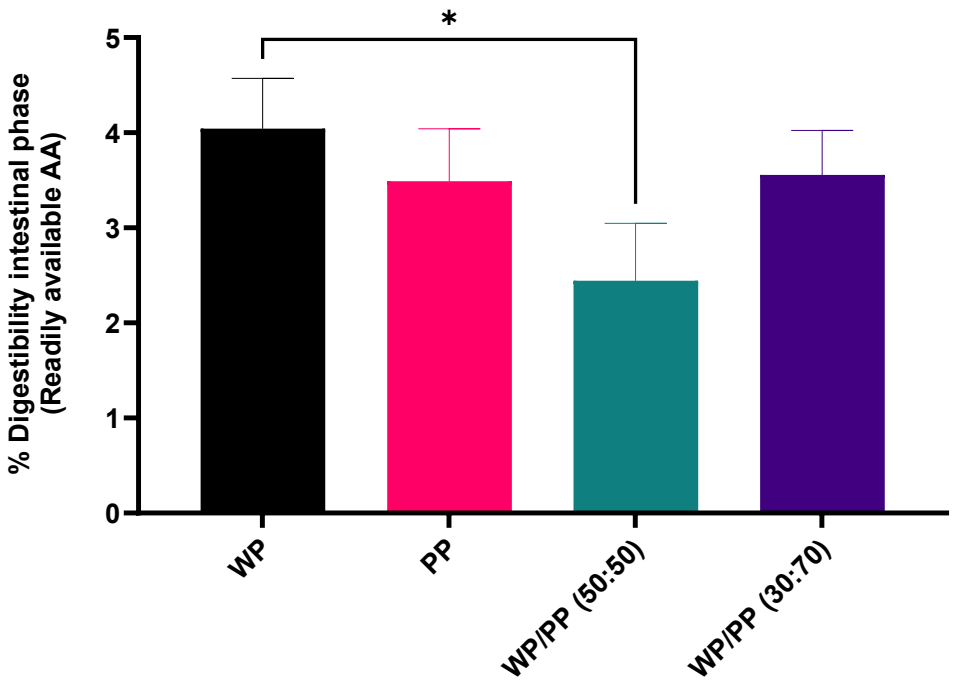


Figure 27. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of blended whey (WP) and potato protein (PP) isolates in a ratio of 50:50 and 30:70, respectively, following intestinal phase digestion in comparison to unblended WP and PP isolates. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly higher in WP vs WP and PP blend in a 50:50 ratio following intestinal phase digestion (* p <0.05).

The analysis of bioaccessible fractions post gastric digestion in WP and PeP isolate blends in a ratio of 50:50 showed that blending WP and PeP isolates resulted in significantly higher concentrations of free AA within the digests compared to unblended WP isolate digests with the same initial concentration with the mean difference of 1.11% (CI 1.86 to 0.36, p = 0.0013). There were no significant differences observed between the digests of WP and PP isolate blends in a ratio of 30:70 and unblended WP digests; however, there was a significant reduction observed compared to the digests of unblended PeP isolates, with the mean difference of 0.86% (CI 0.11 to 1.61, p = 0.0176) (**Figure *28***). No significant differences between the digests of blended WP and PeP isolates and unblended WP and PeP digests were observed in free AA concentration after the intestinal phase digestion.

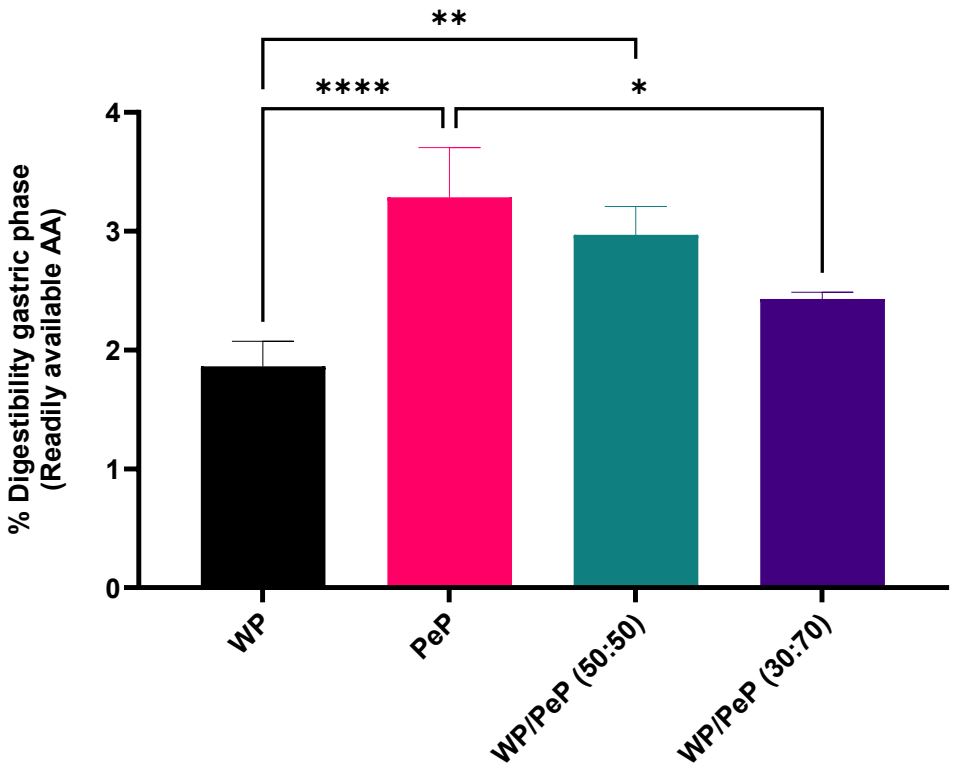


Figure 28. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of blended whey (WP) and pea protein (PeP) isolates in a ratio of 50:50 and 30:70, respectively, following gastric phase digestion in comparison to unblended WP and PeP isolates. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly higher in PeP vs WP and WP/PeP blend in a 30:70 ratio, and in WP/PeP blend in a 50:50 ratio vs WP following gastric phase digestion (**** p <0.0001, ** p < 0.01,* p <0.05).

The free AA concentration in gastric end-point digests of WP and plant-based protein isolate blends in a ratio of 50:50 was significantly lower in digests of WP and RP blends compared to WP with PP and WP with PeP isolate blends with mean differences of 1.39% (CI 0.64 to 2.14, p <0.0001) and 1.07% (CI 1.82 to 0.32, p = 0.0020), respectively. There were no significant differences observed between the digests of WP and PP and WP and PeP isolate blends in a ratio of 50:50 at the end of gastric phase digestion (**Figure *29***).

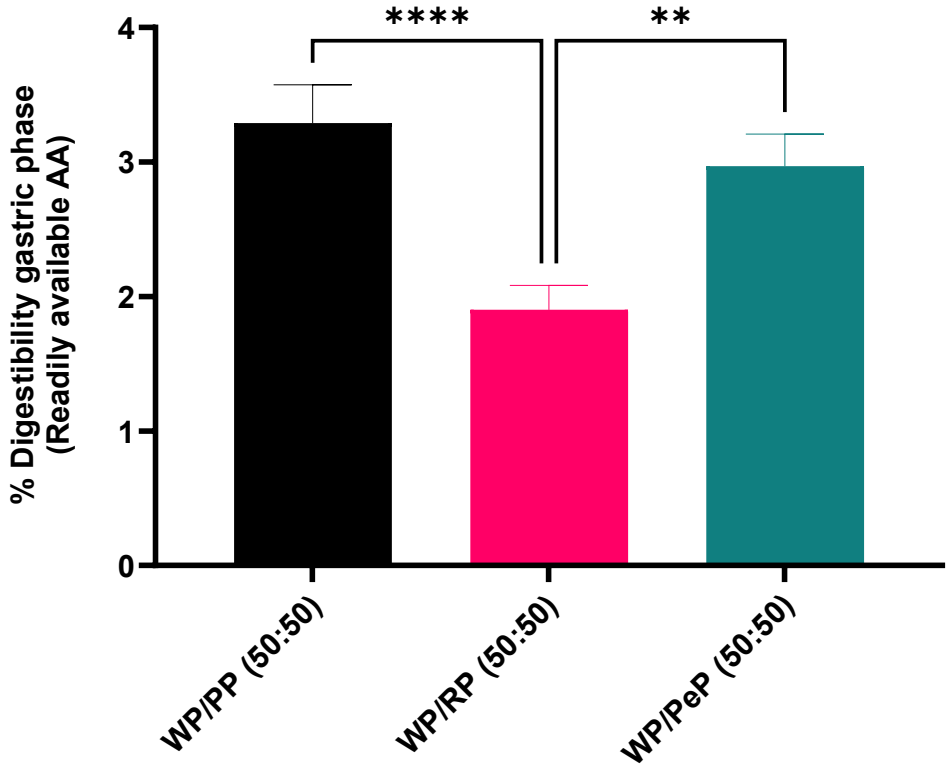


Figure 29. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of whey protein (WP) isolate blended with potato (PP), rice (RP) and pea protein (PeP) isolates in a ratio of 50:50, following gastric phase digestion. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly lower in the WP/RP blend in a 50:50 ratio vs WP/PP and WP/PeP blends in a 50:50 ratio following gastric phase digestion (**** p <0.00001, ** p < 0.01).

The analysis of free AA concentration in the intestinal end-point digests of WP and plant-based protein isolate blends in a ratio of 50:50 showed significantly lower levels in digests of WP and PP blends compared to the digests of WP and RP isolate blends (1.79%, CI 3.14 to 0.40, p = 0.0062). There were no significant differences observed between the digests of WP and PP and WP and PeP isolate blends in a ratio of 50:50 at the end of the intestinal phase digestion (**Figure *30***).

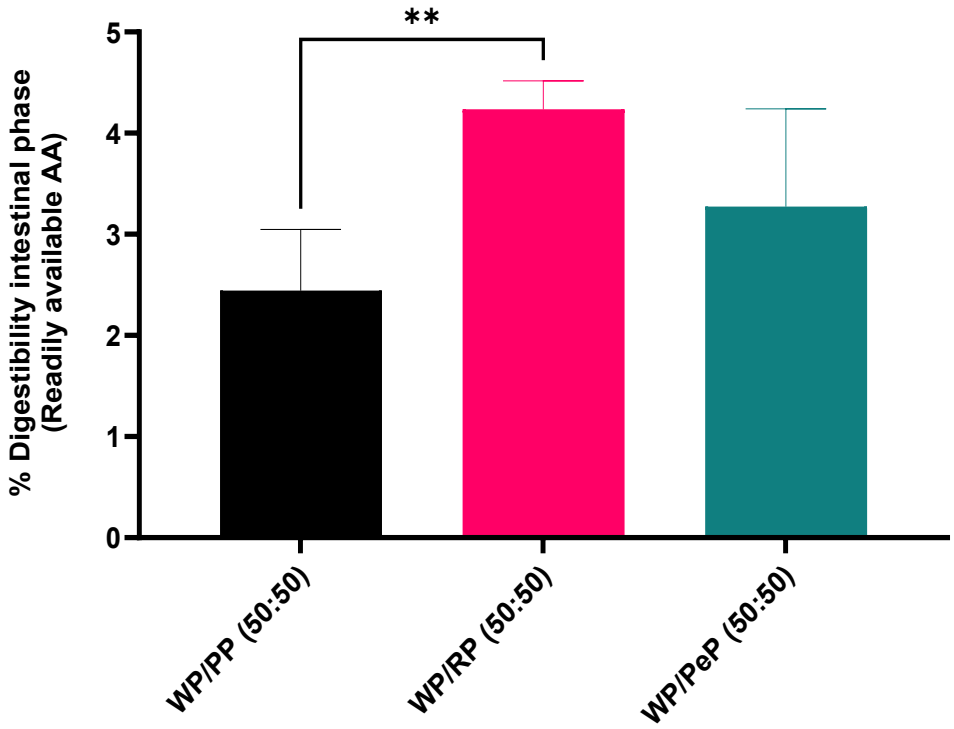


Figure 30. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of whey protein (WP) isolate blended with potato (PP), rice (RP) and pea protein (PeP) isolates in a ratio of 50:50, following intestinal phase digestion. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly higher in the WP/RP 50:50 ratio blend vs the WP/PP 50:50 ratio blend following intestinal phase digestion (** $p < 0.01$).

The analysis of the free AA concentration in gastric end-point digests of WP and plant-based protein isolate blends in a ratio of 30:70 showed significantly lower levels in digests of WP with RP blend compared to WP with PP and WP with PeP isolate blends with mean differences of 2.16% (CI 1.41 to 2.91, p <0.0001) and 0.94% (CI 1.69 to 0.19, p = 0.0074), respectively. Additionally, the digests of WP and PeP isolate blends contained significantly lower concentrations of free AA compared to the digests of WP and PP isolate blends, with a mean difference of 1.22% (CI 0.47 to 1.97, p = 0.0004) at the end of gastric phase digestion (**Figure *31***). There were no significant differences observed in free AA concentration between the digests of blended WP and plant-based protein isolates at the ratio of 30:70 after the intestinal phase digestion.

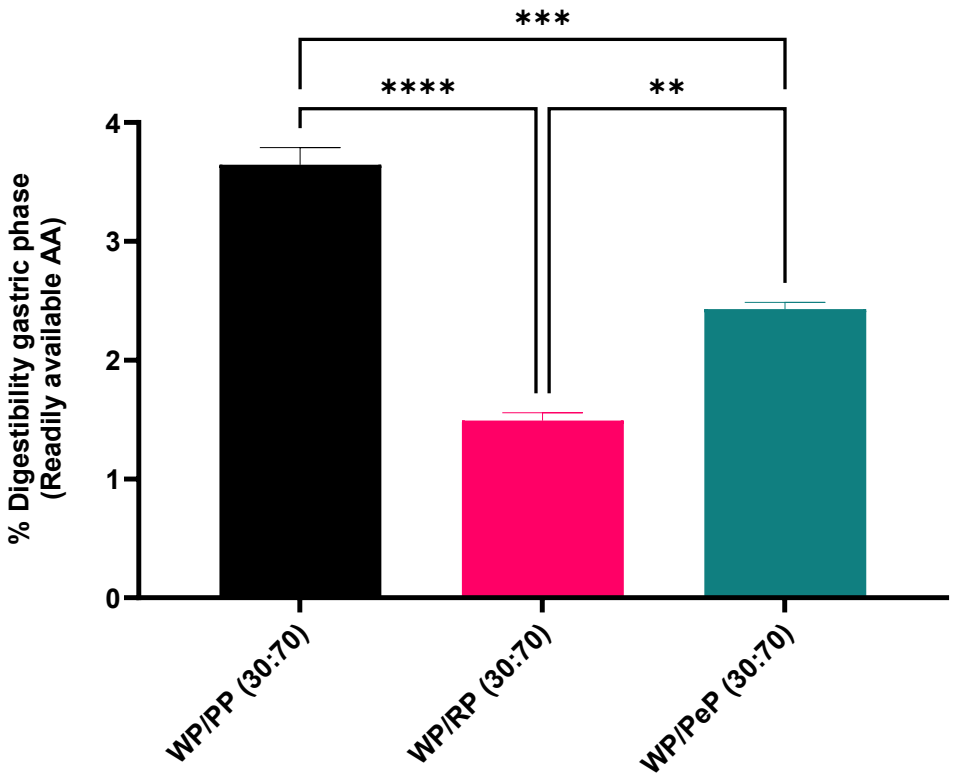


Figure 31. L-Leucine equivalent free amino-acid (AA) concentration (%) in digests of whey protein (WP) isolate blended with potato (PP), rice (RP) and pea protein (PeP) isolates in a ratio of 30:70, respectively, following gastric phase digestion. Results are presented as mean ± standard deviation (n=3). Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Concentration of readily available AA was significantly lower in the WP/RP 30:70 ratio blend vs the WP/PP and WP/PeP 30:70 ratio blends and in WP/PeP 30:70 ratio blend vs WP/PP 30:70 ratio blend following intestinal phase digestion (**** p <0.00001, *** p< 0.001, ** p < 0.01).

The analysis of readily absorbable fractions released from 50:50 ratios blended protein isolates during digestion showed significantly higher concentrations at the intestinal phase end-point compared to the gastric phase end-point, only with the WP and RP isolates blend, with mean differences of 2.18 ± 0.41% (p = 0.0055). No significant differences were observed over time with PP and PeP isolates (**Figure *32***).

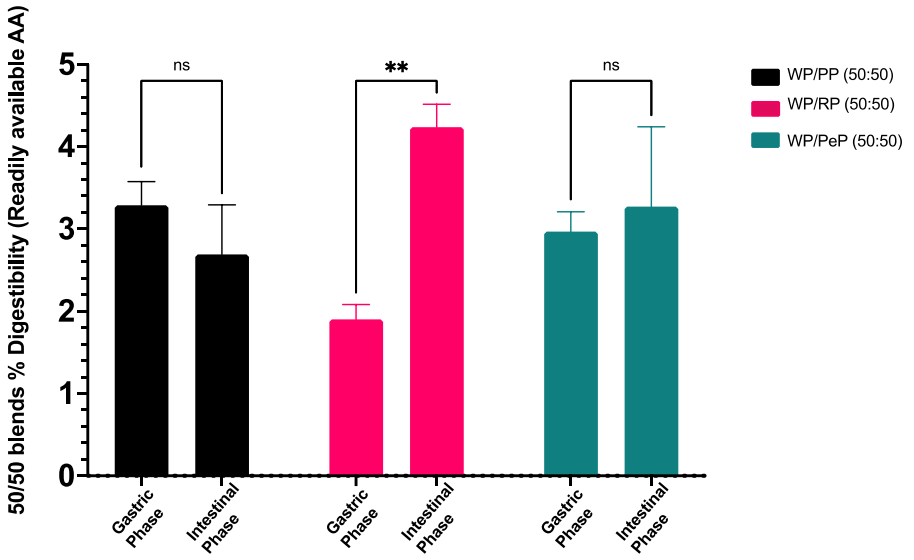


Figure 32. L-Leucine equivalent free amino-acid (AA) concentration in 50:50 blended digested protein samples (%). Results are presented as mean ± standard deviation. Data was analysed using a mixed effect model, Type III test, followed by Tukey's test for multiple comparison correction. Concentration of readily available AA was significantly higher in whey protein (WP) and rice protein (RP) 50:50 ratio blend following intestinal phase digestion vs gastric digestion (** $p < 0.01$, ns $p > 0.05$).

Comparable to 50:50 blends with the 30:70 blended proteins, there was a significant difference observed between gastric and intestinal end-points with WP and RP and additionally with PeP blend, with a mean difference of 2.99 ± 0.21% (p <0.0001) and 1.51 ± 0.21% (p = 0.0010), respectively (**Figure *33***).

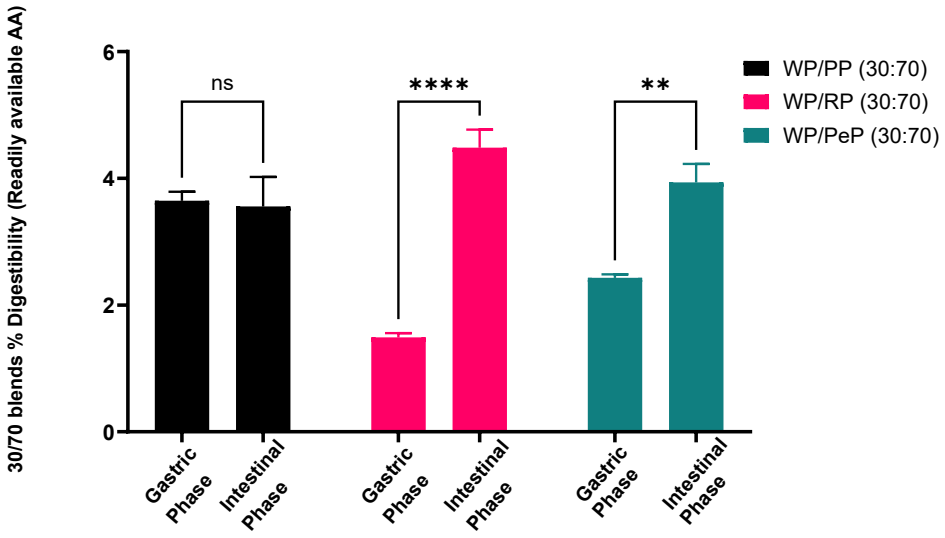


Figure 33. L-Leucine equivalent free amino-acid (AA) concentration in 30:70 blended digested protein samples (%). Results are presented as mean ± standard deviation. Data was analysed using a mixed effect model, Type III test, followed by Tukey's test for multiple comparison correction. Concentration of readily available AA was significantly higher in whey protein (WP) and rice protein (RP) and WP and pea protein (PeP) 30:70 ratio blends following intestinal phase digestion vs gastric digestion (**** $p < 0.0001$, ** $p < 0.01$, ns $p > 0.05$).

3.3. Discussion

With obesity being a global concern due to related health issues such as T2D, cancer, CHD, as well as depression, there has been a major increase in research on obesity management and prevention during the past four decades (Reynolds, 2013). From the dietary interventions studied, meal replacement diets were shown to have the most significant impact on weight reduction and maintenance (Ruban et al., 2019). Meal replacement products such as shakes often contain increased levels of protein from various sources such as dairy-derived WP, casein or plant-based soy, RP, PeP or wheat protein isolates. The total protein content of the meal replacement products can vary between 20 to 40g per meal (Anderson et al., 2007; Mollard et al., 2017), corresponding to the optimal acute anabolic range of protein (which is 20-40g) (Aragon and Schoenfeld, 2013). Protein sparing, as well as increased weight loss effects, have been suggested with a higher 40g protein intake compared to the lower 20g (Contaldo et al., 1980; Layman, 2004; Murphy, Hector and Phillips, 2015). There is an increased interest in plant-sourced proteins, possibly due to consumers' enhanced environmental awareness related to protein production (Medawar et al., 2019). However, appetite regulatory effects of proteins may differ depending on the source due to variations in AA profile as well as digestibility. In support of this, our previous study showed significantly higher appetite hormone secretion, GLP-1, with WP and RP compared to PP (Tiekou Lorinczova et al., 2021). To further assess the reasons for the differing appetite regulatory effects, the current study focused on assessing the digestibility of high-quality commercially available protein isolates: WP (Impact Whey Protein Isolate, Myprotein, Inc, Manchester, UK), PP (Solanic®200, ProteinmiXer.com®, Bonn, Germany), RP (Organic Oryzatein® Silk 90, Axiom Foods/Growing Naturals, Inc., Los Angeles, CA, USA) and PeP (Puris™ Pea 870 H, Cargill, Inc., Minneapolis, MN, US).

The described results support the presented hypothesis by demonstrating differing digestibility characteristics between WP, PP, RP, and PeP, which could go some way in explaining their varying effects on appetite hormone secretion.
The digestibility of WP was observed to be higher with complete digestion by the end of the intestinal phase compared to the plant-based proteins, independent of initial protein concentration within the digests. This finding is in line with the

protein digestibility–corrected AA score (PDCAAS), adopted by WHO (Schaafsma, 2000) and the digestible Indispensable AA Score (DIAAS) (Mathai, Liu and Stein, 2017) of the protein isolates with WP isolate having been scored 1.0 and 0.90, PP 0.87 and 0.85, RP 0.53 and 0.52 and PeP 0.83 and 0.66, respectively (Hertzler et al., 2020). The assessment of free AA following intestinal digestion also supports the overall higher digestibility of WP with differences of 0.56% WP vs PP, 0.17% WP vs RP and 0.67% WP vs PeP.

The observed differences in digestibility may be attributed to some plant-based protein fractions bearing antitrypsin properties, such as protease inhibitors of PP or the serine/trypsin protease inhibitors of PeP. Protease inhibitors are categorised as antinutritional factors (ANFs) due to their presence in food, leading to reduced nutrient utilisation and uptake, specifically proteins, which may lead to impaired gastrointestinal functions and metabolic performance (Salim et al., 2023). Other ANFs found in PeP with similar antitrypsin characteristics are lectins (Salim et al., 2023). ANFs present in RP are albumins, which were shown to reduce glucose absorption (Ina et al., 2016). Although potato protease inhibitors and RP albumins possess specific antinutritional properties, they were also shown to express beneficial bioactive characteristics. PI2 was shown to suppress postprandial appetite significantly in a dose-dependent manner in 44 healthy women in a randomised, placebo-controlled cross-over study when consumed an hour before a standardised meal in two different doses (15 and 30 mg) (Zhu, Lasrado and Hu, 2017). Rice albumin was shown to express copper ion (Cu^{2+}) chelator characteristics, contributing to the reduction of available free copper ions and, therefore, reducing Cu^{2+}-induced oxidation in the intestine (Wei et al., 2007). Furthermore, the antinutritional glucose absorption-reducing effects of rice albumin could be useful in diabetes treatment by preventing the absorption of saccharides from the intestine, potentially leading to effective suppression of hyperglycaemia when starch or glucose-based foods are ingested (Ina et al., 2016).

The observation of WP hydrolysis by SDS-PAGE demonstrated a more rapid proteolysis of α-lac compared to β-lg, with a complete breakdown of α-lac by the end of the gastric phase. β-lg is assumed to be resistant to pepsin digestion due

to the presence of disulfide bonds stabilising its structure at low pH, preventing access to potential cleavage sites (Singh et al., 2014). However, proteolysis of WP peptides occurred rapidly at the intestinal phase, as evidenced by the complete absence of the protein bands on SDS-PAGE gels after 30 min of exposure to pancreatic enzymes. The significantly higher free AA concentration measured in the intestinal end-point digests compared to the gastric end-point digests of WP supports the degree of digestion observed via the breakdown of specific protein fractions. These observations are in agreement with previously reported digestion rates of milk proteins (Mandalari et al., 2009; Egger et al., 2016). Furthermore, gastric and intestinal samples collected from pigs after milk ingestion also showed comparable breakdown of WP peptides, with rapid proteolysis of α-lac and higher resistance of β-lg during gastric digestion and both being fully hydrolysed within the small intestine (Barbé et al., 2014).

Compared to WP, the *in vitro* digestion of PP was observed to be at a lower rate. The hydrolysis of patatin, the main constituent of PP, was evident, with only traces detectable at the end of gastric phase digestion with a representative band size of about 43 kDa (lane 4). By the end of the intestinal phase, complete proteolysis of patatin was observed (lane 9). However, protease inhibitors (7 to 21 kDa) were detectable at the end of the gastric and intestinal phases, suggesting resistance to proteolysis by digestive enzymes. Protease inhibitors' resistance to digestion is due to being mostly stabilised by disulfide bonds, similar to β-lg of WP (Turra and Lorito, 2011). Comparable digestion patterns of PP were observed by Jiménez-Munoz, Tsochatzis and Corredig (2022), with traces of patatin observable at the last stages of gastric digestion and trypsin inhibitor bands remaining constant at all intestinal points.

While with WP and PP, all relevant protein bands were observable in their undigested dispersions with electrophoresis, only prolamin was detectable within the undigested RP dispersions. The absence of a glutelin-representing band at this stage could be explained by the fact that glutelin has completely water-insoluble characteristics (Loveday, 2019). Both prolamin and glutelin, representing protein bands, were observable in the gastric phase digesta samples with little change in the electrophoretic pattern over time, indicating that

these protein fractions of RP were resistant to pepsin proteolysis (Li et al., 2020). Within the intestinal phase samples, the glutelin was undetectable. However, prolamin bands seemed to be unaffected by pancreatic enzyme proteolysis. Poor digestibility of prolamin has been previously demonstrated (Wang et al., 2016; Jiménez-Munoz et al., 2021).

Similarly to WP and PP, all relevant PeP protein bands were observable in the undigested dispersion, indicating better water solubility than RP. In the PeP digesta, a decrease in the molecular weight of convicilin from ~ 77 kDa to ~ 40 kDa is observed during the gastric phase. Comparable observations were reported by Jiménez-Munoz et al. (2021). The digestion of vicilin, legumin and PA1 and PA2 fractions by pepsin was also evident, with only traces of the bands remaining detectable within the end-point sample of the gastric phase digesta. Although the pepsin-cleaved penicillin detectable at ~40 kDa was still distinctive at 60 min of the gastric digestion, intestinal digesta samples showed no detectable levels of digested protein. This observation may indicate rapid proteolysis during the intestinal phase of digestion, also suggested by others (Amagliani et al., 2017; Jiménez-Munoz et al., 2021).

The measurement of the concentration of free AA using the OPA assay showed no significant differences between the intestinal end-point samples of unblended protein digests. However, the results indicated potential interaction between WP and PP when digested in a blended format, where the WP and PP blend in the ratio of 50:50 had a significantly lower free AA concentration compared to WP and RP blends (1.79%, p = 0.0062) with the same ratio in the intestinal phase end-point samples. Furthermore, a significant reduction in the concentration of free AA with WP:PP (50:50) was also observed compared to unblended WP intestinal end-point digested, suggesting a potential inhibition of WP fractions' proteolysis by potato protease inhibitors. However, the same phenomenon was not observed with the WP:PP (30:70) blends intestinal end-points digest. Moreover, no significant differences were observed in the percentage digestibility based on the concentration of free AA at the end of the intestinal phase digestion between any of the WP and plant-based protein blends in the ratio of 30:70, respectively. These results indicate a potential preference for blends with a ratio

of 30:70 above 50:50 when aiming to enhance the AA profile of plant-based proteins with a potential maximised effect on appetite suppression. Although the assessment of free AA concentration using the OPA assay provided an indication of the digestibility of unblended and blended proteins, it is unable to provide a complete picture of digestibility (Santos-Sánchez et al., 2024). The precision of this method to assess the degree of protein hydrolysis is affected by the OPA's weak reaction with cysteine, leading to unreliable quantification of this AA (Church et al., 1985; Spellman et al., 2003). Furthermore, OPA does not react with secondary AAs such as proline or hydroxyproline either (Herbert, Santos and Alves, 2001). The application of reverse-phase high-performance liquid chromatography combined with mass spectrometry (RP-HPLC-MS/MS) could hold a solution for the related challenges, enabling the identification and quantification of AAs as well as potential bioactive protein fractions, such as PI2, which may remain intact following *in vitro* digestion (Hernández-Ledesma et al., 2005).

In conclusion, the study results support the hypothesis that the digestibility of proteins may differ depending on the source, showing a more complete protein hydrolysis of WP compared to plant-based proteins. This study also highlights the importance of considering the potential interaction between plant and dairy-based proteins due to ANFs found in plant proteins reducing proteolysis. This study indicates that blending WP with PP to achieve a complete protein profile is preferable in the ratio of 30:70 compared to 50:50 due to enhanced digestibility. The application of this blend could also reduce related environmental impacts while maintaining maximal appetite regulatory effects. However, there is also an emphasis on considering specific ANFs for their potential positive bioactive characteristics, such as the appetite-suppressive effects of PI2. Further studies are required to identify the complete AA and bioactive peptide profiles of the unblended and blended protein digests and to identify their effects on appetite-regulatory gut hormones to aid understanding of their most appropriate application for appetite and weight management.

4. CHAPTER 4 - Effects of protein digest treatments on Caco-2 and STC-1 cells

4.1. Introduction

The global epidemic of obesity and related metabolic diseases, such as T2D, is a major health concern which have affected over 16% (2022) (WHO, 2024) and 8% (2014) (WHO, 2023) of the adult population worldwide. The most effective treatment for these conditions is RYGB, leading to a loss of about 20 % to 30 % of pre-surgery weight and T2D remission observed in 72% of the patients 2 years post-surgery (Sjöström et al., 2007). The success of the surgery can be related to bypassing the duodenum and a large section of the jejunum, leading to direct nutrient delivery to the distal part of the intestine, where the appetite regulatory EECs are more densely located (Sinclair, Brennan and le Roux, 2018). The consequently increased levels of anorexigenic hormones such as PYY, GLP-1, GIP and CCK aid in appetite reduction, satiety increase and blood glucose regulation (Akalestou et al., 2022). However, surgery is viewed as the last possible approach to weight loss due to related cost and side effects, such as malabsorption of vitamins and minerals (Schweitzer and Posthuma, 2008), vomiting, abdominal pain, constipation (Kalarchian et al., 2014), depression, suicidal thoughts and attempts (Müller et al., 2019). Nevertheless, establishing the mechanisms underlying weight loss and T2D remission following surgery has prompted the search for and development of enteroendocrine cell-stimulating or anorexigenic hormone-mimicking agents. The most successful examples are GLP-1 receptor agonists, such as Semaglutide and Trizepatide, a dual GLP-1 and GIP receptor agonist (Chavda et al., 2022). Both pharmacological agents were shown to lead to a clinically significant weight loss of 17.4% and 20.9% from the initial weight following 68 and 72 weeks of subcutaneous administration, respectively (Jastreboff et al., 2022; Bergmann et al., 2023).

Nevertheless, the relevance of nutritional intervention as a first-line approach to target anorexigenic gut hormone release is still undeniable due to numerous advantages, such as minimal adverse side effects compared to pharmacological agents (Tran et al., 2017; Gentilella et al., 2019). Treatment with Semaglutide, as well as Tirzepatide, was related to gastrointestinal side effects such as nausea,

vomiting and diarrhoea (Frías et al., 2021). Additionally, GLP-1 and GIP receptor agonists may reduce appetite as a result of sole direct interaction with the brain (Sisley et al., 2014), unlike the effects of endogenous hormones GLP-1 and GIP, which are also exerted locally via receptors on the vagus nerve, and indirectly through gastric emptying (Krieger, Langhans and Lee, 2015; Krieger et al., 2016). It was suggested that the natural rise and fall in the levels of endogenous gut hormones may play a crucial part in maintaining receptor sensitivity; in contrast, the continuous stimulation by the pharmacological receptor agonists may lead to reduced sensitivity of these receptors (Jonathan D. Watkins, Koumanov and Gonzalez, 2021).

One of the most effective nutritional interventions for weight loss and consecutive weight maintenance is the increase in protein intake within the diet, often via supplementation (Luhovyy, Akhavan and Anderson, 2007) or via the application of meal replacements with high protein content (Ruban et al., 2019). The total protein content of the meal replacement products can vary between 20 to 40g per meal (Anderson et al., 2007; Mollard et al., 2017), corresponding to the optimal acute anabolic range of protein (which is 20-40g) (Aragon and Schoenfeld, 2013). Nevertheless, observed weight loss effects seemed greater with a higher 40g protein intake compared to the lower 20g (Layman, 2004; Murphy, Hector and Phillips, 2015). Weight loss following a high-protein diet may be attributed to the significant increase in circulatory PYY and GLP-1 levels to a greater extent than that observed following carbohydrate or fat consumption (Batterham et al., 2006; Van Der Klaauw et al., 2013; Moon and Koh, 2020). Therefore, numerous supplements and meal replacements have been developed and marketed for weight loss and maintenance, which are easily accessible at high street retailers. The most accepted and widely utilised protein in such nutritional aids is WP, which is high in BCAAs and contains bioactive components such as αlac (Bendtsen et al., 2013). The αlac fraction makes up 20% of WP and has a high content of essential AA such as leucine, lysine and tryptophan (Chatterton et al., 2006). While tryptophan is a precursor of the anorexigenic signalling molecule serotonin (Haleem, 2017), leucine and lysine levels were shown to be related to appetite reduction under ketogenic conditions (Gloaguen et al., 2012). Some novel plant-based proteins also show potential in satiety and appetite regulation.

The consumption of 15 and 30 mg PI2, the main fraction of PP (50% of total protein) (Waglay, Karboune and Alli, 2014), in 44 healthy women an hour prior to breakfast led to significantly lower postprandial hunger and significantly higher postprandial fullness compared to placebo (Zhu, Lasrado and Hu, 2017). Additionally, brown RP isolate consumption led to a significant increase in GLP-1 levels at 120 min post-prandial, with no significant differences observed compared to WP (Tiekou Lorinczova et al., 2021). However, it was suggested that different proteins may have unique characteristics depending on their source, AA content and absorption kinetics. Thus, proteins from different sources might have dissimilar satiety and appetite regulatory characteristics (Bendtsen et al., 2013; He et al., 2013; Tiekou Lorinczova et al., 2021). The differences may partially be related to varying effects on anorexigenic hormone secretion from EECs in the gut.

The EECs represent <1% of all cells within the epithelial layer of the GI tract and are key components of the gut-brain axis (Jonathan D. Watkins, Koumanov and Gonzalez, 2021). These cells sense nutrients present in the gut lumen and secrete gut peptides, activating neurons of the enteric nervous system, which may lead to activation of the vagal and spinal afferents, transferring nutrient-derived signals to the brain (Shobatake et al., 2019). The EECs were observed to express several types of receptors and transporters (e.g. GPCRs and PEPT1/2) with location-specific responses to meal-related stimuli, such as the GIP-secreting K cells are located along the proximal intestine, while the PYY and GLP-1-secreting L-cells reside in the ileum and colon (Gutierrez-Aguilar and Woods, 2011). However, the molecular mechanisms underlying nutrient sensing leading to the secretion of specific peptides are still poorly understood (Barton et al., 2023). Thus, a good body of research has focused on food-intestine interaction studies. Immortalised intestinal cell lines are extensively used models for such studies, presenting higher reliability and reproducibility and lower costs compared to animal models with reduced reproducibility as well as ethical concerns (Ding et al., 2021).

The Caco-2 cell line is one of the most widely utilised cell models and has long been regarded as the pharmaceutical industry's gold standard for absorption

permeability studies (Volpe, 2020). Caco-2 cells are derived from human colorectal adenocarcinoma and spontaneously differentiate to resemble a small intestine-like phenotype after 15 to 21 days of culture (Ding et al., 2021). These cells express intestinal-like brush border structure on their apical surface as well as several receptors, transporters, and enzymes (Antunes et al., 2013), making them an ideal candidate as a small intestinal model (Ponce de León-Rodríguez, Guyot and Laurent-Babot, 2019).

Caco-2 cells are grown as confluent polarised monolayers and have been extensively used to study absorption and transport kinetics, intestinal metabolism, intestinal barrier, bacterial adhesion/invasion or the effects of prebiotic supplementation on intestinal microbiota (Ding et al., 2021). Recently, the potential use of Caco-2 cells for investigating the appetite regulatory effects of nutrients has been investigated. Caco-2 cells were shown to express the appetite hormones PYY (Kosicka, Renshaw and Zariwala, 2013) and GLP-1 (Shobatake et al., 2019) and secrete CCK, PYY and GLP-1 as a result of nutrient stimulation (Song et al., 2015), indicating their potential application as an enteroendocrine as well as an enterocyte model. However, due to the novelty of investigating the use of Caco-2 cells for appetite studies, there is still a limited representation in the current literature.

The STC-1 cell line, derived from a genetically induced murine enteroendocrine tumour in the duodenum, is a widely used cell model for appetite hormone regulation studies (Santos-Hernández et al., 2018). This cell line possesses the characteristics of numerous enteroendocrine cells, expressing as well as secreting many gut hormones (e.g. CCK, GIP, PYY, GLP-1&2 and oxyntomodulin) (Kuhre et al., 2016). STC-1 cells grow into a heterogeneous enteroendocrine cell line, forming a monolayer in a standard growth medium with a relatively long cell cycle of about 54 hours (McCarthy et al., 2015; Kuhre et al., 2016; Heffernan et al., 2022; Gorecki et al., 2025). This cell line has been used for nutrient interaction (Santos-Hernández et al., 2018), immunology (Cheng et al., 2024), as well as cancer research studies (Ye et al., 2024). It has been suggested that this cell line provides a reliable and reproducible cell model.

However, variations are observed between laboratories and experiments, potentially due to their heterogeneity (McCarthy et al., 2015).

The well-established profile of the Caco-2 cell line as a model of the human intestine for absorption permeability studies, in combination with the recent data indicating their ability to express key appetite hormones, renders them particularly applicable as a potential model for gut-nutrient sensing studies. Therefore, this study aims to investigate the regulatory effects of WP, PP, RP and PeP digests on the expression and secretion of appetite hormones GIP, GLP-1 and PYY in differentiated Caco-2 cell model as a model of intestinal cells *in vitro*, as well as comparing the observed effects to those observed in the more frequently utilised STC-1 cells. The results of this study may provide further insights into establishing Caco-2 cells as a model for nutrient-induced appetite hormone regulatory studies.

4.2. Results

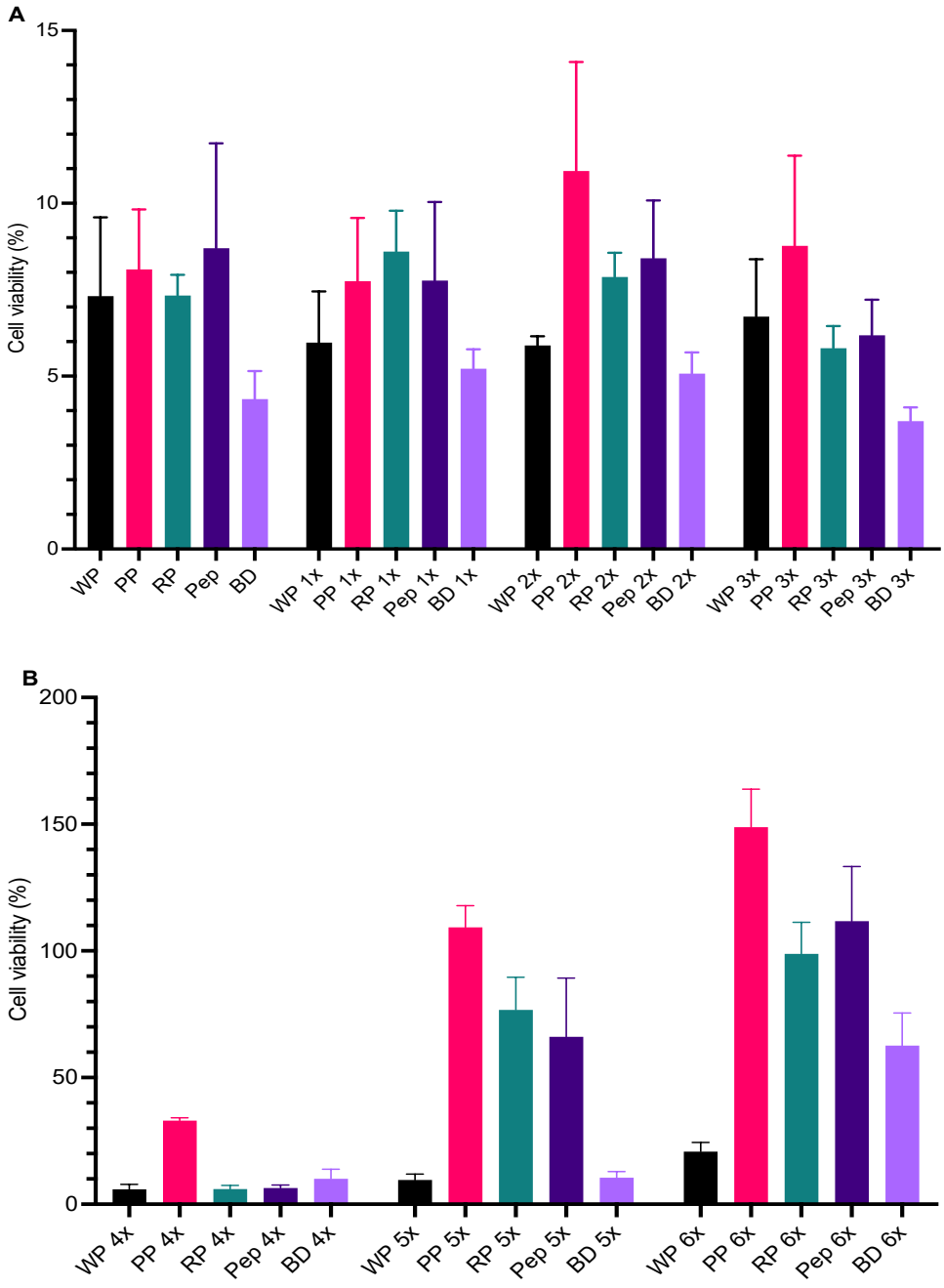
4.2.1. Cell viability

Viability of cells was measured using the MTT assay to evaluate the cytotoxicity of WP, PP, RP and PeP digests on Caco-2 and STC-1 cells, as well as WP protein blends with plant-based proteins in the ratio of 50:50 and 30:70, respectively, on Caco-2 cells at various concentrations and durations of exposure. A cell viability value of less than 50% indicates reduced mitochondrial activity, while a higher cell viability of >80% ensures that the protein isolate digest concentrations are non-toxic to the cells (Wahlang, Pawar and Bansal, 2011).

4.2.1.1. Caco-2 cell viability in response to protein digests exposure

As shown in **Figure 34**, when Caco-2 cells were exposed to unheated and unblended protein digests, viability increased with the reduction in concentration of the treatments. When protein digest concentrations reached about 3.13 mg/mL (7x dilution, **Figure 34C**), the mean cell viability ± standard deviation (±SD) was observed to be between 149.85% (±20.77), 141.58% (±18.72), 104.44% (±8.90) and 122.16 (±19.80) for unheated WP, PP, RP and PeP digests, respectively. The BD included as a control at the same concentration led to a cell viability of 29.50% (±7.26). Further between-group analysis at this concentration indicated significant differences between WP vs RP and WP vs BD of 45.41% (95% CI

14.45 to 76.37, p <0.0001) and 120.3% (95% CI 89.38 to 151.3, p <0.0001), respectively. The one-way ANOVA test summary was F (55, 280) = 120.3 (p <0.0001) (**Figure 34C**).



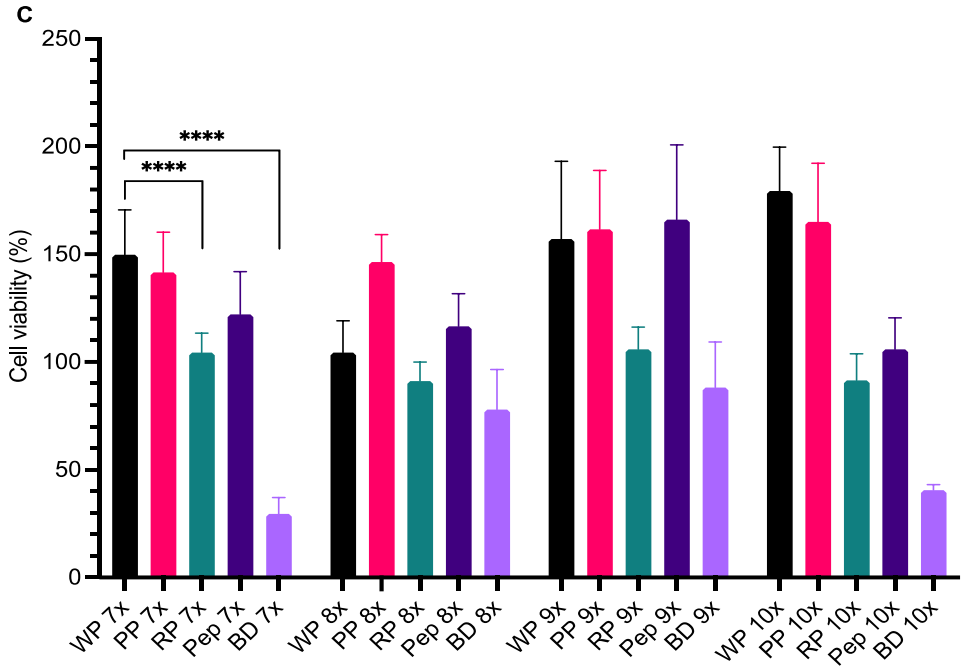


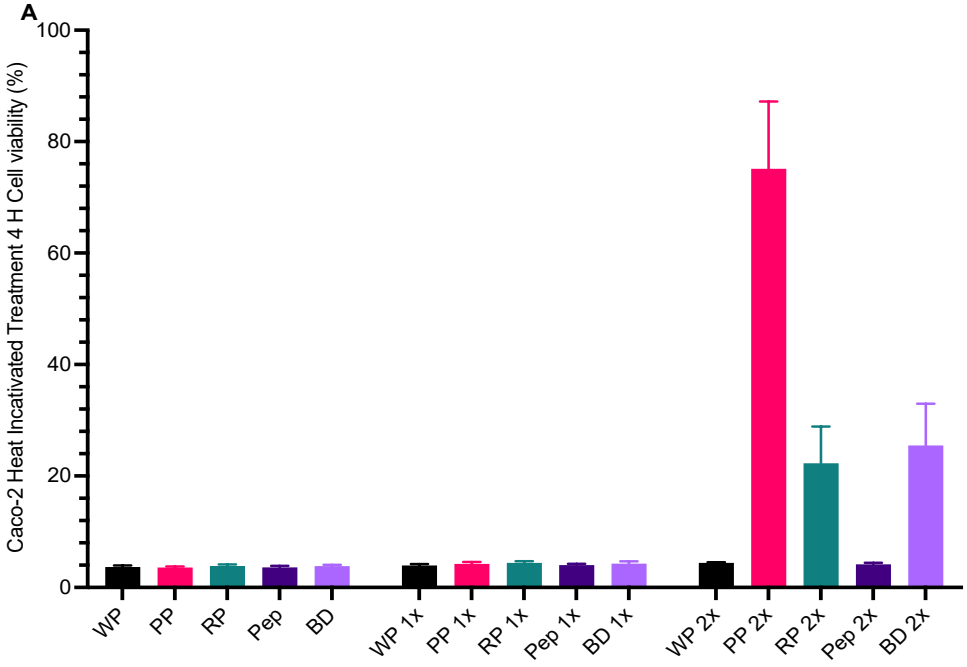
Figure 34. Cytotoxicity results of protein digests at different concentrations in Caco-2 cells using the MTT assay. Values are represented as % cell viability ± SD for unheated protein digests at 24h incubation (n =6), **(A)** represents undiluted, 1x, 2x, 3x diluted digests, **(B)** represents 4x, 5x, 6x diluted digests, **(C)** represents 7x, 8x, 9x, 10x diluted digests. **** Indicates significant differences when comparing the different conditions with the same concentration of 7x (p <0.0001).

When investigating the effects of further digestive enzyme inactivation via heating within the digests, it was observed that the unblended protein digest treatments, except for PeP, achieved the desired at least 80% cell viability at 3x dilution. This dilution corresponds to the estimated protein concentration of 6.25 mg/mL. Significant differences were observed between groups at this concentration, with PeP with a mean cell viability of 10.07% (±2.55) being significantly lower than every other treatment, with the significance of p <0.0001. Additionally, WP, although reaching 101.38 ±20.41% cell viability, was significantly lower compared to PP, RP and BD with mean differences of -44.10% (95% CI -63.85 to -24.36), -48.05% (95% CI -67.80 to -28.30) and -54.89% (95% CI -74.64 to -35.14), respectively all with the significance of p <0.0001 (**Figure 35B**).

The heat-inactivated PeP digest treatments reached the desirable >80% cell viability at 5x dilution, which contained an estimated concentration of 4.17mg/mL protein. Between-group analysis revealed that at this concentration, the mean cell viability of 156.01% (±7.32) of WP treatment was significantly higher compared to any other treatment. The observed mean differences were 25.03%

(95% CI 5.281 to 44.78, *p* =0.0011), 41.44% (95% CI 21.70 to 61.19, *p* <0.0001), 49.32% (95% CI 29.57 to 69.07, *p* <0.0001) and 39.75% (95% CI 20.00 to 59.49, *p* <0.0001) with WP vs PP, RP, PeP and BD, respectively. There was also a significant difference (*p* =0.0020) present between PP and PeP, with a mean difference of 24.29% (4.54 to 44.04) at this concentration.

The one-way ANOVA test summary was F (30, 150) = 295.8 (*p* <0.0001) (**Figure 35B**).



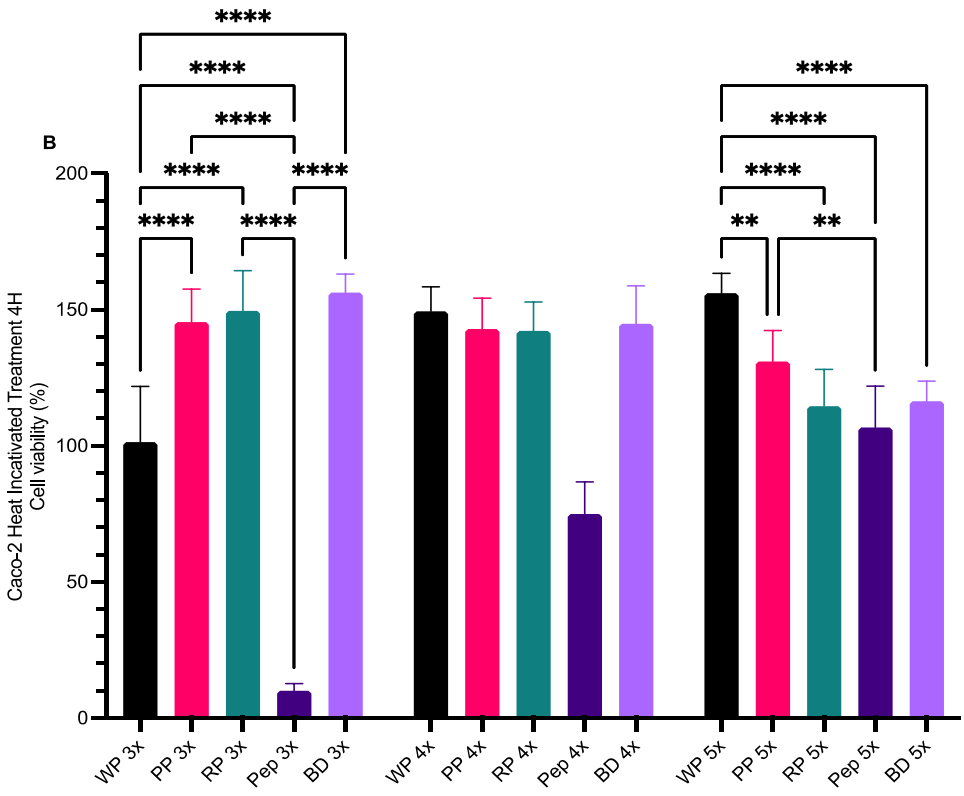


Figure 35. Cytotoxicity results of protein digests after heat inactivation at different concentrations in Caco-2 cells using the MTT assay. Values are represented as % cell viability ±SD for heat-inactivated protein digests at 4h incubation (n =6, except PeP 2x, BD 2x n =5 and WP 2x n =4), **(A)** represents undiluted, 1x, 2x diluted, heat-inactivated digests, **(B)** represents 3x 4x and 5x diluted heat-inactivated digests. * Indicates significant differences when comparing the different conditions with the same concentration of 3x and 5x dilution (**p <0.01, ****p <0.0001).

As shown in **Figure 36**, when Caco-2 cells were treated with heat-inactivated protein blend digests of WP with plant-based proteins in a 50:50 ratio WP/PP and WP/RP conditions with 3x dilution presented 117.15 ± 8.22% and 158.55 ± 13.64% cell viability, respectively. Treatment with WP/PeP with the same concentration also achieved an acceptable cell viability of 79.91 ± 8.82%. Between-group analysis at this concentration revealed that treatment with WP/RP (50:50) for 4 hours led to significantly higher cell viability compared to WP/PP (50:50) and WP/PeP (50:50) with mean differences of 41.40% (95% CI 69.35 to 13.45, p <0.0001) and 78.65% (95% CI 49.46 to 107.8, p <0.0001), respectively. Additionally, WP/PP (50:50) also presented significantly higher cell viability vs treatment with WP/PeP (50:50) with a mean difference of 37.24% (95% CI 9.30 to 65.19, p =0.0005). The one-way ANOVA test summary was F (24, 119) = 116.6 (p <0.0001) (**Figure 36**).

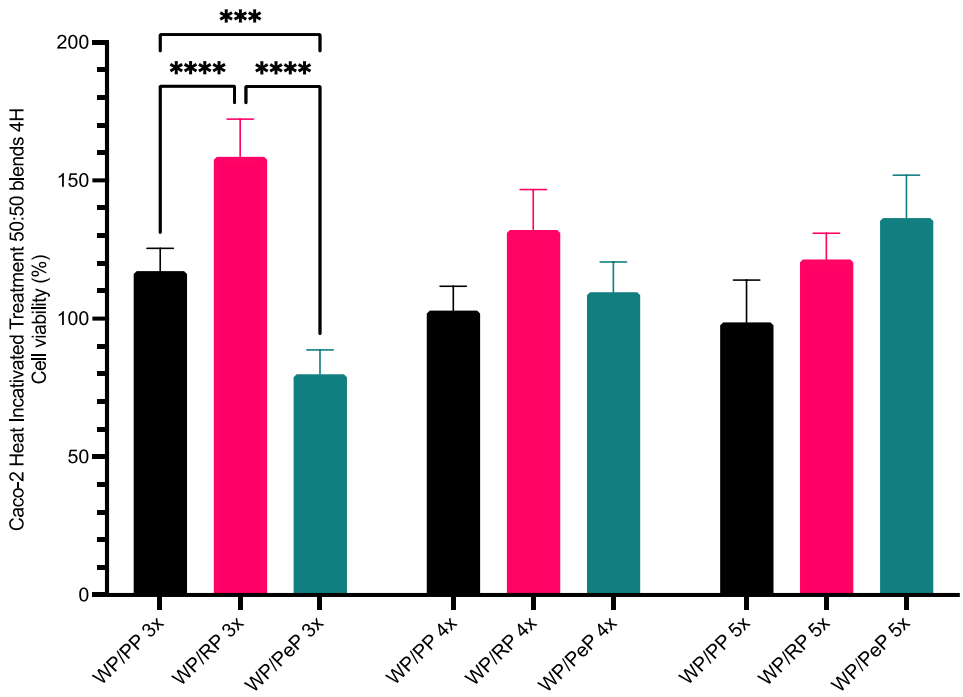


Figure 36. Cytotoxicity results of blended protein digests in 50:50 ratio after heat inactivation at different concentrations in Caco-2 cells using the MTT assay. Values are represented as % cell viability ±SD for heat-inactivated protein digests at 4h incubation (n =6, except WP/RP 3x and WP/PeP 3x n =5), represents 3x 4x and 5x diluted heat-inactivated digests of blended protein isolates in a 50:50 ratio. * Indicates significant differences when comparing the different conditions with the same dilution of 3x (****p <0.0001, ***p <0.001).

As shown in **Figure 37**, when Caco-2 cells were treated with heat-inactivated protein blend digests of WP with plant-based proteins in a 30:70 ratio WP/PP and WP/RP conditions with 3x dilution presented 133.53 ± 19.53% and 149.01 ± 4.97% cell viability, respectively. Treatment with WP/PeP (30:70) with the same concentration presented a cell viability of 3.86 ± 0.12%. Between-group analysis at this concentration revealed that cell viability with WP/PeP (30:70) condition for 4 hours was significantly lower compared to WP/PP (30:70) and WP/RP (30:70) with mean differences of 129.70% (95% CI 96.45 to 162.90, p <0.0001) and 145.20% (95% CI 111.90 to 178.40, p <0.0001), respectively. The one-way ANOVA test summary was F (14, 78) = 25.56 (p <0.0001) (**Figure 37**).

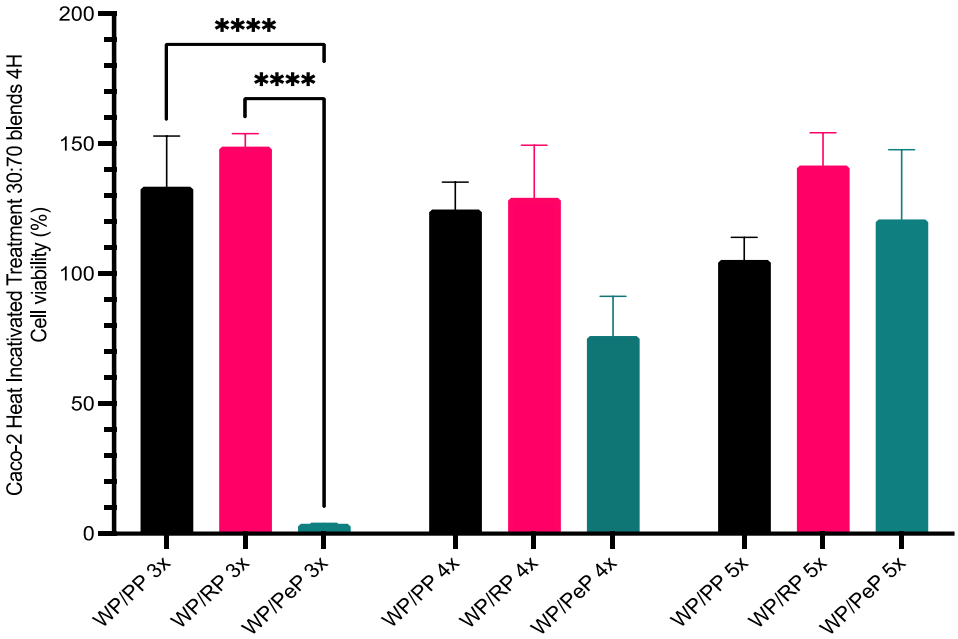


Figure 37. Cytotoxicity results of WP and plant-based protein blend digests in 30:70 ratio, respectively, after heat inactivation at different concentrations in Caco-2 cells using the MTT assay. Values are represented as % cell viability ±SD for heat-inactivated protein digests at 4h incubation (n =6, except WP/PeP 3x n =4), representing 3x, 4x and 5x diluted heat-inactivated digests of blended protein isolates. * Indicates significant differences when comparing the different conditions with the same 3x dilutions (****p <0.0001).

4.2.1.2. STC-1 cell viability in response to protein digestate exposure

When assessing the effects of the 4-hour treatments with different concentrations of the heat-inactivated unblended digests on STC-1 cells, PP presented the highest 114.15 ±7.72% cell viability at 5x dilution. Treatment with WP and BD at this concentration also led to an acceptable cell viability of 89.60 ±11.65% and 99.33 ±9.57%, respectively. However, RP and the PeP conditions at 5x dilution were significantly lower compared to all other treatment conditions, with p <0.0001. There were no significant differences observed at this concentration between WP, PP and BD conditions (**Figure 38**).

Treatment for 4 hours with RP conditions achieved ideal cell viability only at 7x dilution (80.44 ±13.52%). However, there was a reduction of 11.09% cell viability observed with the WP condition at that concentration, resulting in 78.51 ±9.13%. Additionally, the PeP condition still presented cell viability below the desirable 80% (10.74 ±4.66%) at 7x dilution. The one-way ANOVA test summary was F (25, 130) = 51.60 (p <0.0001) (**Figure 38**).

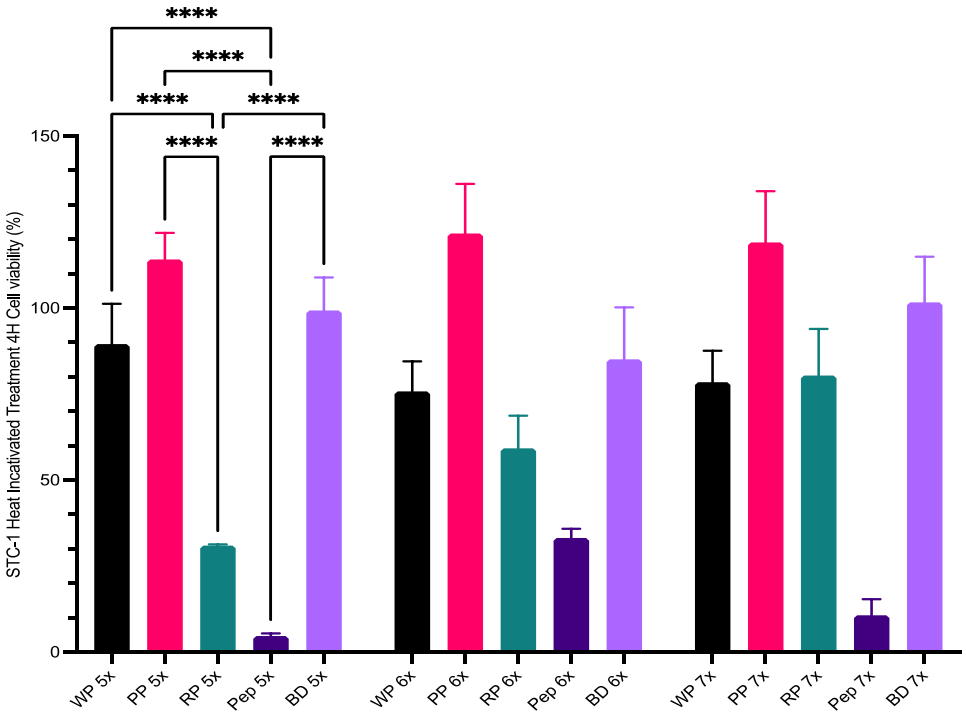


Figure 38. Cytotoxicity results of protein digests after heat inactivation at different concentrations in STC-1 cells using the MTT assay. Values are represented as % cell viability ±SD for protein digests at 4 h incubation (n =6, except RP 5x n =3, PeP 5x, PeP 6x and PeP 7x n =4, WP 6x n =5), representing 5x, 6x and 7x diluted heat-inactivated digests. * Indicates significant differences when comparing the different conditions with the same concentration of 5x dilution (****p <0.0001).

4.2.2. Appetite regulatory hormone measurements

4.2.2.1. PYY levels in Caco-2 with 7x diluted unheated unblended digests

Investigation of the effects of 7x diluted unheated unblended WP, PP, RP, PeP and BD digests on Caco-2 cells revealed detectable levels of intracellular PYY concentrations following 2- and 6-hour treatments.

Statistical inter-group analysis revealed significant differences between the different conditions following 2-hour treatments (F (1, 24) = 267.1, p <0.0001). Significant differences were observed between each treatment condition except PP-2H vs PeP-2H, PP-2H vs DB-2H, RP-2H vs B-2H, Pep-2H vs BD-2H and Pep-2H vs B-2H (**Table 20**).

Table 20. Between-group differences in intracellular PYY levels (pg/mg of total protein) at 2 h.

Treatment conditions	Mean Diff. (pg/mg)	95.00% CI of diff.	Sig.*	Adjusted *p*-Value
WP-2H vs PP-2H	-38.47	-64.04 to -12.89	***	0.0007
WP-2H vs RP-2H	-82.96	-108.5 to -57.39	****	<0.0001
WP-2H vs Pep-2H	-54.26	-79.84 to -28.68	****	<0.0001
WP-2H vs BD-2H	-49.19	-74.76 to -23.61	****	<0.0001
WP-2H vs B-2H	-75.88	-101.5 to -50.30	****	<0.0001
PP-2H vs RP-2H	-44.50	-70.08 to -18.92	****	<0.0001
PP-2H vs PeP-2H	-15.79	-41.37 to 9.784	NS	0.5479
PP-2H vs DB-2H	-10.72	-36.30 to 14.86	NS	0.9232
PP-2H vs B-2H	-37.42	-62.99 to -11.84	**	0.0010
RP-2H vs Pep-2H	28.70	3.126 to 54.28	*	0.0187
RP-2H vs BD-2H	33.78	8.200 to 59.36	**	0.0035
RP-2H vs B-2H	7.082	-18.50 to 32.66	NS	0.9962
Pep-2H vs BD-2H	5.075	-20.50 to 30.65	NS	0.9998
Pep-2H vs B-2H	-21.62	-47.20 to 3.956	NS	0.1546
BD-2H vs B-2H	-26.70	-52.27 to -1.118	*	0.0354

Results presented as mean difference (Mean Diff) and 95.00% Confidence Intervals of difference standard error of difference (95.00% CI of diff). Sig.* represents significance values when comparing treatments at the same timepoint (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, NS p >0.05).

When assessing the inter-group effects of 7x diluted unheated and unblended WP, PP, RP, PeP and BD digests on the intracellular PYY levels following 6 hours of treatment, an overall significant difference (F (1, 24) = 267.1, p <0.0001) was detected. Statistically significant differences were observed between each treatment condition except PP-6H vs. PeP-6H (**Table 21**).

Table 21. Between-group differences in intracellular PYY levels (pg/mg of total protein) at 6 h.

Treatment conditions	Mean Diff. (pg/mg)	95.00% CI of diff.	Sig.*	Adjusted *p*-Value
WP-6H vs PP-6H	-43.92	-69.50 to -18.34	***	0.0001
WP-6H vs RP-6H	-99.32	-124.9 to -73.74	****	<0.0001
WP-6H vs Pep-6H	-39.93	-65.50 to -14.35	***	0.0004
WP-6H vs BD-6H	-169.9	-195.5 to -144.3	****	<0.0001
WP-6H vs B-6H	-234.6	-260.2 to -209.0	****	<0.0001
PP-6H vs RP-6H	-55.40	-80.98 to -29.82	****	<0.0001
PP-6H vs PeP-6H	3.993	-21.85 to 29.57	NS	>0.9999
PP-6H vs BD-6H	-126.0	-151.6 to -100.4	****	<0.0001
PP-6H vs B-6H	-190.7	-216.2 to -165.1	****	<0.0001
RP-6H vs Pep-6H	59.39	33.82 to 84.97	****	<0.0001
RP-6H vs BD-6H	-70.58	-96.16 to -45.00	****	<0.0001
RP-6H vs B-6H	-135.3	-160.8 to -109.7	****	<0.0001
Pep-6H vs BD-6H	-130.0	-155.6 to -104.4	****	<0.0001
Pep-6H vs B-6H	-194.7	-220.2 to -169.1	****	<0.0001
BD-6H vs B-6H	-64.68	-90.26 to -39.10	****	<0.0001

Results presented as mean difference (Mean Diff) and 95.00% Confidence Intervals of difference standard error of difference (95.00% CI of diff). Sig.* represents significance values when comparing treatments at the same time point (***p <0.001, ****p <0.0001, ^{NS} p >0.05).

When assessing the effects of each treatment on intracellular PYY levels adjusted to total protein content after 2- and 6-hours, an intra-group significant difference (F (5, 24) = 245.3, p <0.0001) was detected. Statistically significant differences were observed with BD control (-120.20 pg/mg, 95%CI -145.80 to -94.65, p <0.0001) and with the diluent control MEM (B) (-158.20 pg/mg, 95%CI -183.80 to -132.60, p <0.0001) over time. There were no significant differences detected within active treatment groups (WP, PP, RP and PeP) over time when comparing levels expressed at 2H vs 6H at this dilution (**Figure 39**). There was no detectable concentration of PYY secreted with the application of treatments at 7x dilution.

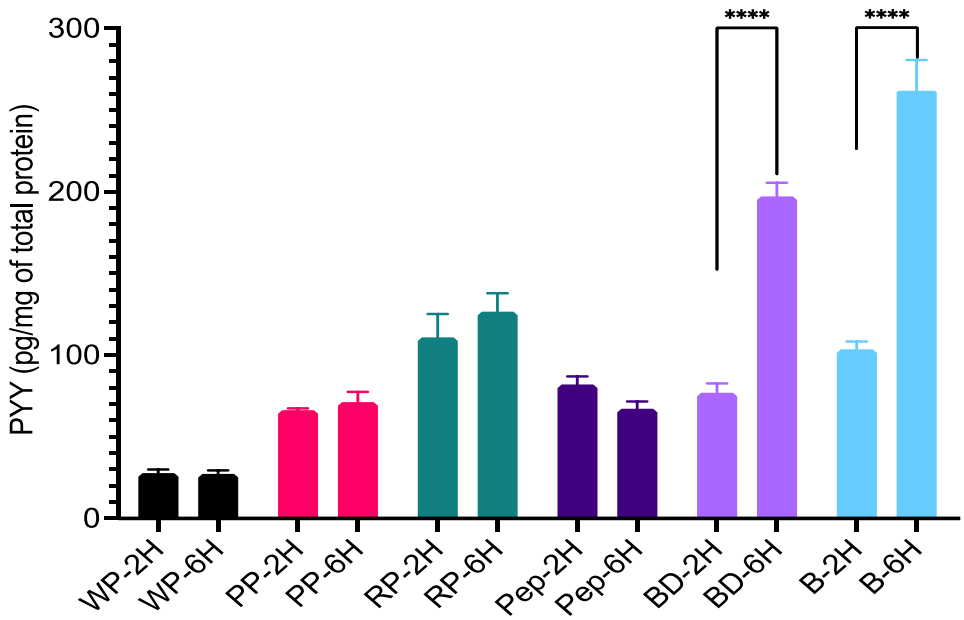


Figure 39. Intracellular PYY levels in Caco-2 cells after 2- and 6-hour treatment with digests. Results are presented as mean ±SD (pg/mg of total protein). **** Indicates significant differences when comparing time points within the same conditions (p <0.0001).

4.2.2.2. Gut hormone levels in Caco-2 with 3x and 5x diluted heat-inactivated unblended treatments

Multiplex immunoassay analysis of Caco-2 cell lysates and concentrated supernatants after exposure to heat-inactivated unblended digests at 3x and 5x dilutions revealed detectable levels of GLP-1 and PPY both intra- and extracellularly with all active treatments. However, secreted GIP levels were only detectable with 3x diluted treatments.

When Caco-2 cells were treated with 3x diluted heat-inactivated unblended digests, significant differences were observed between the conditions after both 2- and 4-hour treatments with intracellular GIP levels. Both at 2- and 4 hours, WP was significantly higher vs PP with mean differences of 0.54 pg/mg (95% CI 0.05 to 1.04, p = 0.0259) and 0.63 pg/mg (95% CI 0.06 to 1.20, p = 0.0273), respectively. However, both WP and PP conditions lead to significantly higher intracellular GIP expression compared to RP and BD after 2 hours of treatment. Mean differences observed were 1.37 pg/mg (95% CI 0.85 to 1.88, p <0.0001) and WP vs RP, 1.46 pg/mg (95% CI 0.91 to 2.00, p <0.0001) WP vs BD, 0.82 pg/mg (95% CI 0.33 to 1.32, p = 0.0004) PP vs RP and 0.91 pg/mg (95% CI 0.39 to 1.44, p = 0.0002) PP vs BD, respectively. No other significant differences were observed in intracellular GIP levels following 2-and 4-hour treatments (**Figure *40*A**).

When analysing the secreted GIP levels, no significant differences were detected between treatments at 2 hours. A significant increase in secreted GIP levels was observed with RP condition alone following 4 hours of treatment which was significantly higher compared to all other conditions with mean differences of 23.73 pg/g (95% CI 20.77 to 26.69, p <0.0001) vs WP, 25.49 pg/g (95% CI 22.50 to 28.47, p <0.0001) vs PP and 24.70 pg/g (95% CI 21.85 to 27.54, p <0.0001) vs BD (**Figure *40*B**).

When analysing the effects of 3x diluted heat-inactivated unblended digests on GLP-1 levels expressed in Caco-2 cells, an overall significant (p = 0.0308) treatment effect was observed only at 2 hours of treatment. Application of Tukey's post-hoc analysis revealed that BD was significantly higher compared to all other conditions with mean differences of 2.53 pg/mg (95% CI 0.93 to 4.13, p = 0.0011) vs WP, 2.98 pg/mg (95% CI 1.38 to 4.59, p = 0.0002) vs PP and 3.73 pg/mg (95% CI 2.04 to 5.42, p <0.0001) vs RP. No other significant differences were observed in levels of intracellular GLP-1 measured (**Figure *40*C**).

Analysis of the secreted GLP-1 levels revealed significant differences between treatments both at 2 hours as well as 4 hours. The secreted levels of GLP-1 with PP condition were significantly higher compared to WP conditions both at 2 and 4 hours, with mean differences of 235.80 pg/g (95% CI 75.64 to 395.90, p = 0.0047) and 229.00 pg/g (95% CI 107.5 to 350.60, p = 0.0009), respectively. GLP-1 secretion with PP condition at 2 hours was also significantly higher compared to RP and BD with mean differences of 155.50 pg/g (95% CI 31.44 to 279.50, p = 0.0140) and 283.60 pg/g (95% CI 152.90 to 414.40, p = 0.0002), respectively. The RP condition also resulted in significantly higher GLP-1 secretion than BD (MD 128.10 pg/g, 95% CI 4.10 to 242.20, p = 0.0424). However, following 4 hours of treatment with the digests, GLP-1 levels observed with RP condition were significantly higher than WP and PP, with mean differences of 758.30 pg/g (95% CI 640.60 to 875.90) and 529.20 pg/g (95% CI 428.50 to 630.00), respectively with significance of p <0.0001 at both comparisons. There were no measurable levels of secreted GLP-1 detected when Caco-2 cells were treated with heat-inactivated BD at 3x dilution for 4 hours (**Figure *40*D**).

Assessing the effects of 3x diluted heat-inactivated unblended digests on intracellular PYY levels expressed in Caco-2 cells revealed an overall significant time*treatment effect of p =0.0297. Tukey's post-hoc analysis revealed that treatment with PP condition led to significantly higher intracellular PYY levels compared to RP condition at 2 hours with a mean difference of 27.29 pg/mg (95% CI 6.07 to 48.50, p =0.0074). No other significant differences were observed in levels of intracellular PYY measured following either 2 or 4 hours of treatment (**Figure *40*E**).

The analysis of the secreted PYY levels revealed significant differences between treatments both at 2 as well as 4 hours. The secreted levels of PYY with PP condition were significantly higher compared to WP and BD conditions both at 2 and 4 hours, with mean differences of 852.20 pg/g (95% CI 334.60 to 1370.00, p =0.0140) vs WP, 1165.00 pg/g (95% CI 629.90 to 1701.00, p <0.0001) vs BD and 714.50 pg/g (95% CI 179.00 to 1250.00, p =0.0052) vs WP, 685.50 pg/g (95% CI 196.00 to 1175.00, p =0.0032) vs BD, respectively. PYY secretion with PP condition at 2 hours was also significantly higher compared to RP, with a mean difference of 621.60 pg/g (95% CI 103.90 to 1139.00, p =0.0135). However, the analysis of secreted PYY levels at 4 hours revealed the opposite effect, with RP condition presenting significantly higher concentrations compared to PP (MD 1117.00 pg/g, 95% CI 627.90 to 1607.00, p <0.0001). Additionally, RP condition also resulted in significantly higher PYY secretion compared to WP at 4 hours with a mean difference of 1832.00 pg/g (95% CI 1317.00 to 2347.00, p <0.0001). Significant differences in secreted PYY levels were also present between RP and BD conditions both at 2 and 4 hours treatments, where mean differences RP vs BD were 543.80 pg/g (95% CI 57.26 to 1030.00, p =0.0236) and 1803 pg/g (95% CI 1336.00 to 2270.00, p <0.0001), respectively (**Figure *40*F**).

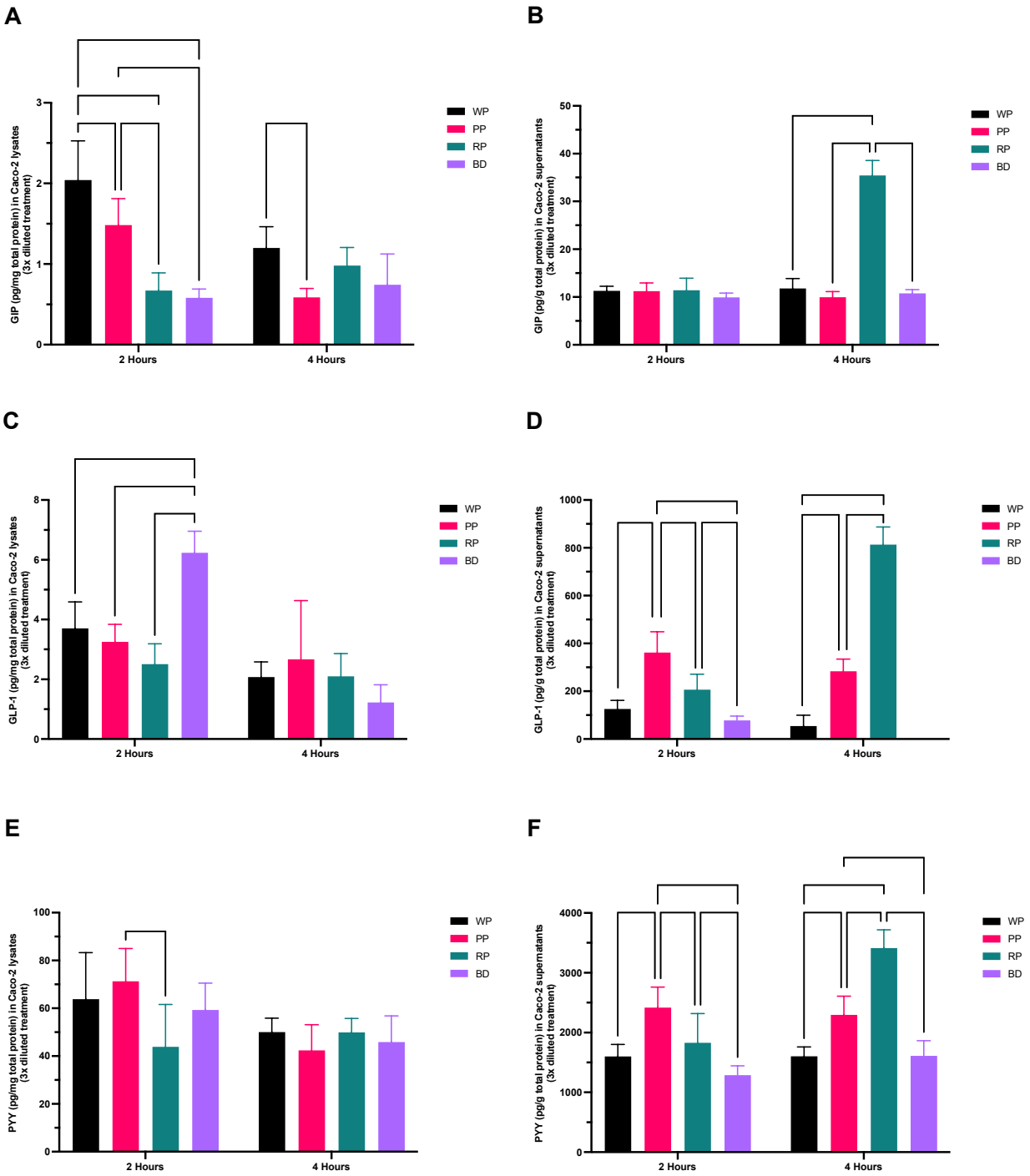


Figure 40. Intracellular and secreted gut hormone levels in Caco-2 cell after 2- and 4-hour treatment with 3x diluted heat-inactivated unblended digests. Results are presented as mean ± SD (pg/mg of total protein for intracellular and pg/g of total protein for secreted levels) and number of replicates (6) unless otherwise specified. Whey condition (WP), Potato condition (PP), Rice condition (RP) and control Blank digest condition (BD). **(A) Intracellular GIP levels** [WP 2H (5); PP 4H (3); RP 2H (5); BD 2H (4), 4H (5)], **(B) Secreted GIP levels** [WP 4H (5); PP 2H (4), 4H (5); RP 2H (5); BD 2H (5)], **(C) Intracellular GLP-1 levels** [WP 2H (5), 4H (4); PP 2H (5), 4H (3); RP 2H (4), 4H (5); BD 2H (4), 4H (3)], **(D) Secreted GLP-1 levels** [WP 2H (3), 4H (3); PP 2H (4), 4H (5); RP 2H (5); BD 2H(4)], **(E) Intracellular PYY levels** [WP 2H (5); PP 2H (5), 4H (5); RP 2H (5); BD 4H (4)], **(F) Secreted PYY levels** [WP 4H (4); PP 2H (4), 4H (5); BD 2H (5)]. *Indicates significant differences when comparing the different conditions at the same timepoint (*p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001).

Analysing the effects of 3x diluted heat-inactivated unblended WP, PP, RP and BD digests on the intra- and extracellular gut hormone concentrations with Caco-2 cells revealed significance with time at each condition (p <0.05).

Within-group analysis revealed significant differences over time with WP (p = 0.0010) and PP (p = 0.0021) in intracellular and with RP (p <0.0001) in extracellular GIP levels. With GLP-1 levels, significant over-time differences were observed with BD intracellularly and RP extracellularly, both with a significance of p <0.0001. The analysis of PYY levels showed significant over-time differences with the PP condition (p = 0.0063) in cell lysates and with the RP condition (p <0.0001) in supernatants (**Table 22**).

Table 22. Within-group differences in intracellular and secreted gut hormone (GIP, GLP-1 and PYY) levels (pg/mg and pg/g of total protein, respectively) when Caco-2 treated with 3x diluted heat-inactivated unblended protein digests.

Gut hormone	Detection condition (unit)	Treatment conditions 2H vs 4H	N 2H, N 4H	Mean Diff.	95.00% CI of diff.	Sig.*	Adjusted *p*-value
GIP	Lysate (pg/mg)	WP 3x	5, 6	0.83	0.35 to 1.30	**	0.0010
		PP 3x	6, 3	0.91	0.34 to 1.48	**	0.0021
		RP 3x	5, 6	-0.32	-0.79 to 0.15	NS	0.2567
		BD 3x	4, 5	-0.18	-0.72 to 0.36	NS	0.8251
	Supernatant (pg/g)	WP 3x	6, 5	-0.44	-2.87 to 1.99	NS	0.9790
		PP 3x	4, 5	1.33	-1.37 to 4.03	NS	0.5564
		RP 3x	5, 6	-24.19	-26.62 to -21.76	****	<0.0001
		BD 3x	5, 6	-0.86	-3.29 to 1.57	NS	0.7997
GLP-1	Lysate (pg/mg)	WP 3x	5, 4	1.63	-0.07 to 3.20	NS	0.0382
		PP 3x	5, 3	0.59	-1.12 to 2.29	NS	0.8380
		RP 3x	4, 5	0.41	-1.16 to 1.97	NS	0.9336
		BD 3x	4, 3	5.01	3.23 to 6.79	****	<0.0001
	Supernatant (pg/g)	WP 3x	3, 3	71.18	-86.05 to 228.40	NS	0.5793
		PP 3x	4, 5	77.91	-37.63 to 193.50	NS	0.2555
		RP 3x	5, 6	-606.80	-711.10 to -502.50	****	<0.0001
		BD 3x	N/A				
PYY	Lysate (pg/mg)	WP 3x	5, 6	13.61	-6.28 to 33.51	NS	0.2556
		PP 3x	5, 5	28.72	7.75 to 49.68	**	0.0063
		RP 3x	5, 6	-6.10	-26.00 to 13.79	NS	0.8670
		BD 3x	6, 4	13.82	-7.57 to 35.20	NS	0.3034
	Supernatant (pg/g)	WP 3x	6, 4	18.22	-432.00 to 468.4	NS	>0.9999
		PP 3x	4, 5	155.90	-304.80 to 616.60	NS	0.8257
		RP 3x	6, 6	-1583.00	-1972.00 to -1194.00	****	<0.0001
		BD 3x	5, 6	-324.00	-738.30 to 90.41	NS	0.1614

Results presented as mean difference (Mean Diff) and 95.00% Confidence Intervals of difference (95.00% CI of diff). N 2H and N 4H represent the number of replicates at 2 and 4 hours, respectively. Whey condition (WP), Potato condition (PP), Rice condition (RP) and control Blank digest condition (BD). Sig.* represents significance values when comparing treatments across the two different time point 2H vs 4H (**p <0.01, ****p <0.0001 and ^{NS} p >0.05).

Treating Caco-2 cells with 5x diluted heat-inactivated unblended digests led to significant differences between the conditions after the 4-hour exposure with intracellular GIP levels. Treatment with PeP condition lead to the lowest intracellular GIP expression (0.10 ± 0.02 pg/mg) and it was significantly lower compared to every other treatment with mean differences of -0.30 pg/mg (95% CI -0.39 to -0.21, p <0.0001) vs WP, -0.23 pg/mg (95% CI -0.32 to -0.14, p

<0.0001) vs PP, -0.34 pg/mg (95% CI -0.43 to -0.25, *p* <0.0001) vs RP and -0.35 pg/mg (95% CI -0.45 to -0.26, *p* <0.0001) vs BD. The highest GIP expression from the active treatments was observed with the RP condition, which, in addition to PeP, was significantly higher compared to PP (MD 0.11 pg/mg, 95% CI 0.02 to 0.20, *p* = 0.0105). BD was also significantly higher than PP (MD 0.12 pg/mg, 95% CI 0.03 to 0.21, *p* = 0.0038) (**Figure *41*A**).

When analysing the effects of 5x diluted heat-inactivated unblended digests on the GLP-1 levels expressed in Caco-2 cells, an overall significant (*p* <0.0001) treatment effect was observed. Application of Tukey's post-hoc analysis revealed that PeP had a significantly larger positive effect compared to WP and PP conditions with mean differences of 19.55 pg/mg (95% CI 10.54 to 28.55, *p* <0.0001) and 9.87 pg/mg (95% CI 1.32 to 18.41, *p* = 0.0188), respectively. Furthermore, every other treatment led to significantly higher GLP-1 expression compared to WP with mean differences of 9.68 pg/mg (95% CI 1.14 to 18.22, *p* = 0.0216) PP vs WP, 13.57 pg/mg (95% CI 5.35 to 21.79, *p* = 0.0007) RP vs WP and 15.14 pg/mg (95% CI 6.92 to 23.36, *p* = 0.0002) BD vs WP (**Figure *41*B**).

Analysis of the secreted GLP-1 levels revealed significant overall differences between treatments (*p* <0.0001). The secreted levels of GLP-1 were highest with the PP condition, and the differences were significant vs every other condition with mean differences of 0.10 pg/g (95% CI 0.03 to 0.17, *p* = 0.0031) vs WP, 0.11 pg/g (95% CI 0.04 to 0.19, *p* = 0.0006) vs RP, 0.11 pg/g (95% CI 0.04 to 0.18, *p* = 0.0008) vs PeP and 0.14 pg/g (95% CI 0.07 to 0.21, *p* <0.0001) vs BD. No other significant differences between groups were observed in extracellular GLP-1 levels (**Figure *41*C**).

Assessing the effects of 5x diluted heat-inactivated unblended digests on intracellular PYY levels expressed in Caco-2 cells revealed an overall significant effect of *p* <0.0001. Tukey's post-hoc analysis revealed that treatment with RP condition led to significantly higher intracellular PYY levels compared to every other active treatment condition with mean differences of 5.37 pg/mg (95% CI 0.09 to 10.68, *p* = 0.0448) vs WP, 7.05 pg/mg (95% CI 1.75 to 12.34, *p* = 0.0052) vs PP and 24.00 pg/mg (95% CI 18.71 to 29.30, *p* <0.0001) vs PeP, respectively.

Further significant differences were observed between WP and PP compared to PeP, with mean differences of 18.62 pg/mg (95% CI 13.32 to 23.91, p <0.0001) and 16.96 pg/mg (95% CI 11.66 to 22.25, p <0.0001), respectively. Moreover, treatments with the BD control resulted in significantly higher PPY expression compared to WP (MD 5.75 pg/mg, 95% CI 0.45 to 11.04, p = 0.0286), PP (MD 7.41 pg/mg, 95% CI 2.12 to 12.70, p = 0.0031) and PeP (MD 24.36 pg/mg, 95% CI 19.07 to 29.66, p <0.0001) conditions (**Figure *41*D**).

The analysis of the secreted PYY levels revealed significant differences between treatments (p = 0.0020) at 4 hours. The secreted levels of PYY with PP condition were significantly higher compared to RP and BD conditions, with mean differences of 0.73 pg/g (95% CI 0.23 to 1.23, p = 0.0022) and 0.69 pg/g (95% CI 0.19 to 1.194, p = 0.0038), respectively. No other significant between-group differences were observed in extracellular PYY levels (**Figure *41*E**).

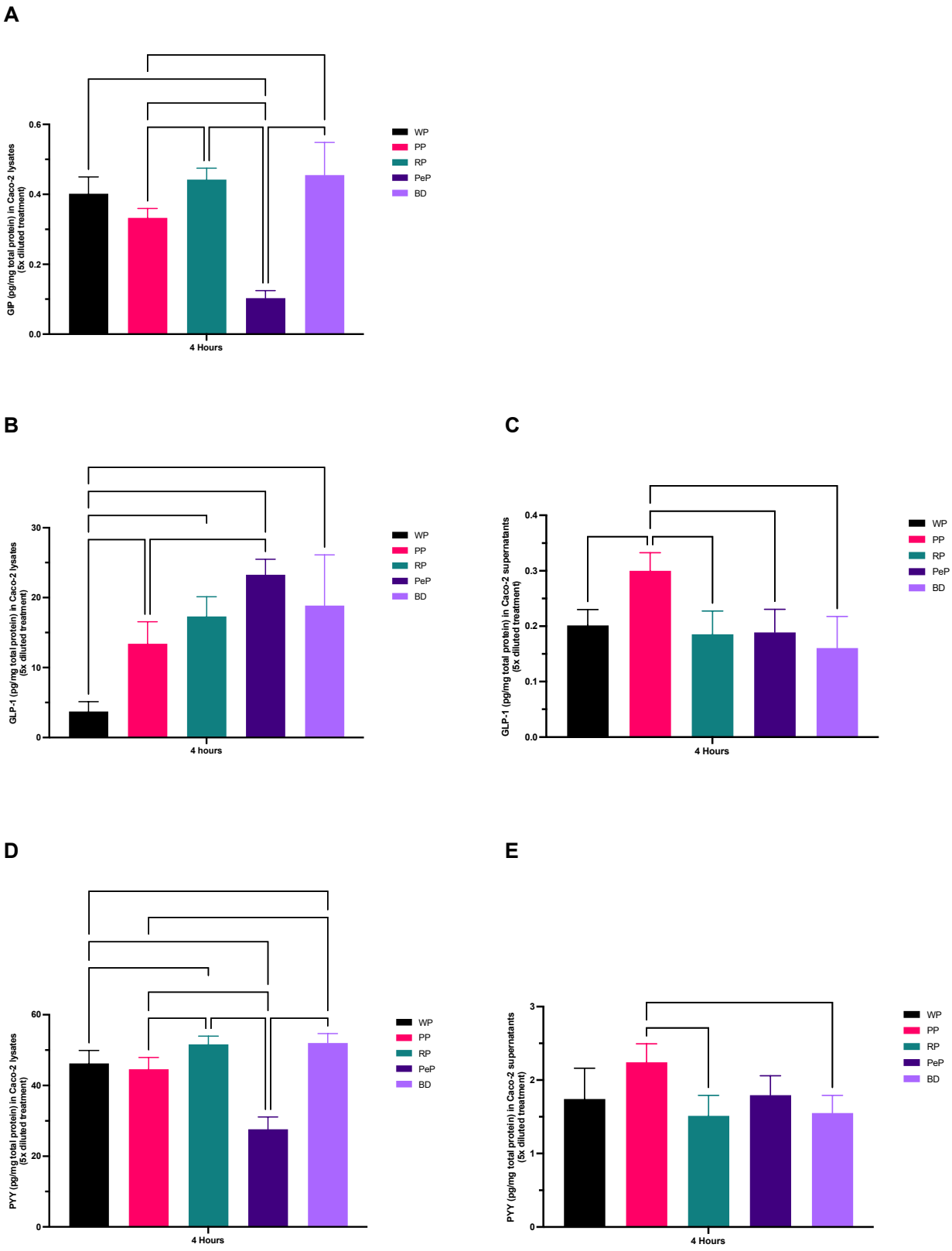


Figure 41. Intracellular and secreted gut hormone levels in Caco-2 cell after 4-hour treatment with 5x diluted heat-inactivated unblended digests. Results are presented as mean ±SD (pg/mg of total protein), number of replicates equals (6) unless otherwise specified. Whey condition (WP), Potato condition (PP), Rice condition (RP), Pea condition (PeP) and control Blank digest condition (BD). **(A) Intracellular GIP levels** [PeP (5)], **(B) Intracellular GLP-1 levels** [WP (4), PP (5), Pep (4)], **(C) Secreted GLP-1 levels**, **(E) Intracellular PYY levels** and **(F) Secreted PYY levels**. *Indicates significant differences when comparing the different conditions at the same timepoint (*p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001).

4.2.2.3. Gut hormone levels in Caco-2 vs STC-1 with 5x diluted heat-inactivated unblended treatments

When comparing the intra- and extracellular levels of gut peptides, GLP-1 and PYY, between the two different cell lines following 4 hours of treatment with 5x diluted heat-inactivated WP, PP and RP, there were significantly higher levels of intra- and extracellular GLP-1 levels measured in STC-1 compared to Caco-2 (p <0.0001). The secreted levels of PYY were also significantly higher with all 3 conditions in STC-1 vs Caco-2 cells (p <0.0001). However, intracellular PYY levels measured in STC-1 were comparable to Caco-2, with only WP treatment leading to significantly higher expression (p = 0.0009) (**Table 23**).

Table 23. Intra- and extracellular GLP-1 and PYY levels (pg/mg of total protein) in Caco-2 vs STC-1 when treated with 5x diluted heat-inactivated protein digests for 4 hours.

Gut hormone	Detection condition	Treatment conditions Caco-2 vs STC-1 (N, N)	Mean Diff. (pg/mg)	95.00% CI of diff.	Sig.*	Adjusted *p*-value
GLP-1	Lysate	WP 5x (4, 5)	-1246.00	-1628.00 to -863.20	****	<0.0001
		PP 5x (5, 6)	-932.80	-1278.00 to -587.40	****	<0.0001
		RP 5x (6, 6)	-1143.00	-1472.00 to -813.60	****	<0.0001
	Supernatant	WP 5x (6, 6)	-175.10	-218.10 to -132.00	****	<0.0001
		PP 5x (6, 6)	-691.40	-734.50 to -648.30	****	<0.0001
		RP 5x (6, 6)	-143.80	-186.90 to -100.80	****	<0.0001
PYY	Lysate	WP 5x (6, 5)	-21.64	-33.40 to -9.87	***	0.0009
		PP 5x (6, 5)	-4.71	-16.47 to 7.06	NS	0.6337
		RP 5x (6, 4)	-3.19	-15.83 to 9.46	NS	0.8701
	Supernatant	WP 5x (6, 5)	-42.34	-49.05 to -35.62	****	<0.0001
		PP 5x (6, 6)	-47.54	-53.94 to -41.14	****	<0.0001
		RP 5x (6, 4)	-40.50	-47.67 to -33.33	****	<0.0001

Results presented as mean difference (Mean Diff) and 95.00% Confidence Intervals of difference (95.00% CI of diff). (N, N) represent the number of replicates in Caco-2 and STC-1 cells, respectively. WP condition (WP), Potato condition (PP) and Rice condition (RP). Sig.* represents significance values when comparing treatment effects between the two different cell lines (***p <0.001, ****p <0.0001 and ^{NS} p >0.05).

Observing the trends of intracellular expression of GLP-1 and PYY in Caco-2 and STC-1 cells revealed that the plant-based protein treatments PP and RP had

comparable effects on the intracellular gut hormone expression. RP condition resulted in higher intracellular GLP-1 and PYY levels compared to PP (**Figure 42A & C)**. While the differences in GLP-1 levels between the two plant-based conditions were not significant neither with Caco-2 (p = 0.5681) or STC-1 (p = 0.6152) (**Figure 42A**), the differences with intracellular PYY levels were significant in Caco-2 cell (p = 0.0052) but not in STC-1 cells (p = 0.6661) (**Figure 42C**). Additionally, WP seemed to have different effects in the two different cell lines on the intracellularly expressed gut hormone levels. While in the Caco-2 cells, the WP condition resulted in significantly lower GLP-1 expression vs both PP and RP (p = 0.0216 and p = 0.0007, respectively), in the STC-1 cells, it led to the highest hormone expression out of the three treatments, although the differences were not significant (**Figure 42A**). The contradictive effects of the WP treatment in the two different cell lines were evident, with it resulting in significantly lower intracellular PYY levels compared to RP in Caco-2 (p = 0.0448) vs significantly higher levels compared to RP in STC-1 cells (p = 0.0373) (**Figure 42C**).

However, when observing the effects of the three digest treatments on gut hormone secretion in the two different cell lines, identical trends were present. PP condition led to the highest GLP-1 and PYY secretion in both Caco-2 and STC-1 cell lines (**Figure 42B & D**). Additionally, the secreted GLP-1 levels with PP treatment were significantly higher compared to the other two, WP and RP, conditions in Caco-2 (p = 0.0031 and p = 0.0006) as well as STC-1 (p <0.0001 and p <0.0001), respectively (**Figure 42B**). Secreted PYY levels were significantly higher with the PP condition compared to RP in both Caco-2 (p = 0.0022) and STC-1 (p = 0.0162) cell lines (**Figure 42D**).

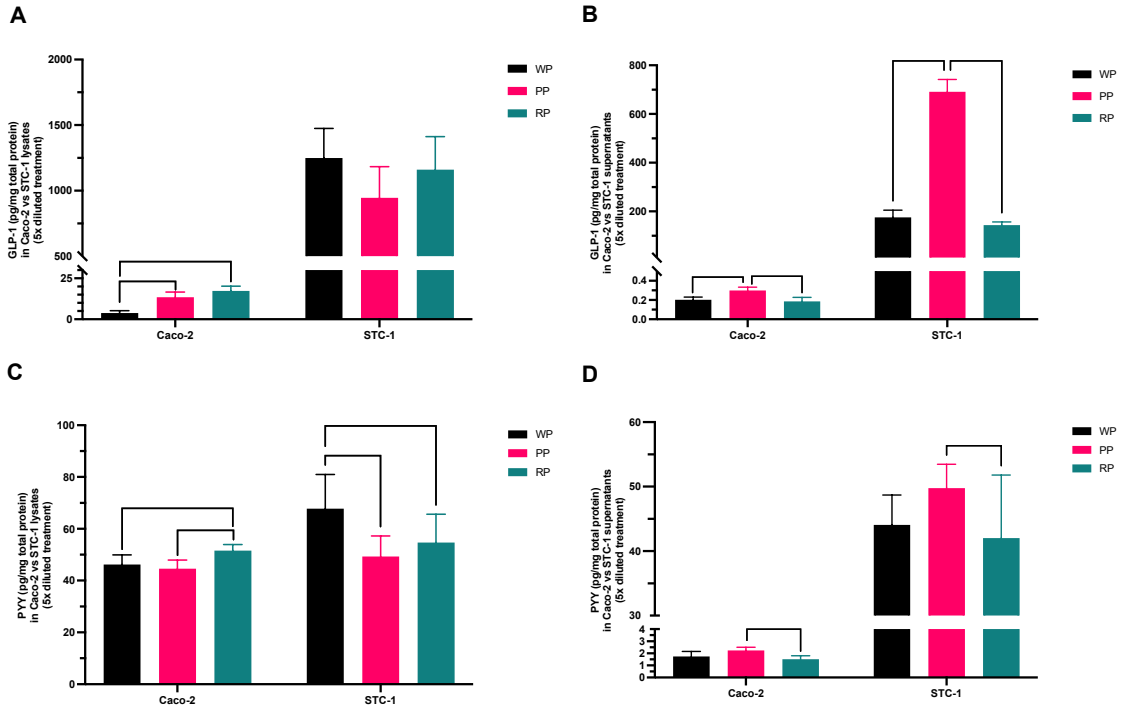


Figure 42. Representation of the expression and secretion trends of gut hormones GLP-1 and PYY in Caco-2 vs STC-1 cells after 4-hour treatment with 5x diluted heat-inactivated unblended digests. Results are presented as mean ±SD (pg/mg of total protein) and number of replicates (6) unless otherwise specified. Whey condition (WP), Potato condition (PP) and Rice condition (RP). **(A) Intracellular GLP-1 levels in Caco-2 vs STC-1** [WP Caco-2 (4), WP STC-1 (5), PP Caco-2 (5), BD Caco-2 (4), BD STC-1 (3)], **(B) Secreted GLP-1 levels in Caco-2 vs STC-1**, **(C) Intracellular PYY levels in Caco-2 vs STC-1** [WP STC-1 (4), PP STC-1 (5), RP STC-1 (4), BD STC-1 (5)], **(F) Secreted PYY levels in Caco-2 vs STC-1** [WP STC-1 (5), RP STC-1 (4), BD STC-1 (5)]. * Indicates significant differences between the treatment conditions within the same cell line (*p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001).

4.2.2.4. Gut hormone levels in Caco-2 with 3x diluted heat-inactivated blended treatments

Multiplex analysis of Caco-2 cell lysates and concentrated supernatants after exposure to heat-inactivated blended digests at 3x dilution revealed detectible levels of intracellular GIP, GLP-1 and PPY with all conditions following both 2 and 4 h treatment times. Secreted levels of GIP and PYY hormones were also detected with all conditions at both time points (**Figure 43**). However, secreted GLP-1 levels with all conditions were observed at 2 h only. Following 4 h treatment secreted GLP-1 levels were only detectable with WP/PP blend conditions, both with 50:50 and 30:70 ratios (**Figure 43D**).

When Caco-2 cells were treated with 3x diluted heat-inactivated protein blend digests, significant differences were observed between the conditions after both 2 and 4-hour treatments with intracellular GIP levels. Both at 2 and 4h, WP/PP (30:70) was significantly higher vs BD with mean differences of 1.38 pg/mg (95% CI 0.40 to 2.35, p = 0.0026) and 2.82 pg/mg (95% CI 1.48 to 4.16, p <0.0001), respectively. Treatment with WP/RP (30:70) for 2h also led to significantly higher intracellular GIP levels vs BD (MD 2.08 pg/mg, 95% CI 0.89 to 3.28, p = 0.0002), in addition significantly higher levels were observed compared to WP/RP (50:50) (MD 1.55 pg/mg, 95% CI 0.46 to 2.64, p = 0.0024). Further significant differences at 4h were present between BD vs WP/PP (50:50) and WP/RP (50:50), with mean differences of -1.34 pg/mg (95% CI -2.68 to -0.01, p = 0.0482) and -1.41 pg/mg (95% CI -2.74 to -0.07, p = 0.0354), respectively (**Figure 43A**).

When analysing the secreted GIP levels following 2h treatment, all but WP/RP (50:50) conditions lead to significantly higher levels compared to BD, with mean differences of WP/PP (50:50) vs BD 4.76 pg/g (95% CI 2.13 to 7.39, p <0.0001), WP/PP (30:70) vs BD 3.94 pg/g (95% CI 1.31 to 6.57, p = 0.0012) and WP/RP (30:70) vs BD 5.31 pg/g (95% CI 2.83 to 7.79, p <0.0001). While the secreted GIP levels at 2h with WP/RP (50:50) were significantly lower compared to WP/PP (50:50) and WP/RP (30:70) with mean differences of 2.84 pg/g (95% CI 0.08 to 5.60, p = 0.0415) and 3.39 pg/g (95% CI 0.77 to 6.01, p = 0.0061), respectively. Significantly higher secreted GIP levels were observed with all conditions compared to BD following 4h treatment, with mean differences of 4.28 pg/g (95% CI 1.65 to 6.92, p = 0.0004) with WP/PP (50:50), 4.74 pg/g (95% CI 2.37 to 7.12, p <0.0001) with WP/RP (50:50), 3.20 pg/g (95% CI 0.35 to 6.06, p = 0.0216) with WP/PP (30:70) and 4.87 pg/g (95% CI 1.62 to 8.11, p =0.0012) with WP/RP (30:70) compared to BD (**Figure 43B**).

Analysing the effects of 3x diluted heat-inactivated blended digests on GLP-1 levels expressed in Caco-2 cells revealed overall significant treatment and time*treatment effects with significance of p <0.0001 and p = 0.0221, respectively. Application of Tukey's post-hoc analysis revealed that 2h treatment with WP/RP (30:70) lead to significantly higher intracellular GLP-1 levels compared to all other active conditions with mean differences of 7.15 pg/mg (95%

CI 3.50 to 10.79, p <0.0001) vs WP/PP (50:50), 3.81 pg/mg (95% CI 0.42 to 7.21, p = 0.0222) vs WP/RP (50:50) and 4.91 pg/mg (95% CI 1.82 to 8.01, p = 0.0008) vs WP/PP (30:70). Furthermore, BD treatment also led to significantly higher levels compared to WP/PP (50:50) (MD 5.73 pg/mg, 95% CI 1.42 to 10.04, p =0.0052). However, after 4h treatment, BD condition resulted in significantly higher GLP-1 expression compared to all but WP/RP (30:70) treatment with mean differences of 7.22 pg/mg (95% CI 3.72 to 10.72, p <0.0001) vs WP/PP (50:50), 11.47 pg/mg (95% CI 7.51 to 15.44, p <0.0001) vs WP/RP (50:50) and 7.97 pg/mg (95% CI 2.87 to 13.08, p = 0.0009) vs WP/PP (30:70). Furthermore WP/RP (30:70) treatment lead to significantly higher GLP-1 expression at 4h compared to every other active condition with mean difference of 5.10 pg/mg (95% CI 1.61 to 8.60, p = 0.0020) vs WP/PP (50:50), 9.36 pg/mg (95% CI 5.39 to 13.32, p <0.0001) vs WP/RP (50:50) and 5.85 pg/mg (95% CI 0.75 to 10.96, p = 0.0189) vs WP/PP (30:70) (**Figure 43C**).

Secreted GLP-1 levels with all conditions were detected only at 2h, with WP/RP (50:50) and BD conditions leading to significantly lower levels compared to every other active treatment. The mean differences observed between WP/RP (50:50) vs WP/PP (50:50) -212.20 pg/mg (95% CI -328.90 to -95.50, p = 0.0010), vs WP/PP (30:70) -291.70 pg/mg (95% CI -408.40 to -175.00, p <0.0001) and vs WP/RP (30:70) -150.60 pg/mg (95% CI -267.30 to -33.83, p = 0.0115). The mean differences observed between BD vs WP/PP (50:50) -228.10 pg/mg (95% CI -378.80 to -77.38, p = 0.0039), vs WP/PP (30:70) -307.50 pg/mg (95% CI -458.20 to -156.80, p = 0.0004) and vs WP/RP (30:70) -166.40 pg/mg (95% CI -317.10 to -15.72, p = 0.0293). Secreted GLP-1 levels at 4 hours were only detectable with WP/PP (50:50) and WP/PP (30:70), with no significant differences between them (**Figure 43D**).

Assessing the effects of 3x diluted heat-inactivated blended digests on intracellular PYY levels expressed in Caco-2 cells revealed an overall significant treatment and time*treatment effect of p = 0.0216 and p = 0.0002, respectively. Tukey's post-hoc analysis revealed that treatment with WP/RP (50:50) condition led to significantly higher intracellular PYY levels compared to WP/PP (50:50) and WP/RP (30:70) condition at 2h with a mean difference of 44.75 pg/mg (95%

CI 6.92 to 82.58, $p = 0.0142$) and 34.60 pg/mg (95% CI 1.84 to 67.36, $p = 0.0344$), respectively. When observing the treatments effects on the intracellular PYY levels following 4h, it was evident that WP/PP (50:50) lead to significantly greater positive effect compared to WP/RP (50:50) and BD, with mean differences of 51.68 pg/mg (95% CI 13.85 to 89.51, $p = 0.0036$) and 59.24 pg/mg (95% CI 11.95 to 106.5, $p = 0.0085$), respectively. No other significant differences were observed with intracellular PYY levels (**Figure 43E**).

The analysis of the secreted PYY revealed that the highest levels were achieved following WP/PP at both rations of 50:50 and 30:70 and at both time points. At 2h levels following WP/PP (30:70) treatment were higher compared to every other condition with mean differences 557.10 pg/g (95% CI 7.94 to 1160.00, $p = 0.0454$) vs WP/PP (50:50), 1669.00 pg/g (95% CI 1120.00 to 2218.00, $p < 0.0001$) vs WP/RP (50:50), 1050.00 pg/g (95% CI 528.80 to 1571.00, $p < 0.0001$) vs WP/RP (30:70) and 1679.00 pg/g (95% CI 1158.00 to 2200.00, $p < 0.0001$) vs BD. The WP/PP (50:50) was found to have led to significantly higher levels compared to WP/RP (50:50) and BD, with mean differences of 1112.00 pg/g (95% CI 563.00 to 1661.00) and 1122.00 pg/g (95% CI 601.20 to 1643.00), respectively, both with the significance of $p < 0.0001$. There was also a significant difference between WP/RP (30:70) vs BD (MD 629.50 pg/g, 95% CI 138.30 to 1121.00, $p = 0.0065$). Similarly to the observations at 2h, following 4h treatment, WP/PP (50:50) and (30:70) also led to the highest PYY secretion. Both treatments presented significant differences compared to WP/RP (50:50) (MD 788.30 pg/g, 95% CI 286.90 to 1290.00, $p = 0.0006$ and MD 669.40 pg/g, 95% CI 199.10 to 1140.00, $p = 0.0021$) BD (MD 983.10 pg/g, 95% CI 462.10 to 1504.00, $p < 0.0001$ and MD 864.20 pg/g, 95% CI 373.00 to 1355.00, $p = 0.0001$), respectively. No other significant differences were observed with secreted PYY levels (**Figure 43F**).

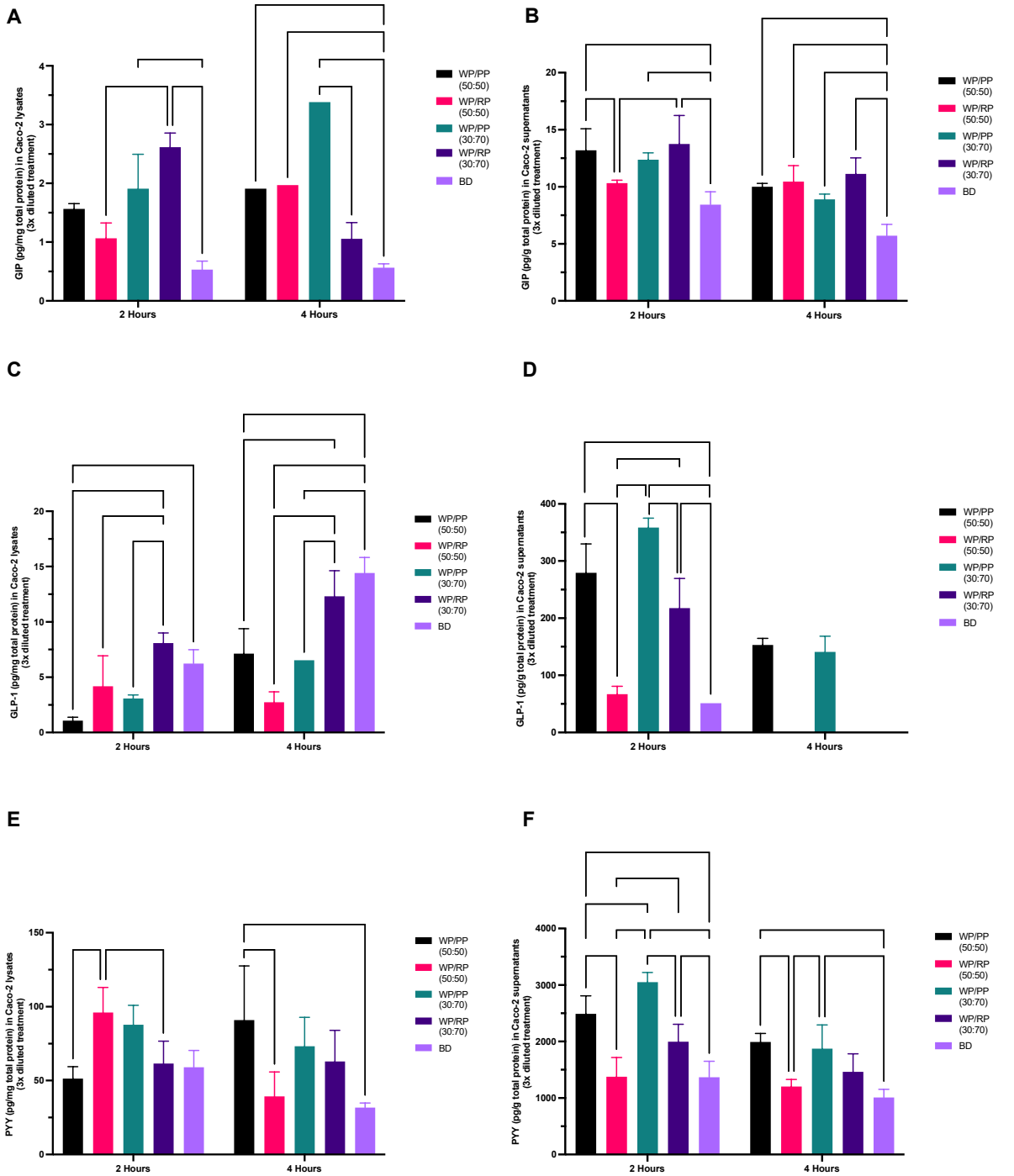


Figure 43. Intracellular and secreted gut hormone levels in Caco-2 cell after 2- and 4-hour treatment with 3x diluted heat-inactivated blended digests. Results are presented as mean ±SD (pg/mg of total protein for intracellular and pg/mg of total protein for secreted levels), number of replicates (6) unless otherwise specified. Whey/Potato protein blend 50:50 ratio digest (WP/PP 50:50), Whey/Rice protein blend 50:50 ratio digest (WP/RP 50:50), Whey/Potato protein blend 30:70 ratio digest (WP/PP 30:70), Whey/Rice protein blend 30:70 ratio digest (WP/RP 30:70) and control Blank digest (BD). **(A) Intracellular GIP levels** [WP/PP (50:50) 2H (2), 4H (1); WP/RP (50:50) 2H (3), 4H (1); WP/PP (30:70) 4H (1), WP/RP (30:70) 2H (2), 4H (2); BD 2H (2), 4H (4)], **(B) Secreted GIP levels** [WP/PP (50:50) 2H (4), 4H (4); WP/RP (50:50) 2H (4); WP/PP (30:70) 2H (4), 4H (3); WP/RP (30:70) 2H (5), 4H (2); BD 2H (5), 4H (5)], **(C) Intracellular GLP-1 levels** [WP/PP (50:50) 2H (3), 4H (3); WP/RP (50:50) 2H (4), 4H (2); WP/PP (30:70) 4H (1); WP/RP (30:70) 2H (4), 4H (5); BD 2H (2), 4H (5)], **(D) Secreted GLP-1 levels** [WP/PP (50:50) 2H (4), 4H (4); WP/RP (50:50) 2H (2); WP/PP (30:70) 2H (4), 4H (3); WP/RP (30:70) 2H (4); BD 2H (1)], **(E) Intracellular PYY levels** [WP/PP (50:50) 2H (3), 4H (3); WP/RP (50:50) 2H (5), 4H (5); WP/PP (30:70) 4H (4); WP/RP (30:70) 2H (5), 4H (4); BD 2H (3), 4H (2)], **(F) Secreted PYY levels** (WP/PP (50:50) 2H (4), 4H (4); WP/RP (50:50) 2H (4); WP/PP (30:70) 2H (4), 4H (5); WP/RP (30:70) 2H (5), 4H (2); BD 2H (5), 4H (5)]. *Indicates significant differences when comparing the different conditions at the same timepoint (*p <0.05, **p <0.01, ***p <0.001 and ****p <0.0001).

The analysis of the expressed and secreted gut hormone levels in Caco-2 cells when treated with 3x diluted heat-inactivated blended WP/PP (50:50), WP/PP (30:70), WP/RP (50:50), WP/RP (30:70) and BD digests revealed varying differences with the same treatments over time.

Within-group analysis showed that there was a significant difference over time with WP/PP (30:70) (p = 0.0102) in intracellular GIP levels. With secreted GIP levels, significant differences over time were observed with WP/PP (50:50) (p = 0.0112, WP/PP (30:70) (p = 0.0124), WP/RP (30:70) (p = 0.0479) and BD (p = 0.0159).

Significant differences were present over time in intracellular GLP-1 levels with WP/PP (50:50) (p = 0.0191), WP/RP (30:70) (p = 0.0283) and BD (p = 0.0066). The analysis of extracellular GLP-1 levels revealed significant differences over time with both treatments, WP/PP (50:50) (p = 0.0002) and WP/PP (30:70) (p <0.0001) detectable at 2h as well as 4h timepoints.

Analysis of intracellular PYY levels revealed a significant difference over time with WP/RP (50:50) (p = 0.0001) condition only. Similarly, with secreted PYY levels, a significant over-time difference was observed with one treatment condition, WP/PP (30:70) (p <0.0001) (**Table 24**).

Table 24. Within-group differences in intracellular and secreted gut hormone (GIP, GLP-1 and PYY) levels (pg/mg and pg/g of total protein, respectively) when Caco-2 treated with 3x diluted heat-inactivated protein blend digests.

Gut hormone	Detection condition (unit)	Treatment condition 2H vs 4H	N 2H, N 4H	Mean Diff.	95.00% CI of diff.	Sig.*	Adjusted *p*-value
GIP	Lysate (pg/mg)	WP/PP (50:50)	2, 1	-0.35	-1.70 to 1.01	NS	0.9785
		WP/RP (50:50)	3, 1	-0.91	-2.18 to 0.38	NS	0.2837
		WP/PP (30:70)	6, 1	-1.47	-2.67 to -0.27	*	0.0102
		WP/RP (30:70)	2, 2	1.56	0.45 to 2.67	NS	0.0029
		BD	2, 4	-0.03	-0.99 to 0.93	NS	>0.9999
	Supernatant (pg/g)	WP/PP (50:50)	4, 4	3.20	0.66 to 5.73	*	0.0112
		WP/RP (50:50)	4, 6	-0.10	-2.47 to 2.26	NS	>0.9999
		WP/PP (30:70)	4, 3	3.46	0.68 to 6.24	*	0.0124
		WP/RP (30:70)	5, 2	3.17	0.02 to 6.31	*	0.0479
		BD	5, 5	2.72	0.4567 to 4.990	*	0.0159
GLP-1	Lysate (pg/mg)	WP/PP (50:50)	3, 3	-6.35	-11.36 to -1.33	*	0.0191
		WP/RP (50:50)	4, 2	1.24	-3.74 to 6.22	NS	0.8961
		WP/PP (30:70)	6, 1	-3.36	-9.90 to 3.17	NS	0.3891
		WP/RP (30:70)	4, 5	-4.30	-8.04 to -0.57	*	0.0283
		BD	2, 5	-7.84	-12.71 to -2.96	**	0.0066
	Supernatant (pg/g)	WP/PP (50:50)	4, 4	126.30	70.15 to 182.40	***	0.0002
		WP/RP (50:50)	N/A				
		WP/PP (30:70)	4, 3	217.60	157.00 to 278.20	****	<0.0001
		WP/RP (30:70)	N/A				
		BD	N/A				
PYY	Lysate (pg/mg)	WP/PP (50:50)	3, 3	-39.59	-79.56 to 0.39	NS	0.0533
		WP/RP (50:50)	5, 5	56.84	25.87 to 87.81	***	0.0001
		WP/PP (30:70)	6, 4	14.54	-17.06 to 46.15	NS	0.7054
		WP/RP (30:70)	5, 4	-1.40	-34.25 to 31.44	NS	>0.9999
		BD	3, 2	27.28	-17.42 to 71.98	NS	0.4247
	Supernatant (pg/g)	WP/PP (50:50)	4, 4	497.20	-60.39 to 1055.00	NS	0.0924
		WP/RP (50:50)	4, 6	173.40	-337.10 to 683.80	NS	0.8658
		WP/PP (30:70)	4, 5	1173.00	643.40 to 1703.00	****	<0.0001
		WP/RP (30:70)	5, 2	540.70	-123.20 to 1204.00	NS	0.1409
		BD	5, 5	358.10	-140.60 to 856.90	NS	0.2318

Results presented as mean difference (Mean Diff) and 95.00% Confidence Intervals of difference (95.00% CI of diff). N 2H and N 4H represent the number of replicates at 2 and 4 hours, respectively. Whey/Potato protein blend 50:50 ratio digest (WP/PP 50:50), Whey/Rice protein blend 50:50 ratio digest (WP/RP 50:50), Whey/Potato protein blend 30:70 ratio digest (WP/PP 30:70), Whey/Rice protein blend 30:70 ratio digest (WP/RP 30:70) and control Blank digest (BD). Sig.* represents significance values when comparing treatments across the two different time points, 2H vs 4H (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, ^{NS} p >0.05).

4.3. Discussion

This study demonstrated differing appetite hormone regulatory properties between WP, PP, RP, and PeP in cell line models following in vitro digestion. These effects were observed using the highest treatment concentrations deemed suitable based on cell viability tests in Caco-2 and STC-1 cells, in order to maximise the potential observable effects. The GLP-1 and PYY responses with RP and PP with lower treatment dilution (6.25 mg/mL protein concentration, the highest viable concentration in the Caco-2 cell line) were more substantial than WP following the longer 4h exposure time. Conversely, with a shorter 2h exposure time at lower dilution, as well as with the higher dilution (4.17mg/mL protein concentration, the highest viable concentration in the STC-1 cell line) following 4h treatment, only PP showed increased potency for appetite hormone regulation compared to WP with significantly higher GLP-1 secretion. The observed trends in GLP-1 and PYY secretion with the higher dilution of digested WP, PP, and RP were identical between the Caco-2 and STC-1 cell lines. Together, this suggests that the characteristics of each protein, such as digestibility and absorption rate, AA and peptide profile, and the applied concentration, may result in differing appetite hormone regulatory responses, independent of the cell line models (Caco-2 or STC-1) used.

When establishing the suitability of different treatment dilutions for the specific cell lines, there were significant differences observed between treatments at the same concentration. The observed digestibility of the tested protein isolates may go some way to explaining the significant differences present in cell viability between unheated WP and RP at 7x dilution. As all WP fractions were observed to be digested, the availability of their glutamine content may have been maximised during the treatment. While the glutamine content of RP is similar to WP with 17.22 and 18.1 g / 100g protein, RP-derived prolamin, which contains the highest amount of glutamine (19.9 mol%) of all RP protein fractions (Hibino et al., 1989), showed resistance to digestion possibly leading to reduced levels of free glutamine within the digest. Glutamine is known to provide energy for proliferating cells in addition to its utilisation in protein synthesis and is transported into the cell via the SLC1A5 AA transporter, where it undergoes

deamination facilitated by glutaminase within the mitochondria. Glutamate is further converted to α-ketoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle and a key metabolite for energy production. Glutamate within the cell is also crucial to producing glutathione (GSH), which protects against oxidative stress. The glutamine transport via the SLC7A5 AA transporter also enables the influx of leucine. The influx of leucine activates cell growth mediated by the mechanistic target of rapamycin complex 1 (mTORC1), an essential regulator of cell growth and metabolism (Endicott, Jones and Hull, 2021). Therefore, the absence of glutamine may also explain the significant difference observed in cell viability between WP and BD at the same concentration. Due to the lack of glutamine in the BD digests, the energy source supporting cell growth and proliferation was limited, as well as there was reduced protection against oxidative stress induced by bile acids present in the digesta (Lechner et al., 2002). This observation is further supported by BD showing normal mitochondrial activity (cell viability > 80%) at 9x dilution as with the 1:9 dilution (digesta:MEM), the final concentration of bile acids was 1mM, which was previously shown to be non-toxic to Caco-2 cells (Shekels, Beste and Ho, 1996). Moreover, treatments with plant-based protein digests presenting lower toxicity with higher concentrations (5x dilution PP, 6x RP and PeP) compared to WP digest at the same concentrations could potentially be attributed to their extra antioxidant characteristics as previously presented in the literature (Wang et al., 2016; Hellmann, Goyer and Navarre, 2021; Lefranc-Millot et al., 2022).

The availability of glutamine may also partially explain the detected intracellular PYY levels when Caco-2 cells were treated with 7x diluted unheated digests. The observed levels were the lowest with WP digest treatment after both 2 and 6 h timepoints and significantly lower compared to any other conditions tested, including BD and MEM, with significance levels ranging from $p = 0.0007$ to <0.0001 and $p = 0.0004$ to <0.0001, respectively. Although this may seem contentious, as it is known that proteins, including WP and RP, increase circulatory PYY levels (Sukkar et al., 2013), the significantly lower intracellular PYY levels may be due to increased hormone secretion resulting in higher extracellular PYY levels. It has been shown previously that gut hormone secretion

from the L-cells after protein digestion is stimulated by small oligopeptides (di- or tripeptides) as well as free AAs. The proposed mechanism of gut hormone secretion is mediated by the uptake of peptides and free AAs by Pept1 and AA transporter, respectively. As Pept1 is a proton-coupled and AA is a sodium (Na^+) transporter, these mechanisms may involve membrane depolarisation and a subsequent influx of Ca^{2+} from voltage-gated Ca^{2+} channels (Hjørne, Modvig and Holst, 2022). Moreover, basolateral mechanisms of gut hormone secretion may also be mediated by absorbed AAs via activation of the basolateral CASR and GPRC6A. AA binding to these receptors leads to the activation of protein kinase C and the release of Ca^{2+} from the endoplasmic reticulum via the phosphatidylinositol pathway, which consequently leads to GLP-1 exocytosis (Watkins, Koumanov and Gonzalez, 2021).

Furthermore, glutamine after absorption was also shown to increase the co-secretion of GLP-1 and PYY by activation of an unknown basolateral receptor, leading to an increase in cAMP (Joshi, Tough and Cox, 2013; Hjørne, Modvig and Holst, 2022). Therefore, WP digest containing the highest amount of glutamine may have induced an increased secretion of PYY levels. Although RP contained a similar amount of glutamine, its incomplete digestion, leading to reduced levels of free glutamine, may explain the significantly higher intracellular PYY levels observed, compared to WP after both 2 and 6 treatments, with a mean difference of 38.47, $p = 0.007$ and 43.92, $p = 0.0001$, respectively. The glutamine content of both PP and PeP was lower than that of WP by 7.40 and 4.78 g / 100g total protein, respectively. Although glutamine levels of PP were lower compared to PeP, and its digestion was also observed to be incomplete, there were no significant differences observed in intracellular PYY levels at any time point. This observation may be attributed to possibly enhanced PYY secretion mediated by potato protease inhibitors (Flechtner-Mors et al., 2020). No significant differences were observed over time with the same digest treatments. In contrast, the intracellular PYY levels with BD and MEM increased significantly over time (120.20, $p < 0.0001$; 158.20, $p < 0.0001$, respectively). This may indicate increasing PYY production due to limited AAs but minimal secretion due to the lack of glutamine within these treatments. The significantly lower intracellular PYY

levels with BD vs MEM at 2 and 6 h may result from bile acid-mediated PYY secretion. Bile acids are signalling molecules with key regulatory roles in peripheral metabolism. The effect of bile acids on the L-cells is mediated by the Takeda GPCR 5 (TGR5), which leads to an elevation in intracellular cAMP levels and PKA activation (Kuhre et al. 2018; Goldspink et al. 2018). Acute bile acid exposure was shown to induce GLP-1 and PYY release from L-cells in the small intestine and colon (Kuhre et al., 2018; Christiansen et al., 2019). However, when supernatants were assessed after 2- and 6-hour exposure to 7x diluted unheated digests with a corresponding estimated protein concentration of 3.13mg/mL, no measurable levels of PYY were detected.

As suggested previously, a potential threshold for the required levels of AAs necessary for appetite hormone secretion may exist in intestinal cells (Tiekou Lorinczova et al., 2021). Furthermore, secreted levels of PYY might have been below the detection level of the ELISA kit used in this study. As observed in previous studies, heat-inactivation may cease the potential remanence of protease activity following digestion (Tenenbaum et al., 2023), thus helping to increase cell viability and, therefore, the usable concentration of treatments when applying digests. Indeed, following heat treatment of digests, desirable viability was achieved with 3x dilutions in Caco-2 cells with all except PeP conditions. Protein concentration in 3x diluted digests was estimated to be 6.25 mg/mL, enabling a two-fold increase in the concentration of interest protein in the treatment vs the 7x dilution (3.13 mg/mL). Furthermore, viability of >80% with PeP digest was observed when diluted 5x, corresponding to an estimated protein concentration of 4.17 mg/mL. Thus, in addition to the above-stated antioxidant characteristics of plant-based proteins, enhanced cell viability could also be attributed to antitrypsin properties of plant-based protein fractions, such as potato protease inhibitors (Zhu, Lasrado and Hu, 2017), brown RP-derived Alpha1-antitrypsin (Viglio et al., 2020) or the PeP-derived lectins (Salim et al., 2023). In order to maximise the potential impact of available free AAs and peptides within the digests on appetite hormone regulation, digests achieving desirable cell viability with 3x dilution were used to treat Caco-2 cells for 2 and 4 hours. Treatment with 3x diluted heat-inactivated digests showed that the impact on

appetite hormone expression within the Caco-2 cells differed significantly between active treatments and the positive control (BD) at 2 hours, where WP and PP led to significantly higher intracellular GIP levels vs BD, with significances of p <0.0001 and p = 0.0002, respectively. However, the opposite effects were observed with intracellular GLP-1 levels, where all active treatments WP, PP and RP resulted in significantly lower expression compared to BD, p = 0.0011, p = 0.0002 and p <0.0001, respectively. Furthermore, no significant differences between the active treatments and BD were observed in intracellular PYY levels. Although the observed differences in intracellular GIP levels at 2 hours did not lead to significant differences in hormone secretion between the different treatments, the secreted GLP-1 levels may partially provide an explanation for the differences present in the intracellular levels. The GLP-1 secretion at 2 hours led to significantly (p = 0.0002 vs PP and p = 0.0424 vs RP) higher levels compared to BD with all but the WP condition. Although secreted levels of GLP-1 were also higher with WP treatment (MD 47.85 pg/g, 95% CI -112.3 to 208.0), the difference was not statistically significant (p = 0.8055). This is in agreement with the previous observation by Tenenbaum et al. (2023), where treatment of STC-1 cells with digested WP isolates (WPId) at a concentration of 10 mg/mL did not lead to significantly higher GLP-1 secretion compared to BD (p = 0.08). However, treatment with the WP hydrolysate digest (WPHd) at the same concentration for 2 hours did result in significantly higher GLP-1 secretion compared to both BD and WPId, with significance values of p <0.01 and p <0.0001, respectively (Tenenbaum et al., 2023). It was reasoned that the differences observed in the bioactivities of WPId and WPHd might be attributed to the modified peptide profile following digestion as a result of enzymatic hydrolysis. Indeed, Tenenbaum et al. (2023) presented a higher proportion of small peptides within the WPHd compared to that observed in WPId. Furthermore, such a difference in bioactivities was also observed in humans, where it has been shown that hydrolysed WP increased insulin secretion compared to non-hydrolysed protein (Morifuji et al., 2010).

Furthermore, significantly higher levels of secreted GLP-1 and PYY following 2 hours of treatment with PP compared to WP (p = 0.0047 and p = 0.0140), RP (p

= 0.0140 and p = 0.0135) and BD (p = 0.0002 and p = 0.0032), respectively, could be explained by the presence of potato protease inhibitors, comprising 50% of total PP fractions. Although secreted PYY levels with PP treatment remained significantly higher vs WP (p = 0.0052) and BD (p = 0.0032) and GLP-1 levels compared to WP (p = 0.0009), following 4 hours of treatment, there was a significant (p <0.0001) increase in all the three appetite hormone secretion over time with RP treatment, attaining the most potent bioactive characteristic in appetite hormone secretion between all 4 conditions.

The potential high-quality calcium chelating properties of RP peptides could explain this phenomenon with RP conditions. Although further research is required to understand the underlying mechanisms, Yang et al. (2024) demonstrated that the RP-derived peptide AHVGMSGEEPE (AHV) displayed optimal calcium-binding properties with a calcium-chelating capacity of 55.69 ± 0.66 mg/g in a Caco-2 cell monolayer. Moreover, a potentially enhanced GLP-1 release due to co-ingestion of protein and calcium was also highlighted, related to a probable synergistic effect between phenylalanine and Ca^{2+}, also apparent in the release of PYY and GIP (Jonathan D Watkins, Koumanov and Gonzalez, 2021). The observed delay in appetite hormone secretion with RP condition could be attributed to its slow absorption rate, as it was observed with chickpea and lupin protein isolates in Caco-2 cells, where following 2 hours of incubation, with the transport values of the individual AAs being lower (p <0.01) than those measured after casein or lactalbumin treatments (Rubio and Clemente, 2009). Furthermore, it was observed in humans that total plasma AA for WPI reached its maximum concentration after 69 ±3 min, while RPI was significantly slower (93 ±4 min) with a significance value of p = 0.00, classifying RPI as a medium to slow absorbing protein (Purpura et al., 2014). However, this slower absorption rate could lead to an overall stronger satiating effect, such as observed with slow-absorbed casein when compared to WP when consumed as a preload to a meal (Abou-Samra et al., 2011). This may further contribute to the reasoning behind the significantly higher appetite hormone secretion observed with RP treatment for a duration of 4 hours. Nevertheless, by 4h, the significant differences in intracellular appetite hormones between active and blank digests were

diminished, likely due to the degradation by aminopeptidase DPP-IV and aminopeptidase P cell-surface peptidases present in the small intestine brush border membranes (Mentlein, 2004; Toräng et al., 2016; Agustia et al., 2024)

Despite RP showing superior appetite hormone secretory effects at 3x dilution in Caco-2 cells, with the reduction in concentration required to enable comparison with STC-1 (5x dilution), RP did not show a higher impact than that related to the presence of bile acids in the blank digests. Furthermore, the PeP studied at this concentration in Caco-2 cells did not show an increased impact on appetite hormone regulation compared to BD. However, the potency of PP in inducing gut hormone secretion remained evident, with significantly higher GLP-1 levels vs every other treatment and PYY levels compared to BD and RP. These results further support the observation that a threshold for AA and bioactive peptide concentration may exist for triggering appetite hormone secretion (Tiekou Lorinczova et al., 2021; Tenenbaum et al., 2023), which was reached by PP but not by RP at the lower concentration tested.

Nonetheless, the appetite hormone regulatory trends observed in Caco-2 and STC-1 cells were closely comparable when applying treatments with higher dilution. Although WP seemed to have a higher impact on intracellular gut hormone expression in STC-1 cells than in Caco-2 cells, the trends observed with PP and RP were similar across the two cell lines, with RP leading to higher peptide hormone expression vs PP. Moreover, the GLP-1 and PYY secretion trends were identical, with differences and significances observed between the same treatment comparisons across the two cell lines. The identical observations were present with PP, which led to significantly higher GLP-1 secretion compared to both WP and RP, and significantly higher PYY secretion compared to RP in both cell lines. Furthermore, comparably, PYY secretion was also higher with the PP condition vs WP, while the differences were not statistically significant with any of the cell lines. These comparable observations support the Caco-2 cells' suitability as an alternative cell line to STC-1 for nutrient-induced appetite hormone regulation studies.

Lastly, the previously described effects of plant-derived bioactive fractions on appetite hormone regulation were also evident in our preliminary observations with blended protein isolate digests. The blend of WP/PP in a 50:50 ratio led to significantly higher secretion of GIP, GLP-1 and PYY compared to the WP/RP blend with the same ratio at 2h (p <0.05). The secretion of GLP-1 and PYY with a 30:70 ratio of WP/PP was also significantly higher compared to the blend of WP/RP with the equivalent ratio, with significances of p <0.0001 and p <0.01, respectively. The WP/RP (30:70) blend presented an increased impact only on GIP secretion with significantly higher concentration measured compared to WP/RP (50:50). All secreted appetite hormone levels were significantly higher with active treatments vs BD, except WP/RP (50:50). The previously observed surge in appetite hormone secretion after 4 hours of treatment with RP was not present when RP was blended with WP. The negligible impact of WP/RP (50:50), where only half of the total 6.25 mg/mL protein concentration is derived from RP isolate, and the comparable increased impact of the 30:70 blend to that of RP at the concentration of 4.38 mg/mL further indicates the relevance of a bioactive peptide threshold required for appetite hormone secretion. Although further studies are required to confirm these preliminary results, no advantages derived from blending plant-based proteins with WPI were observed in appetite hormone regulation. However, WPH leading to significantly higher GLP-1 secretion compared to WPI in STC-1 cells (Tenenbaum et al., 2023) may justify further investigation into potential synergistic appetite regulatory effects of WPH and plant-derived proteins when blended to achieve a complete protein source.

This study is the first to show GIP expression and secretion in the Caco-2 cell line and identical trends in GLP-1 and PYY secretion between Caco-2 and STC-1 cells while observing differing effects between WP and plant-based proteins, influenced by concentration. Overall, within the scope of this project, plant-derived PP and RP were observed to be more effective at inducing gut hormone secretion than the unhydrolysed form of WP isolate *in vitro*. These effects were likely due to the presence of plant-derived bioactive peptides following digestion. Although further investigations are required to confirm the peptide profile of digested proteins and their bioactive characteristics, the presented findings shed

more light onto the relative effects of WP, PP, RP and PeP isolates on the dynamics of appetite hormone synthesis and secretion, which seem to be influenced by the rate of digestion and absorption, the presence of EAAs namely glutamine as well as the concentration of bioactive fractions such as potato protease inhibitor. The observed enteroendocrine hormone secretions in Caco-2 compared to STC-1 cells highlighted its suitability as an alternative cell line model for studying nutrient-induced appetite hormone regulation.

5. CHAPTER 5 - Assessing the effects of whey and potato protein-enriched breakfasts on appetite regulation in healthy male participants

5.1. Introduction

5.1.1. Global Prevalence and Health Impact of Obesity

Obesity (excessive body fat accumulation) is a global epidemic, with more than 650 million people classified as obese (WHO, 2021). Obesity is a source of major health concern due to related issues such as T2D, cancer, CHD, as well as depression (Jarolimova, Tagoni and Stern, 2013). Therefore, there has been a major increase in research focusing on obesity during the past four decades, leading to numerous approaches available for its management and prevention (Reynolds, 2013).

5.1.2. Current Clinical Treatments for Obesity

The most effective approaches at present are bariatric surgeries, such as RYGB (Colquitt et al., 2014), and the subcutaneously administered GLP-1Ras Liraglutide and GLP-1 analogue (GLP-1A) Semaglutide (Bergmann et al., 2023). During RYGB, a small pouch (~30 mL) is created from the upper portion of the stomach, and bypassing the duodenum, it is connected to the jejunum (Olbers et al., 2003). The smaller stomach size and the duodenum bypass result in smaller meal portions as well as fewer nutrients absorbed in the small intestine, leading to a significant calorie deficit (Akalestou et al., 2022). In comparison, Liraglutide and Semaglutide are once-a-day and once-a-week administered pharmacological treatments, respectively (Bergmann et al., 2023). The above-described surgical and pharmacological treatments were shown to lead to a mean weight loss of 25 %, 6% and 15%, respectively, as well as aid in the management of obesity-related conditions, such as T2D, with remission observed in 72% of the patients 2 years post-surgery (Sjöström et al., 2007; Bergmann et al., 2023). RYGB, GLP-1Ras and GLP-1A are comparable in their effects on appetite reduction and satiety increase by modulating the regulation of gut-derived satiety hormones, including PYY and GLP-1. Although effective, surgical and pharmacological treatments are expensive, with limited accessibility and

potentially serious or life-long side effects (Livingston, 2005; Malinowski, 2006; NHS, 2019). Therefore, the first line approach to obesity management is dietary modification.

5.1.3. Dietary Interventions and Protein-Based Strategies

From the numerous dietary modifications available (e.g. low-fat, low-carbohydrate and low-calorie diets), meal replacement products were shown to have the most significant impact on weight reduction and maintenance (Ruban et al., 2019). Although the scientific evidence supporting the efficacy of these supplements is sparse at present (Dwyer et al., 2005), meal replacement therapies seem to be successful in supporting weight loss, with 2.1% greater weight reduction compared to a conventional calorie control diet at 40 weeks (Ruban et al., 2019). Meal replacement products such as shakes or dried packaged meals often contain increased levels of protein from various sources such as milk-derived whey, casein or plant-based soy, rice and pea protein isolates. The total protein content of the meal replacement products can vary between 20 to 40g per meal (Anderson et al., 2007; Mollard et al., 2017), corresponding to the optimal acute anabolic range (Aragon and Schoenfeld, 2013). Protein sparing, as well as increased weight loss effects, have been suggested with a higher 40g protein intake compared to the lower 20g (Contaldo et al., 1980; Layman, 2004; Murphy, Hector and Phillips, 2015).

On the whole, an increase in protein in the daily diet has been shown to have the potential to contribute to the management and prevention of obesity (Luhovyy, Akhavan and Anderson, 2007). The increase in protein appears to raise PYY 3-36 levels significantly (Batterham et al., 2006), as well as GLP-1 expression compared to carbohydrate or fat (Van Der Klaauw et al., 2013). It is believed that different proteins have unique characteristics depending on the source of protein, AA content and absorption kinetics, the rate and extent of AA uptake from the digestive tract into the bloodstream (Trommelen, Tomé and van Loon, 2021). Thus, proteins from various sources might have different effects on satiety and appetite regulation (Bendtsen et al., 2013). Dairy products that are a complete source of protein, containing all essential AAs (AAs which must be obtained from

the diet), were found to have promising effects on body weight and composition (Gilbert et al., 2011). Dairy proteins, particularly whey and casein, have been shown to reduce appetite and increase satiety (Pal et al., 2014) via the ileal break effect (Hall et al., 2003) as well as via the involvement of enteroendocrine hormone secretion (PYY, GLP-1 and CCK) (Fromentin et al., 2012). Whey contributes 20%, while casein contributes 80% to the total protein content of dairy protein. Casein and WP were observed to have different physiological effects related to their AA constitution (Faghih et al., 2011; Pennings et al., 2011). WP is high in BCAAs and contains bioactive components such as α-lac. The αlac fraction makes up 20% of WP, and it has been hypothesised to have a major contribution to satiety inductions as it has a high content of essential AAs such as leucine (LEU), lysine (LYS) and tryptophan (TRYP) (Chatterton et al., 2006). Some AAs found in WP were shown to contribute to appetite regulation, such as TRYP, which affects satiety signalling in the brain as it is a precursor of the anorexigenic (loss of appetite) signalling molecule, serotonin (or 5-hydroxytryptamine, 5-HT). Furthermore, LEU and LYS are ketogenic (catabolized to ketone bodies in adipose tissue) AAs, which have been shown to be involved in reduced appetite under ketogenic conditions (Gloaguen et al., 2012). Although casein is also high in TRYP, Westerterp-Plantenga et al. (2009) indicated that TRYP may not play an important role in appetite regulation on its own but rather in combination with other AAs.

5.1.4. Plant-Based Proteins and Environmental Considerations

Some novel plant-based proteins also show potential in relation to satiety and appetite regulation. Zhu et al. (2017) demonstrated in 44 healthy women (in a double-blind placebo-controlled crossover trial) that consumption of 15 mg or 30 mg PI2 one hour prior to a standard breakfast meal resulted in significantly lower postprandial hunger and significantly higher postprandial fullness compared to placebo. Potato protease inhibitors are fractions of PP comprising 50% of the total protein content (Waglay, Karboune and Alli, 2014). Moreover, a recent study showed that administration of brown RP isolate in 9 healthy male participants (in a single-blind, triple-crossover design) led to a significant post-consumption increase in GLP-1 levels at 120 min in comparison to circulating levels observed

at 30 min (p = 0.024) and at 60 min (p = 0.04). Although differences in GLP-1 levels were observed between whey vs rice proteins at 60 min after consumption (with significantly higher circulatory levels in the whey condition, p = 0.001), this difference was not evident in subjective appetite perception (Tiekou Lorinczova et al., 2021). Furthermore, recent studies suggest that the choice of plants as a source of protein may be a more viable option for individuals with an increased risk of developing insulin resistance or diabetes due to lower insulinemic response as well as improved glucose maintenance observed after consumption compared to animal-derived proteins (He et al., 2013; Tiekou Lorinczova et al., 2021).

There appears to be an increased interest in plant-sourced proteins, possibly due to consumers' enhanced environmental awareness related to protein production (Medawar et al., 2019). In 2010, the global CO_2 equivalent greenhouse gas (CO_{2eq} GHG) emission per 100 grams of protein produced was shown to be different depending on the source, with milk, rice and potato contributing 9.50, 6.27 and 2.71 kg CO_{2eq} GHG, respectively (Poore and Nemecek, 2018). Furthermore, it was suggested that approximately 6 kg of plant protein is required to yield 1 kg of high-quality animal protein (Pimentel and Pimentel, 2003). Therefore, direct human consumption of plant-derived proteins may be more environmentally sustainable than the consumption of animal-derived proteins (Tonsor, Lusk and Schroeder, 2022).

5.1.5. Study Rationale and Objectives

Although plant-based proteins may serve as a more environmentally sustainable alternative to animal-derived proteins, research on the appetite regulatory effects of plant-based proteins is still in its early stages. Therefore, this study aimed to identify the appetite regulatory effects of animal and plant-derived proteins using high-quality commercially available protein isolates: WP (Impact Whey Protein Isolate, Myprotein, Inc, Manchester, UK) and PP (Solanic®200, ProteinmiXer.com®, Bonn, Germany), when added to a carbohydrate-rich standard meal to increase the protein content.

5.2. Results

Twelve out of the seventy-three screened participants (Appendix C) were recruited for the study. Two participants withdrew from the study after the first visit due to time commitment issues, resulting in ten participants completing the study with the following characteristics (mean ± SD): age: 28.4 ± 8.15 years; height: 172.22 ± 0.05 cm; bodyweight: 76.3 ± 12.28 kg; BMI 25.47 ± 3.32 kg/m2 and percentage body fat (% BF) 25.06 ± 4.53 %; HOMA-IR 2.48 ± 0.25. Participants were of mixed ethnicity, with 50% identifying as Asian, 30% African and 20% Caucasian.

5.2.1. Glycaemic and insulinaemic response

There was an overall significant difference over time ($p < 0.0001$) and an overall significant Condition * Time interaction ($p = 0.0262$) observed for capillary blood glucose (**Figure *44***). Time-related changes were observed within all three conditions. There was a significant increase in glucose concentration from baseline to 30 min timepoint with all three conditions, specifically: with Control 2.07 ± 0.23 mmol/L ($p < 0.0001$), with Whey condition 1.67 ± 0.32 mmol/L ($p = 0.004$), and with Potato condition 2.14 ± 0.27 mmol/L ($p = 0.0007$) (**Figure *45***).
With the Control condition, glucose levels were still significantly higher at 60 min compared to baseline, with a mean difference of 1.27 ± 0.29 mmol/L ($p = 0.0154$), despite them being significantly reduced compared to the levels measured at 30 min with the mean difference of 0.80 ± 0.17 mmol/L ($p = 0.0107$). Additionally, significantly lower levels of glucose were also observed at 120 and 180 min compared to 30 min, with mean differences of 2.14 ± 0.24 mmol/L ($p < 0.0001$) and 2.23 ± 0.24 mmol/L ($p < 0.0001$), respectively. Glucose levels measured at 120 and 180 min were also significantly lower compared to 60 min with mean differences of 1.34 ± 0.34 mmol/L ($p = 0.0243$) and 1.43 ± 0.33 mmol/L ($p = 0.0159$), respectively (**Figure *45***).

In the Whey condition, there was a significant reduction in glucose levels at 60 min compared to 30 min, with a mean difference of 1.15 ± 0.23 mmol/L ($p = 0.005$). Further significant reductions were observed at 120 and 180 min compared to 30 min, with mean differences of 1.59 ± 0.27 mmol/L ($p = 0.0016$)

and 1.93 ± 0.33 mmol/L (p = 0.0019), respectively. Significant reductions in glucose levels were also observed between 120 and 180 min compared to 60 min, with mean differences of 0.44 ± 0.12 mmol/L (p = 0.0326) and 0.78 ± 0.19 mmol/L (p = 0.0169), respectively (**Figure *45***).

Similarly to control, glucose levels in the Potato condition significantly increased further by 60 min compared to baseline, with the mean difference of 1.6 ± 0.33 mmol/L (p = 0.0107). Significant reductions in glucose concentration were observed by 120 min compared to 30 min and 60 min with a mean difference of 1.55 ± 0.30 mmol/L (p = 0.0073) and 1.01 ± 0.27 mmol/L (p = 0.0379), respectively. There was a further significant reduction observed in glucose concentration with potato condition at 180 min vs the concentrations observed at 30, 60 and 120 min postprandial, with a mean difference of 2.42 ± 0.38 mmol/L (p = 0.0024), 1.88 ± 0.46 mmol/L (p = 0.0261), and 0.86 ± 0.23 mmol/L (p = 0.0429), respectively (**Figure *45***). No significant differences were observed in capillary blood glucose concentrations between the three different conditions at specific time points (**Figure *44***), nor with total iAUC comparisons (p = 0.4785) (**Table *25***).

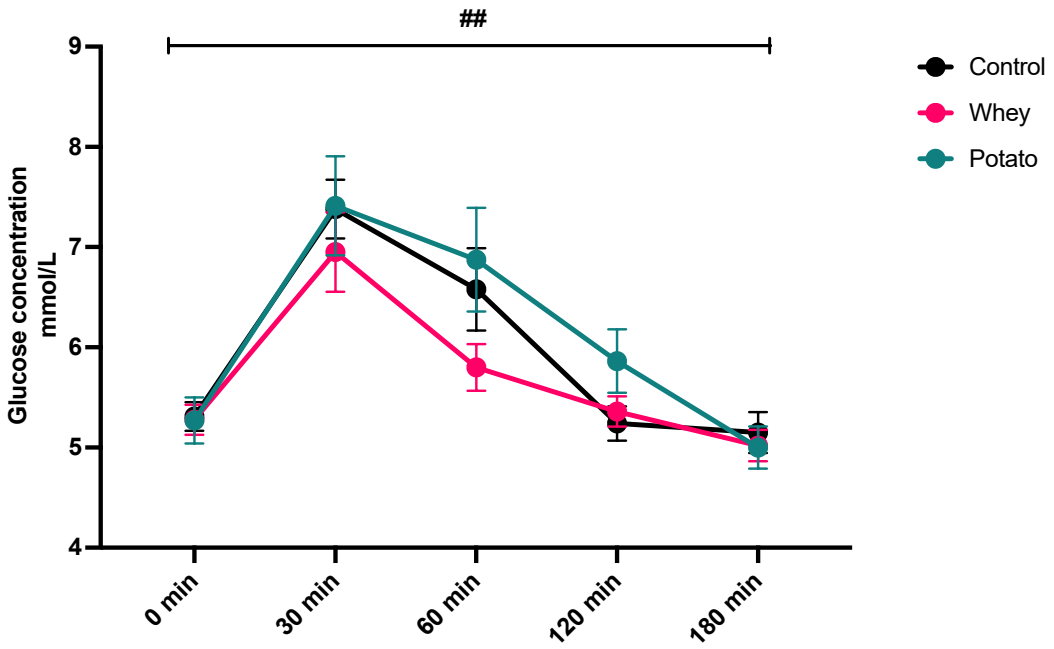


Figure 44. Capillary blood glucose concentration (mmol/L) measured over 3 h following control, whey and potato treatment consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. ## Shows that significance was present with Time and Condition * Time interaction (p < 0.0001, p = 0.0262, respectively).

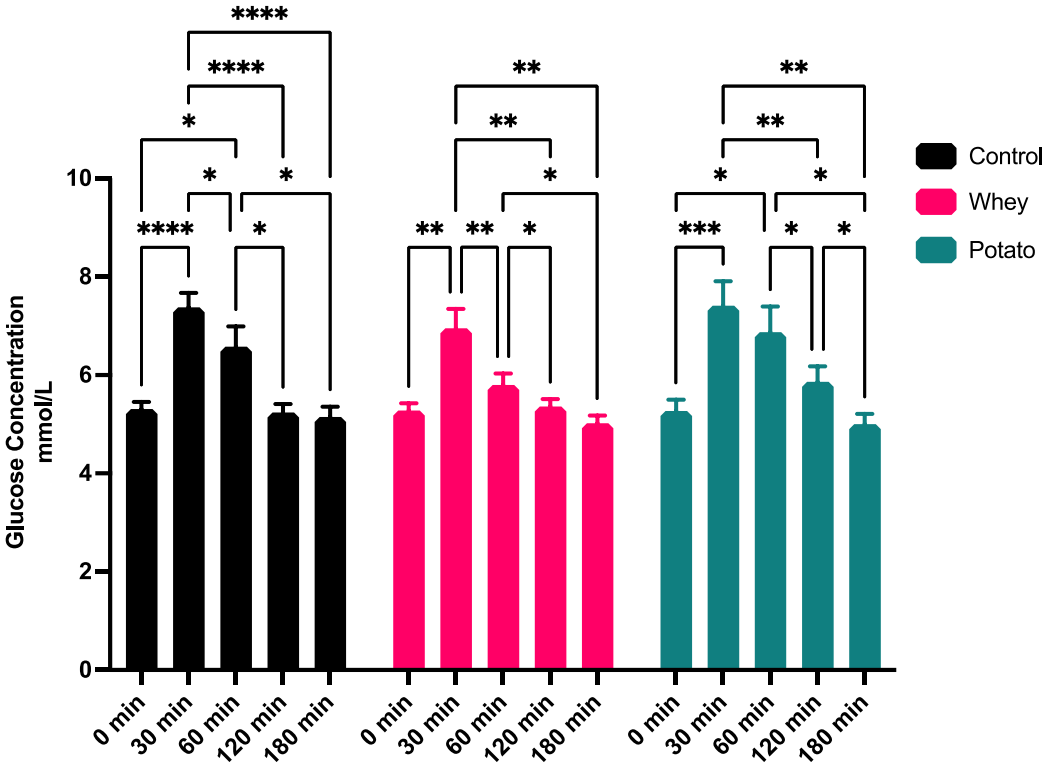


Figure 45. Within-group, capillary glucose concentration (mmol/L) measured over 3 h following control, whey and potato treatment consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Within-group differences were detected with the following significance levels **** p <0.0001, *** p< 0.001, ** p < 0.01 and *p < 0.05.

Blood insulin displayed an overall significant difference over time (p < 0.0001, **Figure 46**), with post hoc analysis revealing a significant increase at 30 min from baseline occurring with all three conditions with mean differences of 415.9 ± 42.70 pmol/L; p < 0.0001 in control, 479.80 ± 36.48 pmol/L; p < 0.0001 in Whey and 381.5 ± 44.10 pmol/L; p = 0.0008 in Potato conditions (**Figure *47***).

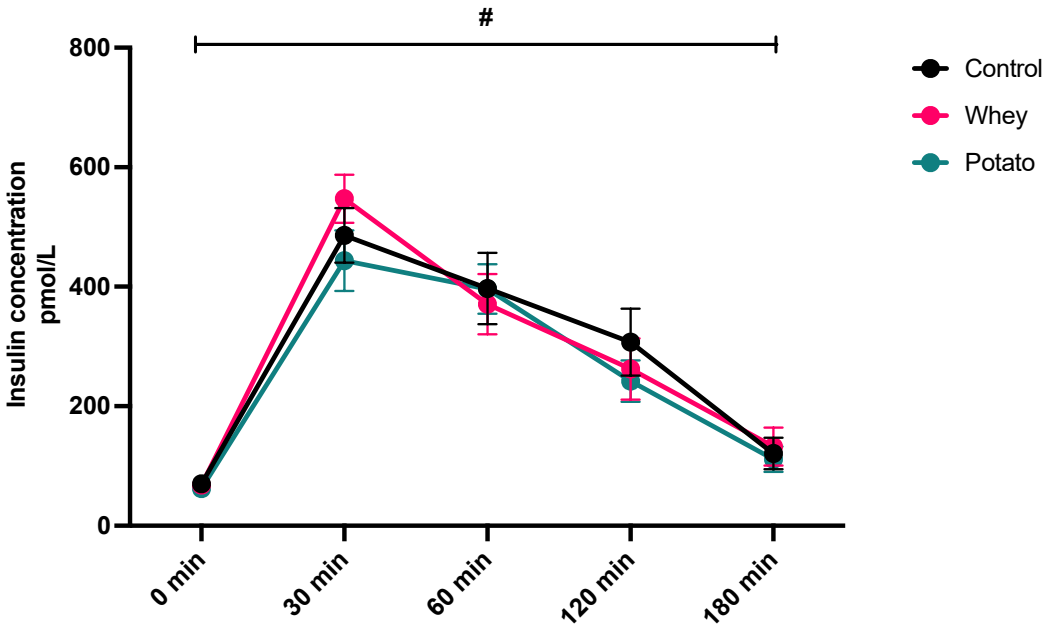


Figure 46. Plasma Insulin concentration (pmol/L) measured over 3 h following control, whey and potato treatment consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. # Shows that significance was present with Time interaction ($p < 0.0001$).

A significant decrease in insulin concentration from 30 min to 60 min was present only in the Whey condition, with a mean difference of 176.7 ± 31.94 pmol/L; p = 0.0025; however, these concentrations were still significantly higher than the baseline levels, with the mean difference of 303.10 ± 51.71 pmol/L; p = 0.0025. Further significant reductions in plasma insulin were observed at 120 min vs 30 min and 120 min vs 60 min with mean differences of 285.3 ± 35.50 pmol/L; p = 0.0002 and 108.6 ± 22.29 pmol/L; p = 0.0059, respectively. Nevertheless, the levels measured at 120 min were still significantly higher compared to baseline levels (194.4 ± 53.77 pmol/L; p = 0.0402). Plasma insulin levels returned to near baseline levels with no significant difference present only by 180 min. The concentration measured at this timepoint was significantly lower than at 30 and 60 min, with mean differences of 415.1 ± 37.91 pmol/L; $p < 0.0001$ and 238.4 ± 46.28 pmol/L; p = 0.0041, respectively (**Figure *47***).

In both Control and Potato conditions, concentrations of plasma insulin increased significantly compared to baseline, by 60 min (327.1 ± 55.01 pmol/L; p = 0.0015; 334.2 ± 39.05 pmol/L; p = 0.0008) and 120 min (237.1 ± 52.41 pmol/L; p = 0.0095; 180.2 ± 28.96 pmol/L; p = 0.0046) respectively. The Control and Potato

conditions showed the first significant reduction in insulin levels at 120 min compared to 30 min with mean differences of 178.8 ± 28.73 pmol/L; $p = 0.0011$ and 201.4 ± 50.46 pmol/L; $p = 0.0302$, respectively. Furthermore, significant reduction in concentrations were observed by 180 min compared to 30min (364.8 ± 42.86 pmol/L; $p < 0.0001$; 332.3 ± 45.26 pmol/L; $p = 0.0010$), 60 min (276.00 ± 47.73 pmol/L; $p = 0.0018$; 285.1 ± 44.12 pmol/L; $p = 0.0022$) and 120 min (186.00 ± 48.50 pmol/L; $p = 0.0252$; 131.00 ± 30.29 pmol/L; $p = 0.0203$) in both Control and Potato conditions, respectively (**Figure *47***).

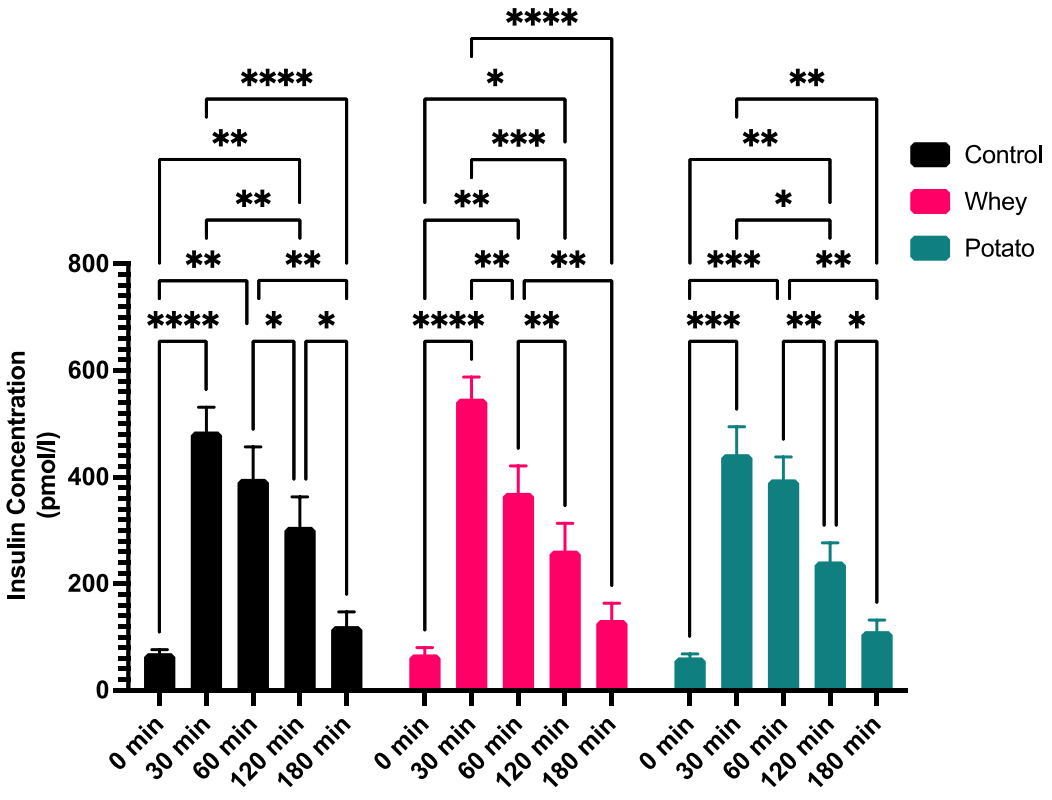


Figure 47. Within-group plasma insulin concentration (pmol/L) measured over 3 h following control, whey and potato treatment consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Within-group differences were detected with the following significance levels **** p <0.0001, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

There were no overall differences observed in plasma insulin concentrations between the three different conditions at specific time points, nor with total iAUC comparisons ($p = 0.9470$) (**Table *25***).

5.2.2. Appetite-Related Hormones

Overall differences in GLP-1 were observed with Time, as well as Condition * Time interaction, with the significance of p <0.0001 in both (**Figure *48***).

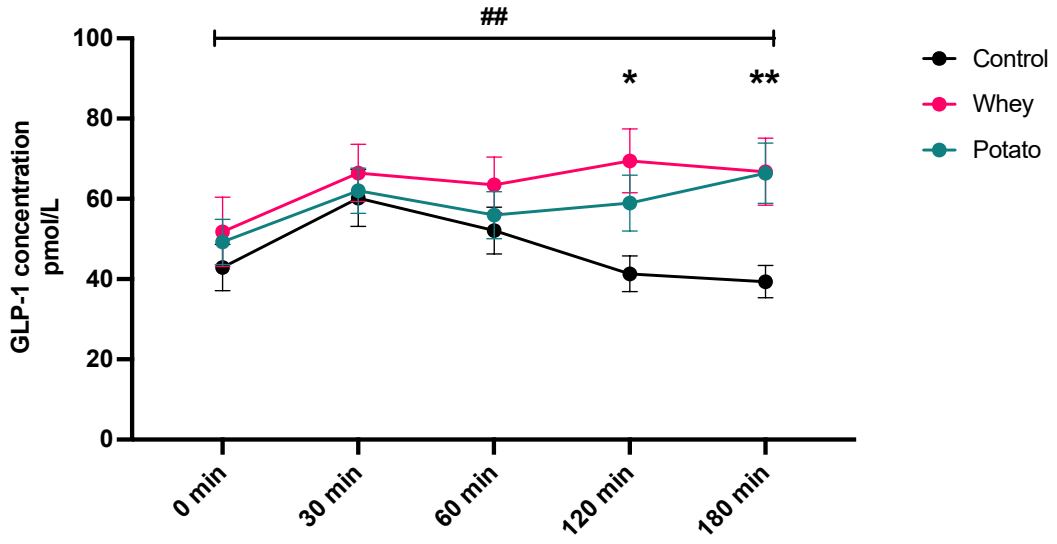


Figure 48. Plasma GLP-1 concentrations (pmol/L) observed with the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Where * Control significantly differs from Whey condition (p < 0.05). ** Control is significantly different to both Whey and Potato conditions (p < 0.05). ## Shows that significance was present with Time and Condition * Time interaction (p < 0.0001, in both).

The between-group analysis revealed significantly higher GLP-1 levels at 120 min postprandial with the Whey condition compared to the Control condition, with the mean difference of 28.14 ± 9.10 pmol/L (p = 0.0220). Additionally, at 180 min postprandial, GLP-1 levels were observed to be significantly higher with both whey and Potato conditions compared to the Control, with mean differences of 27.42 pmol/L (± 9.31, p = 0.0315) and 27.05 pmol/L (± 8.53, p = 0.0178), respectively (**Figure *48***).

Within-group analysis of GLP-1 concentration revealed significant increases in all three conditions from 0 min to 30 min, with mean differences of 17.32 pmol/L (± 4.43; p = 0.0225) in Control, 14.68 pmol/L (± 3.71; p = 0.0255) in Whey and 12.75 pmol/L (± 3.50; p = 0.0330) in the Potato condition (**Figure 49**).

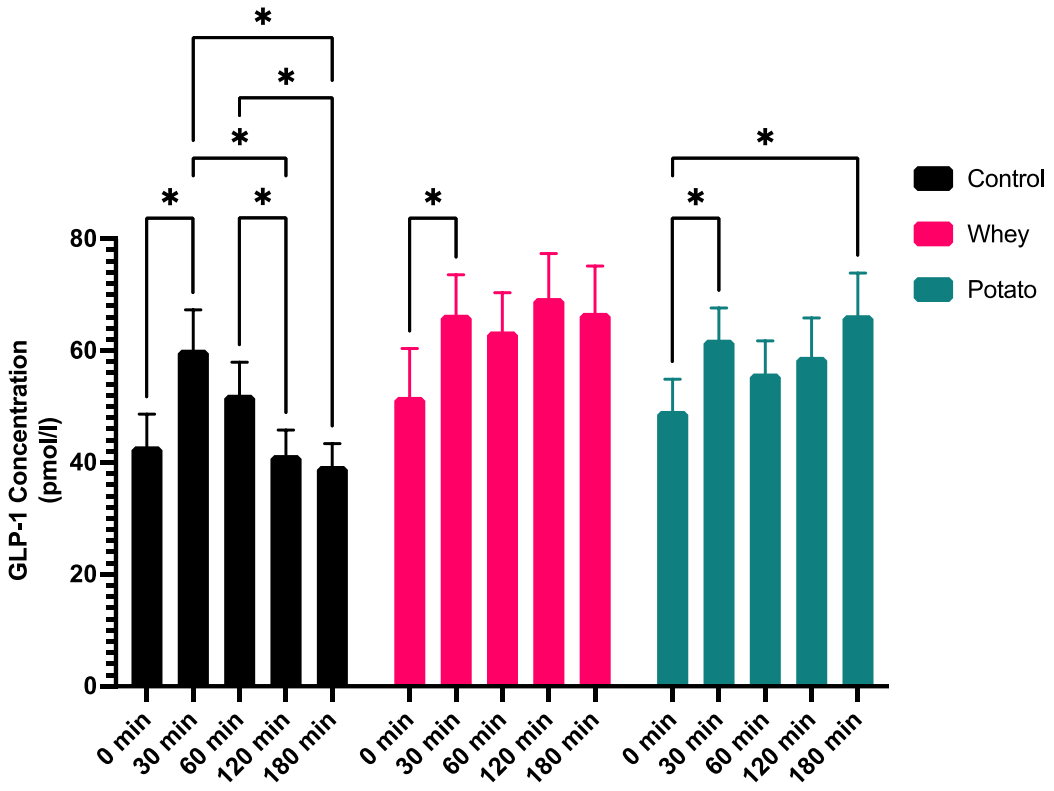


Figure 49. Plasma GLP-1 concentration (pmol/L) within-group analysis across the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Significant differences were observed with * $p < 0.05$.

No further significant increases were observed with the Control condition in GLP-1 levels. Additionally, the concentration of GLP-1 with this condition decreased significantly by 120 min and 180 min compared to 30 min postprandial with mean differences of 18.88 pmol/L (± 4.77; $p = 0.0210$) and 20.85 pmol/L (± 5.54; $p = 0.0278$), respectively. GLP-1 concentrations measured at 120 min and 180 min were also significantly lower compared to concentrations measured at 60 min with mean differences of 10.77 pmol/L (± 2.57; $p = 0.0152$) and 12.74 pmol/L (± 3.09; $p = 0.0167$), respectively (**Figure 49**). While there were no other significant differences observed with the Whey condition, with the Potato condition, there was a further significant difference with GLP-1 concentrations observed compared to baseline, at 180 min postprandial (17.12 ± 4.57 pmol/L; $p = 0.0279$) (**Figure 49**).

No significant differences were observed when comparing the total iAUC of GLP-1 concentrations of the three different conditions ($p = 0.7542$) (**Table *25***).

The analysis of plasma GIP displayed overall significance with Time, Condition and Time * Condition interaction ($p < 0.0001$, $p = 0.0044$ and $p < 0.0001$, respectively) (**Figure 50**).

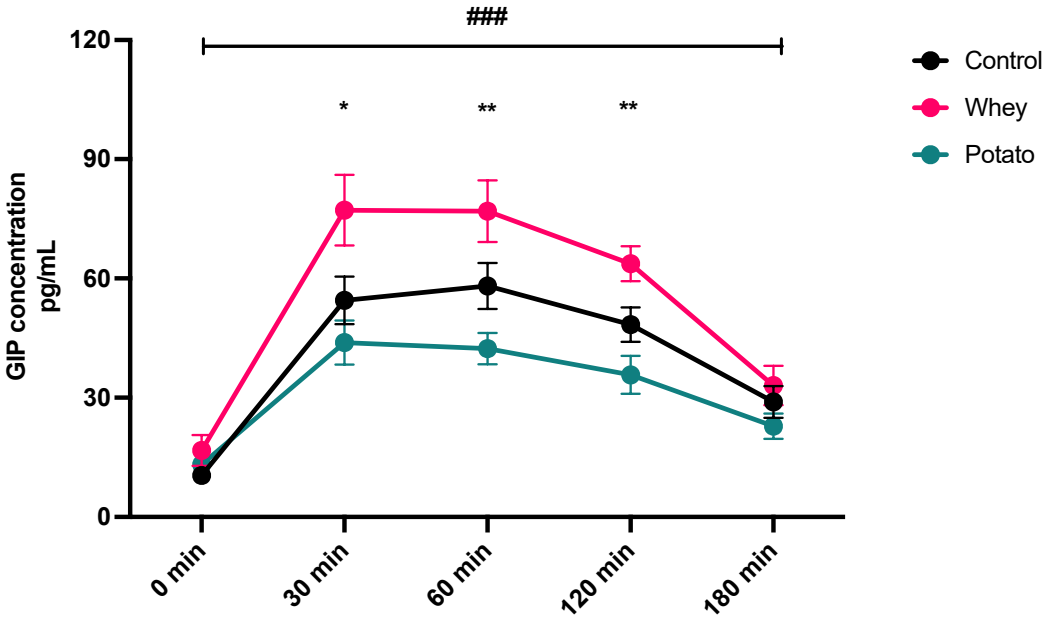


Figure 50. Plasma GIP concentrations (pg/mL) observed with the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test**.** * Whey is significantly different to potato condition (** $p < 0.01$ and * $p < 0.05$). ### Shows that significance was present with Time, Condition and Time * Condition interaction ($p < 0.0001$, $p = 0.0044$ and $p < 0.0001$, respectively).

The analysis of plasma GIP concentrations between the three different intervention groups revealed that Whey condition resulted in a significantly higher concentration at 30 min, 60 min and 120 min postprandial compared to Potato condition with mean differences of 22.67 ± 10.73 pg/mL ($p = 0.0159$), 18.85 ± 9.69 pg/mL ($p = 0.0041$) and 15.33 ± 6.16 pg/mL ($p = 0.0012$), respectively (**Figure 50**). Furthermore, iAUC analysis also revealed significantly higher values with whey condition than potato ($p = 0.0266$, **Table *25***).

Analysing the changes in plasma GIP concentration over time within the three different intervention conditions showed that significant differences were present between identical time points and in identical directions in each condition. Significant increases were observed from baseline to 30 min, 60 min, 120 min

and 180 min with the mean differences of 44.05 pg/mL (± 5.33; $p = 0.0001$), 47.62 pg/mL (± 4.87; $p < 0.0001$), 37.91 ± 3.56 pg/mL ($p < 0.0001$) and 18.49 ± 3.41 pg/mL ($p = 0.0029$) with Control, 60.47 ± 6.27 pg/mL ($p < 0.0001$), 60.23 ± 4.95 pg/mL ($p < 0.0001$), 46.99 ± 4.47 pg/mL ($p < 0.0001$) and 16.35 ± 4.52 pg/mL ($p = 0.0401$) with Whey and 30.67 ± 4.01 pg/mL ($p = 0.0002$), 29.14 ± 2.76 pg/mL ($p < 0.0001$), 22.57 ± 3.77 pg/mL ($p = 0.0014$) and 9.63 ± 2.47 pg/mL ($p = 0.0230$) with Potato conditions, respectively. There was also a statistically significant fall detected in GIP concentration at 180 min compared 30 min 60 min and 120 min with the mean differences of 25.55 pg/mL ± 5.60; ($p = 0.0090$), 29.13 ± 4.01 pg/mL ($p = 0.0003$) and 19.42 ± 1.78 pg/mL ($p < 0.0001$) with Control, 44.12 ± 7.14 pg/mL ($p = 0.0011$), 43.88 ± 6.24 pg/mL ($p = 0.0004$) and 30.64 ± 4.44 pg/mL ($p = 0.0005$) with Whey and 21.03 ± 4.80 pg/mL ($p = 0.0116$), 19.51 ± 3.03 pg/mL ($p = 0.0013$) and 12.94 ± 2.59 pg/mL ($p = 0.0051$) with Potato conditions, respectively (**Figure *51***).

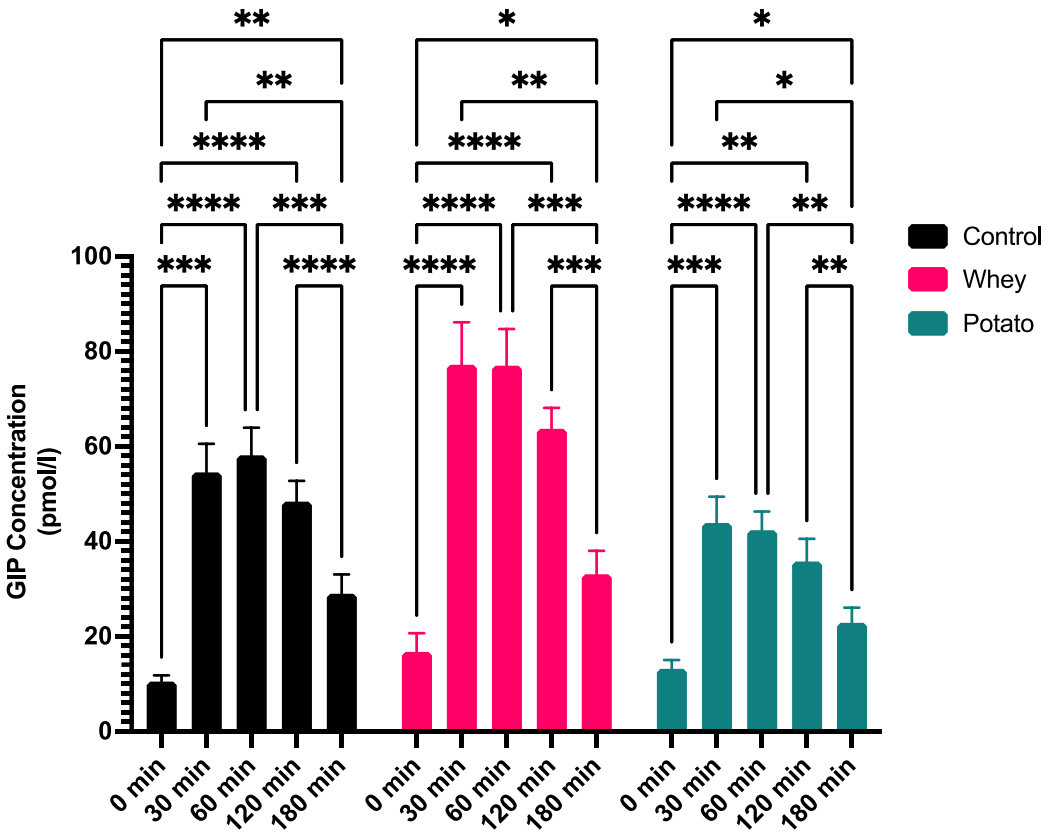


Figure 51. Plasma GIP concentration (pmol/L) within-group analysis across the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Significant within-group differences were observed with significances of **** p <0.0001, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Differences in PYY were not detected by time or condition (**Figure *52***), with any changes throughout the measurement period remaining nonsignificant. Equally, no differences in iAUC of PYY were calculated (p = 0.7915) (**Table *25***).

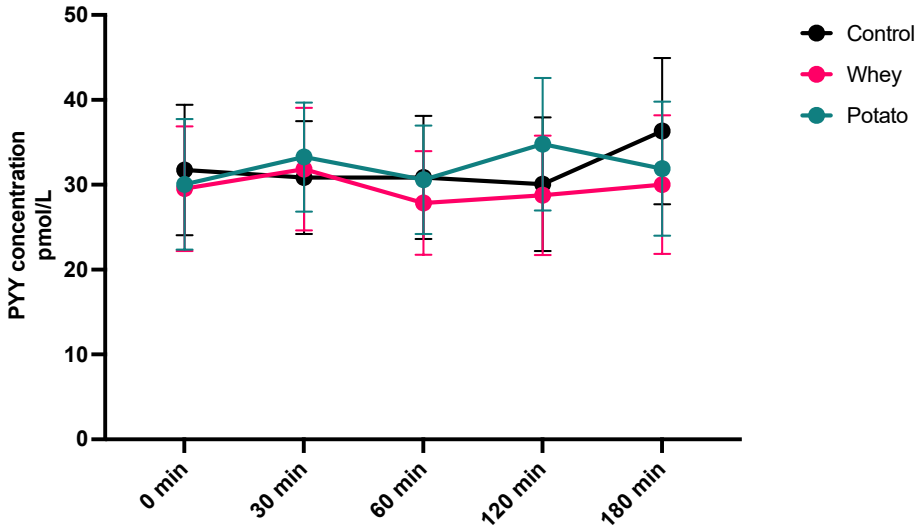


Figure 52. Plasma PYY concentrations (pmol/L) observed with the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test**.** No significant between-group or within-group difference was observed.

Plasma Ghrelin displayed an overall significant change over time (p < 0.0001). However, no significant differences were observed following between-group analysis (**Figure *53***). The iAUC of the Whey condition was observed to be lower than that of the Potato or Control conditions; however, it was not statistically significant (**Table *25***).

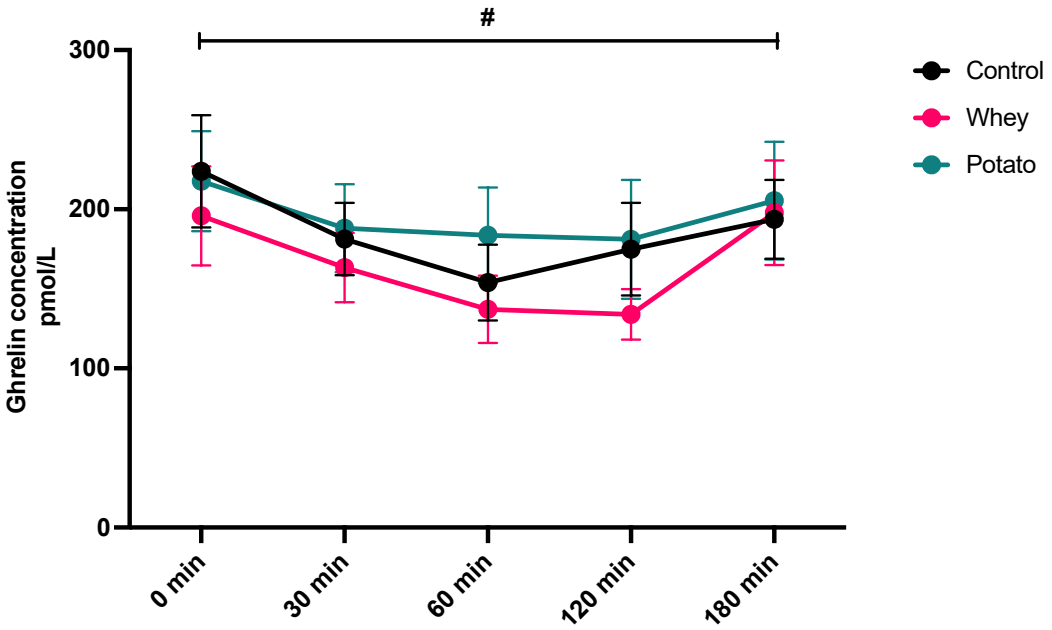


Figure 53. Plasma Ghrelin concentrations (pmol/L) observed with the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test**.** No significant between-group or within-group difference was observed. [#]Shows that significance was present with Time interaction ($p < 0.0001$).

Within-group analysis of plasma ghrelin concentration revealed significant reduction in all three conditions from 0 min to 60 min, with mean differences of 69.95 ± 14.25 pmol/L ($p = 0.0076$) in Control, 58.89 ± 12.75 pmol/L ($p = 0.0084$) in Whey and 33.86 ± 9.81 pmol/L ($p = 0.0439$) in the Potato condition. Significant reduction in ghrelin levels from 0 min to 30 min was seen only with the Potato condition (29.43 ± 7.55 pmol/L; $p = 0.0229$). Whilst significant postprandial reduction from 30 min to 60 min was observed only with the Whey conditions, with a mean difference of 26.24 ± 6.27 pmol/L ($p = 0.0152$). Plasma ghrelin increased significantly by 180 min postprandial, compared to 60 min, in both Control and Whey conditions, with mean differences of 39.72 ± 8.91 pmol/L ($p = 0.0236$) and 60.73 ± 15.62 pmol/L ($p = 0.0233$), respectively. No significant increase was observed in ghrelin levels with the Potato condition (**Figure *54***).

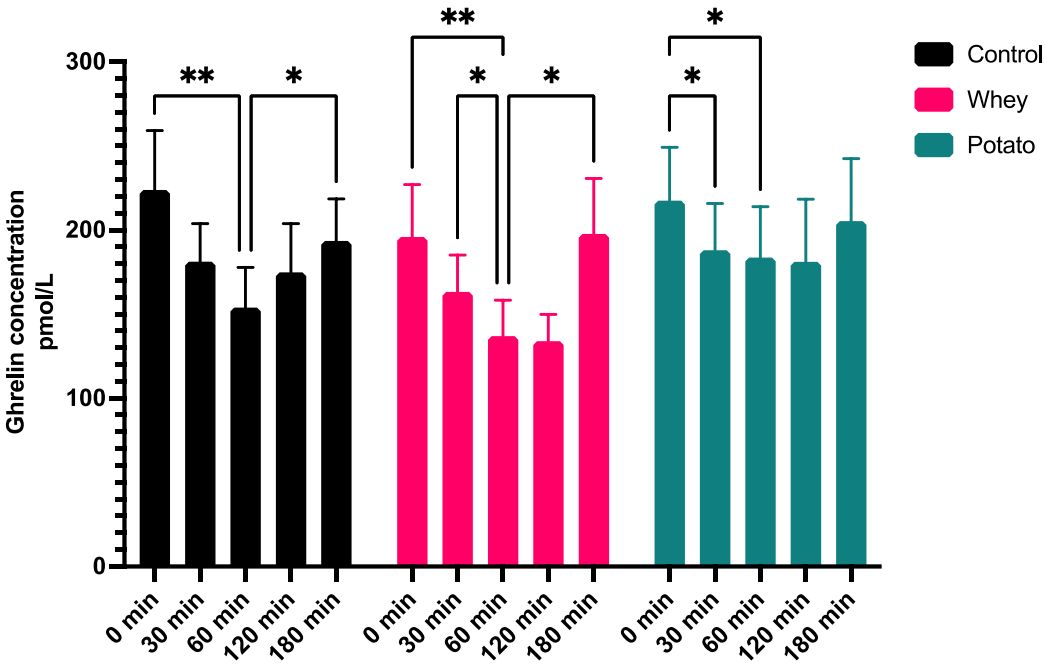


Figure 54. Plasma Ghrelin concentration (pmol/L) within-group analysis across the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. Within-group differences were observed with significances of ** $p < 0.01$ and * $p < 0.05$.

Table 25. Total AUC for capillary blood glucose, plasma insulin, GLP-1, GIP, PYY and ghrelin (mean ± SEM).

	Control	Whey	Potato	Overall sig. (*p*)
Glucose	117 ± 45	77 ± 40	167 ± 67	0.4785
Insulin	42950 ± 10622	41636 ± 9635	38769 ± 6765	0.9470
GLP-1	1054 ± 965	2479 ± 1558	1775 ± 1409	0.7542
GIP	6294 ± 1017	7834 ± 1276*	3874 ± 896	0.0463
PYY	15359 ± 2418	13054 ± 1906	13835 ± 2774	0.7915
Ghrelin	8268 ± 5523	7297 ± 3658	4961 ± 7302	0.9150
Appetite	95 ± 24	91 ± 25	152 ± 25	0.1684

* Whey is statistically significant compared to Potato ($p = 0.0380$)

5.2.3. Appetite and Satiety

The average appetite perception was not significantly different between the three conditions at any time point. However, there was an overall significant trend observed with Time ($p < 0.0001$) (**Figure 55**).

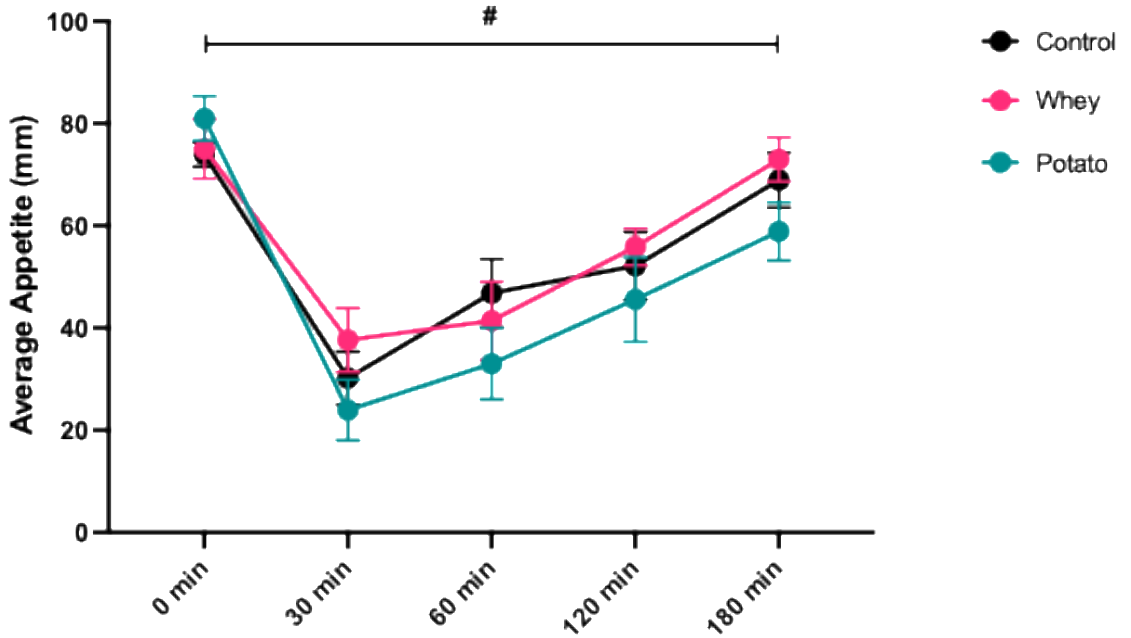


Figure 55. Appetite perception observed over 3h following treatment (control, whey, potato) consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test. # Shows that significance was present with Time interaction ($p < 0.0001$).

Within-group analysis of average appetite perception showed that there was a significant reduction present 30 min postprandial compared to baseline; additionally, the perceived appetite remained significantly lower at 60 min vs 0 min in all three intervention conditions, with mean differences of 43.71 ± 4.90 ($p = 0.0003$) and 27.10 ± 7.17 ($p = 0.0391$) in Control, 37.36 ± 4.51 ($p = 0.0002$) and 33.61 ± 8.00 ($p = 0.0185$) in Whey and 57.04 ± 8.28 ($p = 0.0027$) and 48.00 ± 9.44 ($p = 0.0127$) in the Potato condition, respectively. The earliest significant appetite increase was observed with the Control condition, with mean differences of 16.61 ± 4.80 ($p = 0.0497$) 30 min vs 60 min and 21.94 ± 4.43 ($p = 0.0072$) 30 min vs 120 min. Compared to 30 min later (at 180 min), significantly higher appetite perception was observed in all three intervention conditions. Mean differences observed were 38.72 ± 6.76 ($p = 0.0029$) in Control, 35.31 ± 6.64 ($p = 0.0047$) in Whey and 34.93 ± 8.26 ($p = 0.0299$) in the Potato condition. Appetite

perception observed at 180 min was also significantly larger compared to 60 min in both protein conditions, with mean differences of 31.56 ± 7.91 (p = 0.0245) in Whey and 25.89 ± 6.69 (p = 0.0438) in the Potato condition. With the Whey condition, there was an additional significant difference observed with perceived appetite between 120min and 180 min (-17.10 ± 4.00, p = 0.0215) (**Figure *56***).

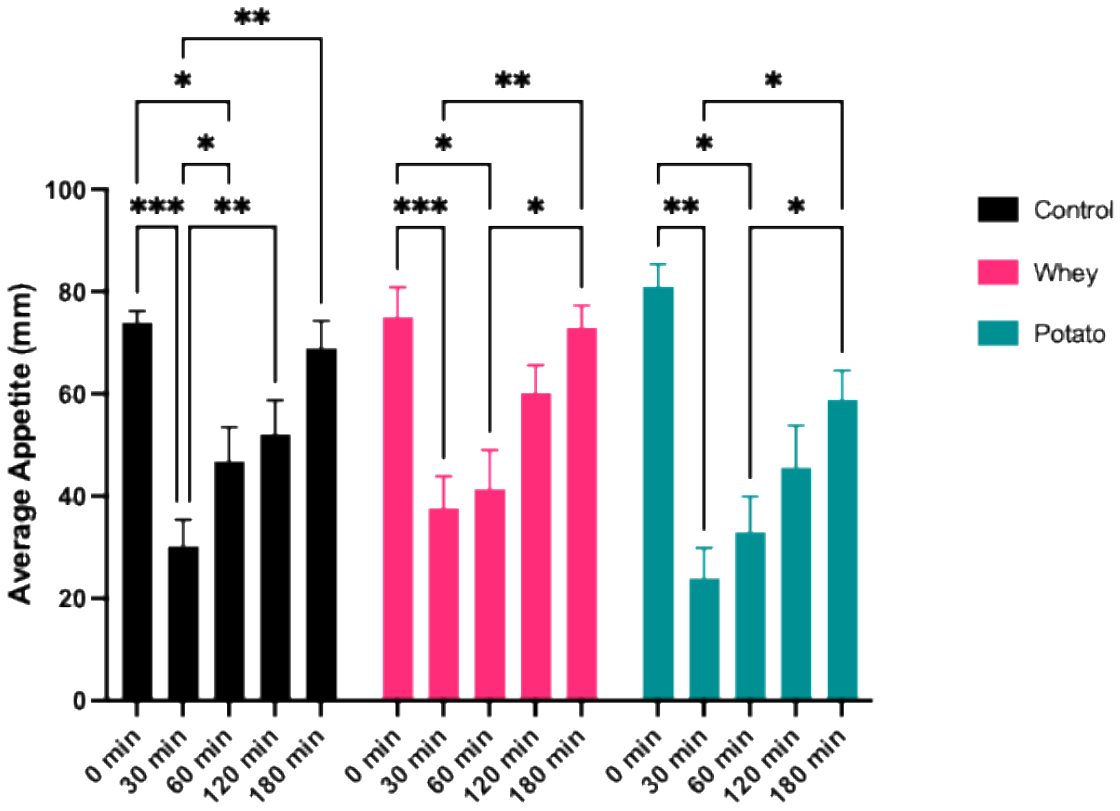


Figure 56. Appetite perception within-group analysis across the three experimental conditions (control, whey and potato) over 3 h following consumption. Data are expressed as mean ± SEM of n=10. Statistical analysis was two-way ANOVA and post-hoc Tukey's multiple comparisons test**.** Within-group differences were observed with significances of *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

A trend for reduced pasta consumption for lunch, 180 min following breakfast, was observed with Whey condition compared to both Control and Potato conditions; although not statistically significant, the mean differences were 15.89 ± 92.59 g (p = 0.9839) and 24.97 ± 95.44 g (p = 0.9630), respectively (**Figure *57***).

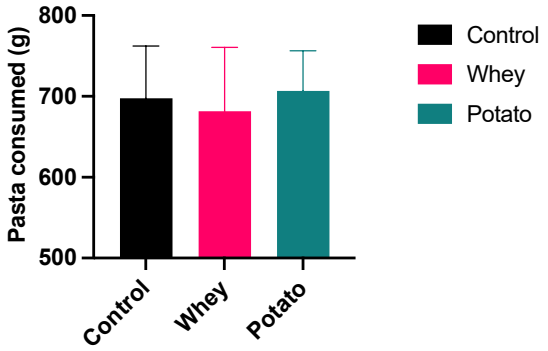


Figure 57. Pasta consumption at lunch expressed in grammes (g) with the three different treatment groups (control, whey, potato). Data are expressed as mean ± SEM of n=10. Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. No significant between-group differences were observed.

5.2.4. Palatability

When assessing the organoleptic properties of the three intervention breakfast meals, participants perceived the Potato condition (porridge with added PP) as significantly worse compared to both Control and Whey conditions in taste (39.00 ± 8.56 mm, p = 0.0035 and 38.50 ± 7.94 mm, p = 0.0023), aftertaste (32.70 ± 9.27 mm, p = 0.0160 and 35.00 ± 8.76 mm, p = 0.0079) and palatability (34.10 ± 10.68 mm, p = 0.0268 and 34.10 ± 11.04 mm, p = 0.0315), respectively. The Potato condition was also reported to possess significantly worse visual appeal (p = 0.0252) than the Control meal. No statistically significant differences in smell were observed between the three conditions (**Figure *58***).

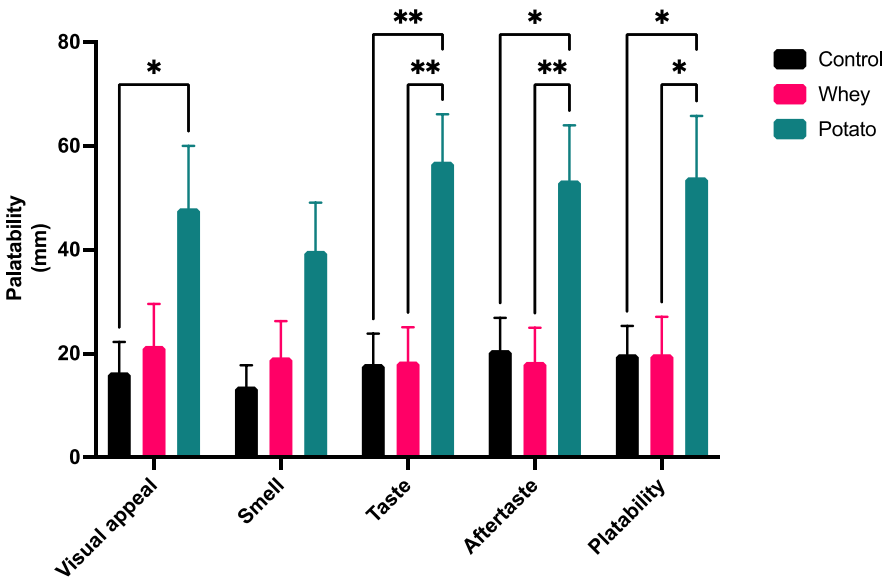


Figure 58. Palatability of the control, whey and potato treatments measured in mm, where the higher the value, the closer the specific aspect is to the worst possible perception. Statistical analysis was one-way ANOVA and post-hoc Tukey's multiple comparisons test. Between-group differences were observed with every assessed condition except smell, with significances of ** p < 0.01 and * p < 0.05.

5.3. Discussion

The results of this study did not demonstrate significant differences in glycaemic and insulinemic properties between WP or PP-enriched breakfast meals compared to Control porridge following ingestion. In our previous study examining the glucogenic and insulinemic properties of WP and PP in the form of protein shakes with a minimal amount of carbohydrate and no fat present, significant differences were observed in both aspects (Tiekou Lorinczova et al., 2021). The differences in observations could be attributed to the presence of the additional nutrient, carbohydrates, causing a significant increase in glucose and insulin levels, masking the protein-related effects (Franz, 1997), providing a more realistic picture when observing the application of protein isolates as part of a mixed diet. However, favourable trends were observed in Whey conditions. Following the initial increase in glucose levels at 30 min after meal ingestion, there was a prompt reduction in concentration at 60 min, to a level that was not statistically significant from the baseline concentrations. A reduction to a comparable extent was observed only by 120 min in the potato or control conditions. The favourable effects of WP on glucose regulation have been widely demonstrated across the literature (Nilsson et al., 2004; Pal and Ellis, 2010; Pal, Ellis and Dhaliwal, 2010; Tsakali et al., 2010). However, the return of the glucose levels close to fasting, within 2 hours post-prandial, as observed with control and potato conditions, is still as expected within normal physiological conditions (Hantzidiamantis, Awosika and Lappin, 2024).

The analysis of the secreted gut hormones postprandial may go some way to explain the observed differences in glucose regulation between the treatments. The concentration of the incretin hormone GIP, secreted from the K cells in the duodenum (Theodorakis et al., 2006), was significantly higher with the Whey condition compared to the Potato condition at 30, 60 and 120 min postprandial. The significance of differences diminished only by 180 min when the concentration of GIP with the Whey as well as Potato conditions returned close to baseline levels. The significant difference between the Whey and Potato conditions was also observed when comparing the overall iAUC of GIP

concentrations measured over time, with a mean difference of 3960 ± 1520 pg/mL*180 min (p = 0.0380).

These observed differences between WP and PP could be related to the slightly lower digestibility of PP, resulting in reduced levels of bioavailable AAs compared to WP. This is reflected by the PDCAAS and DIAAS scores of 0.87 and 0.85 compared to WP, which scored as 1.0 and 0.90, respectively (Hertzler et al., 2020). The lower digestibility of plant-based proteins is usually attributed to the presence of antinutritional factors (ANFs), such as protease inhibitors in PP that lead to a reduction in protein breakdown due to the inhibition of digestive enzymes such as trypsin or chymotrypsin (Habib and Fazili, 2007; Rachel and Sirisha, 2014; Marathe et al., 2019).

Another aspect to consider when assessing the differences in incretin hormone secretion is the AA profile of these proteins, as it has been previously demonstrated that TRYP and phenylalanine induced GIP and GLP-1 secretion when administered orally in mice (Rudenko et al., 2019). The TRYP and PHE content of 1.60% and 3.33% in WP, and 1.10% and 6.10% in PP, respectively, further supports the suggestion that the lower GIP levels observed with the Potato condition might be due to its slightly lower digestibility. Although studies on the effects of GIP in humans are scarce, in rodents, it was observed that increased levels of endogenous postprandial GIP enhanced glucose uptake by ~35% (p < 0.05) in myocytes while insulin levels remained unaffected (Snook et al., 2015). Further studies presented similar observations with adipocytes, highlighting the potential role of GIP in diabetes and obesity management (Song et al., 2007; Kagdi, Lyons and Beaudry, 2024). In addition to the previously described significant increases in GIP levels observed with the Whey condition, favourable results were observed with both protein conditions with circulatory GLP-1 over time. The levels of this appetite regulatory hormone were significantly higher at 120 min with Whey (p = 0.0220) and at 180 min with both Whey and Potato conditions (p = 0.0315 and p = 0.0178, respectively) compared to the Control condition. While with the Whey condition, GLP-1 levels seemed to reach the peak concentration of 69.46 pmol/L (±7.92) at 120 min, with a slight reduction observed

in the concentration by 180 min (66.76 ± 8.39 pmol/L), the Potato condition demonstrated a slower increase reaching the highest observed concentration by the end-point of 180 min postprandial with the concentration of 66.39 pmol/L (±7.52). The release of GLP-1 by the entero-endocrine L cells is triggered by AAs, dipeptides and protein-derived bioactive peptides in the gut lumen (Reimann, Ward and Gribble, 2006; Smith et al., 2020; Kaneko, 2021). L cells are present throughout the human GI tract (Theodorakis et al., 2006), with greater density in the ileum and colon (Larsson et al., 1975). The location of the L cells in the intestine may indicate that the rate of digestion and the appearance of specific AAs may be key to triggering GLP-1 release. The rapid rate of digestion of WP may explain the earlier detection of significantly higher GLP-1 levels vs the Control condition than with the Potato condition towards the end of the study observation period. Furthermore, glutamine, which was associated with GLP-1 secretion to a greater extent than glucose or other AAs (Wang et al., 2015), constituted 20.11% of total whey and 11.82% of total PP. Considering the lower glutamine content of PP, in addition to its marginally lower digestibility, prompts the consideration of the potential appetite regulatory effects of the PP-derived bioactive peptides. Potato protease inhibitors, namely PI2, showed potential appetite regulatory effects via increasing CCK levels in healthy, as well as overweight and obese (otherwise healthy) participants (Komarnytsky, Cook and Raskin, 2011; Peters et al., 2011; Zhu, Lasrado and Hu, 2017; Flechtner-Mors et al., 2020). Conversely, although an initial increase in GLP-1 levels was also observed with the Control conditions, by 120 min postprandial, the concentration was not different from the one measured at baseline. This phenomenon can potentially be attributed to early secretion of GLP-1 from the distal part of the intestine due to sensory signals delivered from the duodenum via a currently unexplored mechanism. The hypothesis was suggested by Veedfald et al. (2018) when they showed that the administration of intraduodenal nutrient-free hyperosmolar saline solution significantly increased human plasma levels of appetite regulatory hormones, including GLP-1. The results of the current study showed that the supplementation of carbohydrate-based meals with WP and PP isolates significantly increased circulatory GLP-1 levels with prolonged duration compared to the control condition. The results align well with the literature that

suggests that a (mixed-macronutrient) diet with a high relative protein composition can enhance GLP-1 release to a greater extent than diets with a high relative proportion of carbohydrate or fat in both healthy and overweight individuals (Watkins, Koumanov and Gonzalez, 2021).

Although no changes were observed in PYY levels over time or between the groups, ghrelin levels reduced significantly by 30 min after the consumption of the meal with added WP, and the observed reduction in concentration was significant with all three conditions by 60 min postprandial. However, ghrelin levels increased to concentrations comparable to baseline levels by 180 min, with no differences observed between the three different conditions. Although ghrelin is secreted from the X/A-like cells in the oxyntic glands of the gastric fundal mucosa, postprandial suppression is not affected by the nutrient content of the stomach. On the contrary, it is regulated by the osmolarity in the lower intestine as well as the increase in insulin levels after food ingestion (Cummings, 2006). Therefore, the comparable insulinotropic effects of the three different test meals in this study may be considered good indicators of the similarities observed in ghrelin regulation.

The trends present over time in glycaemic and insulinemic effects and in the appetite regulatory hormone GIP and GLP-1 levels were reflected by the significant reduction in postprandial appetite perception with all three intervention groups; however, no differences were observed between them. The nutritional composition of the breakfast meals did not seem to affect the amount of lunch consumed post-intervention significantly. Although the Whey condition was indicative of lower pasta consumption compared to both potato and control conditions, the differences were not statistically significant ($p > 0.05$) due to the presence of large within-group variations. This observation indicates that hormonal responses alone may not drive actual food intake and highlights the potential role of hedonic, psychological, and contextual factors in appetite regulation (Bejarano, Hesse and Cushing, 2023). It is therefore recommended that outcome measures of actual food intake be included in appetite studies

alongside appetite hormone levels and VAS scores to enhance the applicability of the findings.

Organoleptic characteristics are known drivers of food choice (Blundell et al., 2010). Although there is an increased interest in plant-based products due to being vegan-friendly and viewed as more environmentally friendly alternatives, they face a strong competitor in dairy products concerning flavour, texture and overall palatability (Nikbakht Nasrabadi, Sedaghat Doost and Mezzenga, 2021; Nemś, Miedzianka and Kita, 2022; Pua et al., 2022). The current study showed that the Potato condition demonstrated significantly worse visual appeal compared to the Control condition and significantly worse taste, aftertaste and palatability than both the Whey and Control conditions. There were no significant differences in smell between the three meal interventions. The variability in palatability between Whey and Potato conditions also suggests that the use of the sugar-free syrup in the intervention meals as a flavouring agent did not sufficiently mask the original sensory and palatability characteristics of the PP. These observations highlight the need for the development of flavour-masking strategies or improved PP processing to enhance sensory characteristics, especially as consumer acceptability remains a barrier for plant protein adoption (Siddiqui et al., 2022).

The current study, therefore, sheds more light onto the area of protein digestion/absorption and suggests that, as well as the AA composition, the rate of digestion/absorption of individual AAs and the presence of protein-derived bioactive peptides may influence the release of incretin hormones GIP and GLP-1. This may suggest that a threshold exists for K cells in the duodenum to simulate GIP secretion in the presence of AA digestion/absorption, and that the PP described in the current study was below this threshold. However, WP exceeds the threshold for GIP secretion, consequently leading to earlier significant postprandial glucose reduction. Nevertheless, both potato and WP triggered GLP-1 release from the L-cells, indicating the potential appetite regulation role of bioactive peptides in PP.

This study is the first to observe differences in the insulinotropic hormone GIP regulation that led to a hyperglycaemic response in the whey condition compared to the potato condition, without effects on insulin levels. The differences in GIP stimulation may be explained by the digestibility of the protein isolates and, particularly, the marginally lower digestibility of PP, potentially leading to lower concentrations of free TRYP and PHE in the PP isolate. While the metabolic effects of WP have been well studied, the results showed that the insulin response was masked when ingested as part of a mixed meal. However, GLP-1 responses were clearly demonstrated in both dairy-based whey and plant-based PP when supplementing a carbohydrate-based meal. The comparable effects of whey and potato conditions on GLP-1 may be explained by the potential bioactive characteristics of protease inhibitors despite the lower concentrations of glutamine in the PP isolate. Taken together, this study sheds light on the implications of the protein source on glycaemic, insulinemic and appetite regulation when applied as part of a mixed macronutrient diet. The metabolic responses of WP as part of a mixed meal may elicit potential benefits for populations where tighter control of glycaemic and insulinemic regulation is required. Furthermore, the findings indicate that the timing of plant-based PP consumption may be a critical factor, with preloading prior to a meal potentially offering superior benefits for glycaemic control compared to consumption as part of a mixed meal. However, the plant-based PP showed the potential to be as effective for appetite regulation as WP with the appropriate carrier.

6. CHAPTER 6 - General discussion and future directions

6.1. General discussion

This thesis focused on assessing the effects of plant and animal-derived proteins in the context of appetite regulation and obesity, developing and utilising novel cell-based models to assess the mechanistics underlying whole-body effects observed in human studies. Firstly, the characteristics of dietary proteins following *in vitro* digestion were analysed and related to their appetite regulatory effects determined in cell line models. Subsequently, the findings from the *in vitro* observations were applied to guide the selection of treatments and to elucidate the outcomes of the human study.

Obesity is a global epidemic that affects more than 2 billion adults worldwide, and it is predicted that by 2050, as much as 60% of the adult population could be classified as obese (WHO, 2024). Obesity increases the risk of several chronic diseases, such as T2D, CHD, stroke, cancer, arthritis and metabolic disease (Kyle, Dhurandhar and Allison, 2016; Powell-Wiley et al., 2021; Lopez-Jimenez et al., 2022). This, in turn, may affect the life expectancy and quality of the individuals suffering from obesity-related conditions. Moreover, the management and treatment of obesity and related diseases place a huge burden on the healthcare system, with costs being predicted to reach £10 billion per year by 2050 (WHO, 2024). The rise in obesity rates is related to the increase in energy-dense foods that are available with ease, reduced physical activity due to the use of motorised vehicles, and increased sedentary lifestyle (Romieu et al., 2017).

Though an ample amount of research has focused on obesity, generating an abundance of advice and interventions for weight loss in the past decades, the rate of obesity has not ceased to increase. The lack of successful outcomes might be related to the complexity of appetite regulation, which is not yet fully understood (Andermann & Lowell, 2017; Casanova et al., 2019; Gan et al., 2024; Gibbons et al., 2019; Moris et al., 2022). To date, the most successful intervention has been gastric bypass surgery (Colquitt et al., 2014). Although resulting in a mean 25% weight loss (Sjöström et al., 2007), it has numerous adverse effects,

such as nutrient malabsorption or potential development of gallstones, ulcers, hernia, chronic nausea and vomiting (Kalarchian et al., 2014). Thus, due to a combination of factors, including side effects, cost and being an invasive procedure, the surgical weight loss intervention is not applicable to the wider population (Wiebe and Tonelli, 2024). Satiety hormone-mimicking pharmacological agents, such as GLP-1 receptor agonists, are another recent intervention which may aid in a mean weight loss of 15% to 20% of initial body weight (Myerson and Paparodis, 2024). Although GLP-1 receptor agonists have increased availability compared to surgery, they may still lead to mainly gastric-related adverse effects, such as nausea and vomiting, diarrhoea and constipation, while their long-term efficacy and safety are yet to be confirmed (Myerson and Paparodis, 2024). Furthermore, the financial implications of these treatments represent limitations in their accessibility (Dellgren, Persad and Emanuel, 2024; Talay and Vickers, 2024).

Thus, dietary modifications may provide the most widely applicable and desirable management approach. Dietary modification is often achieved by increasing the intake of one of the macronutrients (fat, protein, carbohydrates) at the expense of others (Tobias et al., 2015; Hallberg et al., 2018; Ruban et al., 2019). The macronutrient with the most significant impact on appetite regulation has been observed to be protein (Ruban et al., 2019). Increased protein intake at sufficient levels (20g to 40 g/meal) leads to comparable trends in appetite hormone regulation to that observed following gastric bypass surgery via a surge in postprandial GLP-1 and PYY secretion (Van Der Klaauw et al., 2013; Watkins et al., 2024). GLP-1 and PYY are gastric enteroendocrine hormones released in response to nutrient cues, with an increased response to protein stimulation compared to fat or carbohydrates (Van Der Klaauw et al., 2013). GLP-1 and PYY contribute to appetite regulation via stimulation of the vagal afferent neurons as well as direct interaction with central appetite regulation, as the N-terminal within their structure allows them to cross the BBB readily (Hoffman and Adeli, 2024; Xiao, Jin and Zhang, 2024).

The most extensively researched protein is dairy-derived WP (Patel, 2015; Cava et al., 2024; Zeng et al., 2024). It is widely used as a dietary supplement in weight loss aids, such as meal replacements or high protein snack bars, due to its established satiety effects and complete AA profile (Patel, 2015). However, with the increasing interest in exclusively plant-based dietary patterns in recent years, there has been a rising demand for plant-protein-based alternatives (Aschemann-Witzel et al., 2021). Although plant-based proteins, such as wheat, soy, RP and PeP, are widely available on the market, their appetite-regulatory effects are not yet well clarified (Kaneko, 2021). Another novel plant-based protein, PP, has attracted increasing research interest due to its potential appetite-inhibitory effects related to its PI2 content (Flechtner-Mors et al., 2020; Komarnytsky et al., 2011; Peters et al., 2011; Zhang et al., 2022; Zhu et al., 2017). However, more research is required to understand the full extent of appetite regulatory effects of plant-based proteins, as it is believed that different proteins have unique characteristics depending on the source of protein, AA content and absorption kinetics. Thus, proteins from different sources might have different effects on satiety and appetite regulation (Bendtsen et al., 2013; Tiekou Lorinczova et al., 2021).

The appetite regulatory effects of proteins are often studied using *in vitro* cell line models, as they enable the observation of the mechanistics of interaction with a wide range of nutrients in a cost-effective and ethical way (Ding et al., 2021). One of the most often used cell line models for nutrient-induced appetite regulation studies is the intestinal L-cell-like STC-1 cell model derived from a genetically induced murine enteroendocrine tumour in the duodenum (Kuhre et al., 2016; Kondrashina, Papkovsky and Giblin, 2018; Santos-Hernández et al., 2018; Wang, Murthy and Grider, 2019). Although nutrient interactions with STC-1 cells induce expression and secretion of appetite regulatory hormones such as GLP-1, GIP, PYY and CCK with a comparable trend to that present in *in vivo* enteroendocrine cells, observations seem to vary between experiments and laboratories, potentially due to their heterogeneity (McCarthy et al., 2015). The heterogeneity observed in STC-1 cell lines across different laboratories is primarily attributed to clonal variation, genetic drift, and epigenetic modifications

arising from extended passaging, subcloning, and variable culture conditions (Ghandi et al., 2019; Hayford et al., 2021). Such factors contribute to inconsistencies in enteroendocrine hormone expression and secretion, including GLP-1 and PYY, which may partly explain divergent experimental outcomes. Recent studies have reinforced the complexity of enteroendocrine cell phenotypes, highlighting that nutrient-sensing receptor expression and hormone release profiles can vary significantly not only between cell subtypes but also within clonal populations (Li et al., 2020). These findings underscore the critical need for rigorous standardisation of culture protocols and validation across multiple models to ensure reproducibility in enteroendocrine research.

The Caco-2 cell line is one of the most widely utilised cell models and has long been regarded as the pharmaceutical industry's gold standard for absorption permeability studies (Volpe, 2020). Caco-2 cells are derived from human colorectal adenocarcinoma and express small intestine-like characteristics following spontaneous differentiation after 15 to 21 days of culture (Ding et al., 2021). These cells express intestinal-like brush border structure on their apical surface as well as several receptors, transporters, and enzymes (Antunes et al., 2013), making them an ideal candidate as a small intestinal model (Ponce de León-Rodríguez, Guyot and Laurent-Babot, 2019). Thus, recently, the potential use of Caco-2 cells for investigating appetite regulatory effects of nutrients has been investigated. Caco-2 cells have been shown to express the appetite hormones PYY (Kosicka, Renshaw and Zariwala, 2013) and GLP-1 (Shobatake et al., 2019) and to secrete CCK, PYY and GLP-1 as a result of nutrient stimulation (Song et al., 2015), indicating their potential application as an enteroendocrine model.

While secondary cell line-based models may contribute to increasing our knowledge of cellular signalling pathways and improving our understanding of mechanisms underlying appetite hormone regulation, they lack the complexity of human pathophysiology to enable direct translatability of results to humans (Cassotta et al., 2024). Immortalised cell-based *in vitro* models using cells such as Caco-2 and STC-1 are static monolayers lacking mechanical cues such as

peristalsis and biochemical cross-talk with other cell types, such as stem cells, goblet cells, microfold cells, Paneth and tuft cells found in the intestinal lining *in vivo* (Cassotta et al., 2024). The simplicity of *in vitro* cell line models, in combination with the multi-organ level complexity of appetite regulation, warrants the need for human subjects for nutrient-induced appetite regulatory research. This project, therefore, took a multidimensional approach, studying the intricacies of gut nutrient interactions at a mechanistic level, utilising the two cell lines, and assessing global whole-body responses via a human intervention study.

Our results demonstrated that differences in concentration, digestion rate as well as the levels of available AA and bioactive peptides present in the digested fractions of WP, PP, RP and PeP may have affected the appetite regulatory effects of differing protein conditions observed *in vitro* independent of cell lines (Caco-2 and STC-1) used as well as *in vivo*. This study also highlights the importance of considering the potential interaction between plant and dairy-based proteins due to ANFs found in plant proteins, which reduces proteolysis. ANFs are poorly digestible protein fractions, and their presence in food may lead to reduced nutrient utilisation and uptake, specifically proteins, potentially causing impaired gastrointestinal functions and metabolic performance (Salim et al., 2023). However, there is also an emphasis placed on considering specific ANFs for their potential positive bioactive characteristics, such as the appetite-suppressive effects of PI2 and other potential bioactive peptides present following digestion, such as the rice-derived peptide AHV, that may enhance appetite regulatory hormone secretion, due to calcium-binding and chelating characteristics. Furthermore, this study is the first to show GIP expression and secretion in the Caco-2 cell line and identical trends in GLP-1 and PYY secretion between Caco-2 and STC-1 cells while observing differing effects between WP and plant-based proteins, highlighting the suitability of Caco-2 cells as an alternative cell line model for nutrient-induced appetite hormone regulatory studies. Moreover, the results also demonstrated for the first time that consumption of WP leads to a significant increase in GIP postprandial, resulting in insulin-independent glycaemic response in humans. Thus, the metabolic responses of WP as part of a mixed meal may elicit potential benefits for

populations where tighter control of glycaemic and insulinemic regulation is required. Meanwhile, the effects of plant-based PP on GLP-1 secretion indicate that it could be as effective for appetite regulation as WP with the appropriate carrier.

When observing the digestibility of differing protein conditions, the digestibility of WP was observed to be higher, with complete proteolysis detected by the end of the intestinal phase, compared to the plant-based proteins following the INFOGEST *in vitro* digestion. The assessment of free AA concentration following intestinal digestion also showed a similar trend with the overall higher digestibility of WP with differences of 0.56% WP vs PP, 0.17% WP vs RP and 0.67% WP vs PeP. These results are in line with the PDCAAS and DIAAS of the protein isolates, with WP isolate having scored 1.0 and 0.90, PP 0.87 and 0.85, RP 0.53 and 0.52 and PeP 0.83 and 0.66, respectively (Hertzler et al., 2020). Meanwhile, the digested soluble phases of plant-based PP and RP contained protein fractions resistant to pancreatic proteases, such as PI2 and RP-derived prolamin. Protease inhibitors' resistance to digestion is due to being mostly stabilised by disulfide bonds, similar to β-lactoglobulin of WP (Andlinger et al., 2021); meanwhile, prolamin's tightly packed structural composition due to the presence of β-helices is believed to hinder enzymatic hydrolysis (Moreno et al., 2021). Comparable digestion patterns were previously observed with PP and RP by Jiménez-Munoz, Tsochatzis and Corredig (2022), with potato-derived trypsin inhibitor bands detected at all intestinal digestion time points and poor digestibility of prolamin has been previously demonstrated (Wang et al., 2016; Jiménez-Munoz et al., 2021). Although potato protease inhibitors possess specific antinutritional properties, they were also shown to express beneficial bioactive characteristics. PI2 was shown to suppress postprandial appetite significantly in a dose-dependent manner in 44 healthy women in a randomised, placebo-controlled crossover study when consumed an hour before a standardised meal in two different doses (15 and 30 mg) (Zhu, Lasrado and Hu, 2017). Meanwhile, the digestibility of plant-based PeP protein was comparable to that of WP, based on undigested protein bands being observed at the end of intestinal phase digestion using the SDS-PAGE electrophoresis separation, in agreement with the

observation of others (Amagliani et al., 2017; Jiménez-Munoz et al., 2021). Further analysis of the digestibility of WP, PP, RP and PeP by OPA assay measurement of the concentration of free AA within the liquid phase of protein digests revealed no significant differences between the four different protein isolates. Protein blends of WP with the plant-based proteins PP, RP and PeP prepared in 50:50 and 30:70 ratios, respectively, with the aim to enhance the AA profile of plant-based proteins with a potential maximised effect on appetite suppression, were also examined for their breakdown by measuring the concentration of free AA following *in vitro* digestion. The results indicated potential interaction between WP and PP when digested in a blended format, where the WP and PP blend in the ratio of 50:50 had a significantly lower free AA concentration compared to WP and RP blends (1.79%, $p = 0.0062$) with the same ratio in the intestinal phase end-point samples. Additionally, a significant reduction in the concentration of free AA with WP:PP (50:50) was also observed compared to unblended WP intestinal end-point digested, suggesting a potential inhibition of WP fractions' proteolysis by potato protease inhibitors. As the same phenomenon was not observed with the WP:PP (30:70) blends, and no differences were observed in the percentage digestibility between any of the WP and plant-based protein blends at this ratio, the application of 30% WP to 70% plant-based proteins seems to be the preferred blend when enhancing the AA profile of plant-based proteins.

When assessing the appetite hormone regulatory effects of differing protein treatments using suitable concentrations identified via cell viability tests in Caco-2 and STC-1 cells, the digestibility characteristics, AA and peptide profiles, as well as protein concentration, seem to have influenced the appetite hormone secretion trends observed. The GLP-1 and PYY responses with RP and PP with lower treatment dilution (6.25 mg/mL protein concentration, the highest viable concentration in the Caco-2 cell line) were more substantial than WP following the longer 4h exposure time. The measured concentration of secreted PYY levels with PP treatment was significantly higher vs WP ($p = 0.0052$), and BD ($p = 0.0032$) and GLP-1 levels were significantly higher compared to WP ($p = 0.0009$) following 4 hours of treatment. Whereas 4 hours of treatment with RP at the same

concentration led to significantly higher secreted GIP, GLP-1 and PYY levels compared to WP, as well as all other treatments (p <0.0001), attaining the most potent bioactive characteristic in appetite hormone secretion among all 4 conditions. Conversely, with a shorter 2h exposure time at lower dilution, as well as with 4h exposure time at the higher dilution (4.17mg/mL protein concentration, the highest concentration viable in the STC-1 cell line), only PP showed increased potency for appetite hormone regulation compared to WP with significantly higher GLP-1 secretion (p <0.01). The consistently significantly higher impact on appetite regulatory hormone secretion with PP compared to WP could be explained by the presence of potato protease inhibitors, comprising 50% of total PP fractions. Meanwhile, the unique superior appetite hormone secretory effects presented by RP only when applying the higher treatment concentration for the longer 4h treatment period could be explained by the high-quality calcium chelating properties of RP peptides, potentially attributed to peptide AHV, which was shown to display optimal calcium-binding properties with a calcium-chelating capacity of 55.69 ± 0.66 mg/g in a Caco-2 cell monolayer by Yang et al. (2024). Furthermore, enhanced GLP-1 release, potentially due to co-ingestion of protein and calcium, was also highlighted, related to a probable synergistic effect between phenylalanine and Ca^{2+}, also apparent in the release of PYY and GIP (Watkins, Koumanov and Gonzalez, 2021). Whereas the absence of the significant appetite hormone regulatory effects of RP at the shorter 2h treatment time point and the lower treatment concentration could be explained by its slow absorption rate, which was demonstrated to be 24 min slower vs WPI (p = 0.00) in humans (Purpura et al., 2014), and by the 5x dilution of treatment condition potentially leading to a lower concentration of RP-derived bioactive peptides than that required to reach the suggested threshold required for the activation of appetite regulatory hormone secretion (Tiekou Lorinczova et al., 2021; Tenenbaum et al., 2023), respectively.

The study results showed that overall, WP had a negligible impact on the appetite regulatory hormone secretion at both 6.25 mg/mL and 4.17 mg/mL concentrations and both 2h and 4h treatment times compared to the positive control BD. Furthermore, the PeP, with a treatment concentration of 4.17mg/mL, studied in Caco-2 cells, did not show an increased impact on appetite hormone

regulation compared to BD. These results align with the previous observation, where treatment of STC-1 cells with WPId at a concentration of 10 mg/mL did not lead to significantly higher GLP-1 secretion vs BD (p = 0.08). However, treatment with the WPHd at the same concentration for 2 hours led to significantly higher GLP-1 secretion compared to both BD and WPId, with significance values of p <0.01 and p <0.0001, respectively (Tenenbaum et al., 2023). The observed differences in the bioactivities of WPId and WPHd might be due to the presence of a higher proportion of small peptides within the WPHd compared to that observed in WPId (Tenenbaum et al., 2023). Moreover, no advantages were observed in blending WP with PP and RP with the ratios of 50:50 and 30:70, respectively, compared to applying PP and RP treatments only, related to appetite hormone regulation. This may be attributed to the previously observed negligible appetite regulatory effects of WP *in vitro*. Nonetheless, the observed GLP-1 and PYY secretion trends with the higher dilution of digested WP, PP, and RP were identical between the Caco-2 and STC-1 cell lines. Treatment with PP led to significantly higher GLP-1 secretion vs both WP and RP and significantly higher PYY secretion compared to RP in both cell lines. PYY secretion was also higher with the PP condition than with WP. Still, the differences were not statistically significant with any of the cell lines. This finding is especially important as it underscores both the functional comparability and versatility of the Caco-2 model in appetite hormone research. The observation that GLP-1 and PYY secretion trends were consistent across both Caco-2 and STC-1 cell lines, despite their differing origins and phenotypes, suggests that under specific conditions, such as exposure to digested protein fractions, Caco-2 cells may exhibit enteroendocrine cell characteristics (Heffernan et al., 2022). This result enhances the potential utility of Caco-2 cells in preliminary screening studies of nutrient-induced enteroendocrine activity, especially when STC-1 or primary enteroendocrine cells are unavailable or present experimental limitations due to heterogeneity or lack of standardisation (McCarthy et al., 2015; Kuhre et al., 2016; Gorecki et al., 2025). Taken together, these results support a broader application of the Caco-2 model in nutrient-hormone interaction studies, thereby expanding methodological options for in vitro appetite research.

The consistency in significantly higher impact on appetite regulatory hormone secretion observed with PP compared to BD and WP *in vitro* prompted the investigation of its appetite regulatory effects in 10 healthy male participants in comparison to WP when used to increase the protein content of an oat porridge breakfast meal. The results of this study demonstrate favourable trends with WP conditions compared to PP conditions and Control porridge, with a prompt reduction in concentration at 60 min, following the initial increase in glucose levels at 30 min after meal ingestion to a level akin to baseline concentrations. A reduction to a comparable extent was observed only by 120 or 180 min with the PP or control conditions. The favourable effects of WP on glucose regulation have been widely demonstrated across the literature (Nilsson et al., 2004; Pal and Ellis, 2010; Pal, Ellis and Dhaliwal, 2010; Tsakali et al., 2010). The observed significantly higher concentration of GIP with the WP vs the PP condition at 30, 60, and 120 min postprandial may go some way to explain this phenomenon. This significant difference between WP and PP conditions was also observed in the overall iAUC of GIP concentrations with a mean difference of 3960 ±1520 pg/mL*180 min (p = 0.0380). These observed differences between WP and PP could be related to the slightly lower digestibility of PP (Chapter 3), resulting in reduced levels of bioavailable AAs compared to WP, such as TRYP and PHE, which previously induced GIP and GLP-1 secretion when administered orally in mice (Rudenko et al., 2019). Although studies on the effects of GIP in humans are scarce, it was observed in rodents that increased levels of endogenous postprandial GIP enhanced glucose uptake by ~35% (p < 0.05) in myocytes (Snook et al., 2015) and adipocytes (Kagdi et al., 2024; Song et al., 2007) while insulin levels remained unaffected, highlighting the potential role of GIP in diabetes and obesity management. Furthermore, significantly higher circulatory GLP-1 levels were observed at 120 min with WP (p = 0.0220) and at 180 min with both WP and PP conditions (p = 0.0315 and p = 0.0178, respectively) compared to the Control condition. The rapid digestion rate and the glutamine content of WP, which is double that present in PP, may explain the earlier detection of significantly higher GLP-1 secretion by the entero-endocrine L cells predominantly found in the ileum and colon (Theodorakis et al., 2006) with WP vs Control compared to PP vs Control. Previously glutamine was associated with

GLP-1 secretion to a greater extent than glucose or other AAs (Wang et al., 2015). Considering the lower glutamine content of PP, in addition to its marginally lower digestibility, prompts the consideration of the potential appetite regulatory effects of the PP-derived PI2. PI2 showed potential appetite regulatory effects via increasing CCK levels in healthy, as well as overweight and obese participants (Flechtner-Mors et al., 2020; Komarnytsky et al., 2011; Peters et al., 2011; Zhu et al., 2017). The results of the current study showed that the supplementation of carbohydrate-based meals with WP and PP isolates significantly increased circulatory GLP-1 levels with prolonged duration compared to the control condition. The results align well with the literature that suggests that a (mixed-macronutrient) diet with a high relative protein composition can enhance GLP-1 release to a greater extent than diets with a high relative proportion of carbohydrate or fat in both healthy and overweight individuals (Watkins et al., 2021). The observed changes over time in glucose, and the appetite regulatory hormone, GIP and GLP-1 levels, were reflected by the significant reduction in postprandial appetite perception with all three intervention groups. The nutritional composition of the breakfast meals did not seem to affect the amount of lunch consumed post-intervention significantly. Although the WP condition was indicative of lower pasta consumption compared to both PP and control conditions, the differences were not statistically significant ($p > 0.05$) due to the presence of large within-group variations. The assessment of the organoleptic characteristics of the interventions, which are key drivers of food choice (Blundell et al., 2010), showed that the PP condition demonstrated significantly worse visual appeal compared to the Control condition and significantly worse taste, aftertaste and palatability than both the WP and Control conditions. These observations suggest that the use of sugar-free syrup in the intervention meals was insufficient to mask the PP's original sensory and palatability characteristics. This study is the first to observe differences in the insulinotropic hormone GIP regulation leading to a glycaemic response in WP compared to PP condition without effects on insulin levels potentially due to differences in digest rates and AA profiles of digested proteins. Furthermore, the metabolic responses of WP as part of a mixed meal may elicit potential benefits for populations where tighter control of glycaemic and insulinemic regulation is required. Meanwhile, the plant-

based PP showed the potential to be as effective for appetite regulation as WP with the appropriate carrier.

To conclude, this study is the first to show GIP expression and secretion in the Caco-2 cell line and identical trends in GLP-1 and PYY secretion between Caco-2 and STC-1 cells, highlighting the suitability of Caco-2 cells as an alternative cell line model for nutrient-induced appetite hormone regulatory studies. Moreover, the study results also demonstrated for the first time that consumption of WP leads to a significant increase in GIP postprandial, resulting in insulin-independent glycaemic response in humans.

Although the research presented has limitations, this thesis successfully contributes valuable insights into the relative appetite regulatory effects of dietary proteins *in vitro* and *in vivo* to the existing literature. Overall, this study demonstrates how the mechanisms underlying appetite regulation could be influenced by total protein concentration, the rate of digestion and absorption, as well as AA and peptide profiles of milk and plant-based proteins. Nevertheless, this dissertation paves the way for further investigation, presenting possibilities to delve into more precise areas beyond the limits of the current study.

6.2. Future directions

The long-term aim of this study was to provide information applicable to the development of novel nutrient-based appetite regulatory aids while considering ecological sustainability and consumer inclusivity. Thus, potential future research outlined below could provide additional detailed information that will help achieve the long-term study aim.

Firstly, in the current study, although the assessment of protein digestibility via free AA concentration using the OPA assay provided an indication of the digestibility of unblended and blended proteins, it is unable to provide a complete picture of digestibility (Santos-Sánchez et al., 2024). The precision of this method to assess the degree of protein hydrolysis is affected by the OPA's weak reaction with cysteine, leading to unreliable quantification of this AA (Church et al., 1985; Spellman et al., 2003). Furthermore, OPA does not react with secondary AA such as proline or hydroxyproline either (Herbert, Santos and Alves, 2001). In addition to the OPA assay, SDS-Page was also used to assess digestibility, providing a visual representation of protein breakdown and enabling the observation of undigested protein fractions remaining in the digested liquid phases of proteins. This method applies a molecular size-based separation technique; however, it may not effectively resolve proteins with similar molecular weights. Furthermore, the separation of proteins with a molecular weight of less than 10 kDa is not as effective due to potentially migrating too quickly, appearing as a single band or smear on the gel (Gallagher, 2012). The application of reverse-phase ultra-high-performance liquid chromatography combined with mass spectrometry (RP-UHPLC-MS/MS) could hold a solution for the related challenges, enabling the identification and quantification of AA as well as potential bioactive protein fractions, such as PI2, which may remain intact following *in-vitro* digestion (Hernández-Ledesma et al., 2005). This would aid the detailed characterisation of the digestibility and identification of the unblended and blended protein digests' complete AA and bioactive peptide profiles. Thus, it may potentiate the identification of their effects on appetite-regulatory gut hormones to aid in understanding their most appropriate application for appetite and weight management.

Furthermore, the current study observed no advantages of blending plant-based proteins with WPI on appetite hormone regulation. However, the observation that WPH leads to significantly higher GLP-1 secretion compared to WPI in STC-1 cells (Tenenbaum et al., 2023) may justify further investigation into potential synergist appetite regulatory effects and the underlying mechanisms of WPH and plant-derived proteins when blended to achieve a complete protein source. Further *in vitro* studies using cell line models, such as Caco-2, could also investigate the potential synergistic effects and the underlying mechanisms of RP or its blend with WPH in co-supplementation with Ca^{+}. This approach may utilise the high-quality calcium chelating properties of RP peptides, likely mediated by the rice-derived AHV (Yang et al. 2024), leading to a potentially enhanced GLP-1 release as previously observed as a probable synergistic effect between phenylalanine and Ca^{2+}, also apparent in the release of PYY and GIP (Watkins, Koumanov and Gonzalez, 2021).

Moreover, although there is an increased interest in plant-based products due to being vegan-friendly and viewed as more environmentally friendly alternatives, they face a strong competitor in dairy products concerning flavour, texture and overall palatability (Nikbakht Nasrabadi, Sedaghat Doost and Mezzenga, 2021; Nemś, Miedzianka and Kita, 2022; Pua et al., 2022). The current *in vivo* study clearly demonstrated this by the perception of palatability characteristics of WP and PP conditions. Strategies such as masking and complexation for improving the flavour profile and mouthfeel of plant-based proteins have been previously investigated (Nikbakht Nasrabadi, Sedaghat Doost and Mezzenga, 2021; Mirzapour-Kouhdasht et al., 2023). Masking agents, such as salts and sweeteners, were demonstrated to be effective bitter-taste inhibitors when added to protein hydrolysates (Leksrisompong et al., 2012). Furthermore, the complexation of potato and pea proteins with apple pectin (DE 71%) also improved taste with a greater reduction in bitter off-notes with increasing pectin : protein ratios (Zeeb et al., 2018). A significant reduction in the bitterness of PP was also shown by Yavuz-Düzgün et al. (2020) when utilising high-esterified citrus pectin at a protein pectin ratio of 0.33 pectin for complex coacervation. Developing an appropriate carrier system to mask the original

palatability and mouthfeel characteristics could lead to increased acceptance of plant-based proteins among consumers.

Lastly, the nutritional composition of the breakfast meals did not seem to significantly affect the amount of lunch consumed post-intervention in the current study. However, the WP condition indicated lower pasta consumption compared to both PP and control conditions, although the differences were not statistically significant ($p > 0.05$) due to large within-group variations. By increasing the sample size of a future human study, the within-group variations could be reduced, enabling the detection of significance in differences (Mullineaux and Wheat, 2017). Furthermore, a longer-term observation with a duration of 3 to 6 months may provide insight into whether accumulative effects of WP or PP could lead to significant weight loss. Moreover, the listed proposed future *in vitro* and analytical research, taken together, may prompt the investigation of a potentially novel nutrient-based appetite regulatory approach *in vivo*.

Appendices

Appendix A - Ethical approval

Decision - Ethics ETH2223-1377: Mrs Helena Tiekou Lorinczova (Class 2)

Westminster VRE <VRE@westminster.ac.uk>
Wed 19/04/2023 10:48
To:Helena Tiekou Lorinczova <H.Tiekoulorinczova@westminster.ac.uk>

University of Westminster

Project title: Novel nutrient based approaches for appetite regulation and obesity

Application ID: ETH2223-1377

Date: 19 Apr 2023

Dear Helena

Thank you for providing your response to the Conditions set by the Committee.

Your response to Conditions has been considered by the Committee and your proposal is approved.

The expiry date for this proposal is 31 Oct 2024. Please note that any proposed changes to protocol, including time extensions, must be made **before** the expiry date.

We recommend you submit an amendment to protocol, at least 4 weeks prior to the expiry date. You must wait to hear back from REC (Research and KE Ethics Committee) before actioning any proposed changes.

If the expiry date has passed you must submit a new application and wait to hear back from the REC.

If your protocol changes significantly in the meantime, please contact me immediately, in case of further ethical requirements.

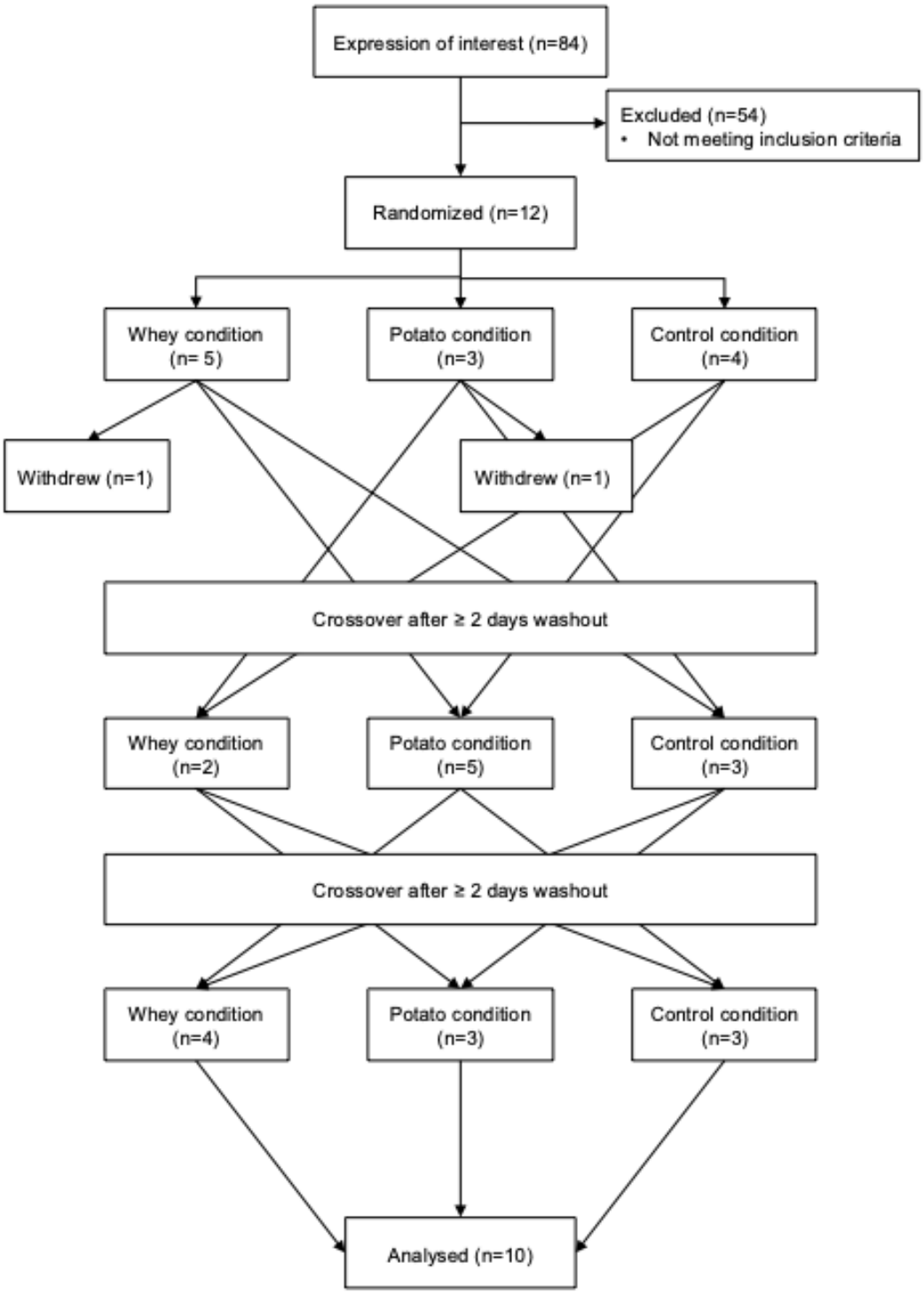
Yours,

Mandy Walton

LAS College Research and Knowledge Exchange Ethics Committee

Appendix B - Recruitment poster

Appendix C - CONSORT flow diagram



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