

The impact of food structure and promoting short chain fatty acids production on energy homeostasis and appetite regulation

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*“It is not how many times you get knocked down that count, it is how many times
you get back up”*

Abstract

The structural elements within a food, known as food structure, are thought to play a critical role in appetite regulation and energy homeostasis. Food structure can refer to the spatial arrangement of cellular structural elements, such as starch granules, or the physical form of a food. Manipulating the starch structure within a food system, such as in peas, can limit starch digestibility and thus improve the quality of the starch eaten. Bypassing upper gastrointestinal (GI) tract digestion can increase microbiota fermentation activity, which is responsible for the beneficial effects associated with resistant starch consumption. Short-chain fatty acids, fermentation by-products, have previously been reported to regulate glucose homeostasis, reduce energy intake and increase peripheral anorexigenic gut hormone concentrations in humans.

A large part of this thesis aimed to investigate the acute effects of manipulating the starch structure within peas by inactivating the starch-branching enzyme of the pea seeds (SBE-deficient peas) on appetite regulation and energy homeostasis in humans. The first study presented in this thesis investigated the effect of food structure within peas on gastric emptying rates using a ^{13}C breath test. The data suggest that manipulating the structure of the peas improves glycaemic response without affecting the gastric emptying rate. The second study presented in this thesis investigated the effect of upper GI digestion on different pea structures. SBE-deficient pea seeds were less available at the small intestinal level to be broken down into glucose. The third study presented in this thesis investigated the effects of lower gut fermentation on different pea structures. SBE-deficient pea seeds produced more short chain fatty acids (SCFAs) as a result of fermentation. However, these pea studies did not cause a change in subjective appetite or in appetite-regulating hormones.

Propionate, one of the main SCFAs produced from microbial fermentation, is thought to play a crucial role in appetite regulation and energy homeostasis. Supplementation with inulin-propionate ester (IPE), which selectively enriches colonic propionate, has been reported to increase peripheral anorexigenic gut hormone concentrations and reduce energy intake acutely in humans. Long-term IPE supplementation prevented weight gain and reduced intra-hepatocellular lipid content. Another part of this thesis aimed to investigate the effects of colonic

propionate on appetite regulation and energy homeostasis in humans following a lower energy diet through IPE supplementation over a 12-week period. IPE reduced subjective appetite and led to significant weight loss compared to baseline. However, these effects were independent of changes in anorexigenic gut hormones.

The majority of the work presented in this thesis supports a role of fermentation in energy and appetite homeostasis. However, the exact underlying mechanisms remain unclear.

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Declaration of Contributors

The majority of the work in this thesis was performed by the author. All collaboration and assistance is detailed below.

Chapter 3:

The study was carried out in collaboration with Dr. Katerina Petropoulou. The [^{13}C] Octanoic acid was provided by Dr. Douglas Morrison, University of Glasgow. Mathematical modelling of [^{13}C] data was performed by Prof. Tom Preston and Dr. Douglas Morrison, University of Glasgow. Radioimmunoassays were performed with the assistance of Dr. Amin Alamshah, Dr. Edward Chambers and Dr. Katerina Petropoulou.

Chapter 4:

The study was carried out in collaboration with Dr. Katerina Petropoulou and Rasha Alshaalan. Nasoduodenal tubes were placed throughout the duration of the study by Dr. Eleutheria Panteliou, Dr. Lilian Mendoza and Dr. Nicolas Penny. Radioimmunoassays were performed with Dr. Katerina Petropoulou and Rasha Alshaalan and with the assistance of Dr. Edward Chambers. Electron microscopic graphs were taken with the assistance of Dr. Louise Salt, Quadram Institute Bioscience, Norwich.

Chapter 5:

The study was carried out in collaboration with Dr. Katerina Petropoulou. Samples analysis and mathematical modeling of ^{13}C plasma glucose and ^{13}C urinary SCFAs was performed by Dr. Douglas Morrison and Prof. Tom Preston, University of Glasgow. Radioimmunoassays were performed with Dr. Katerina Petropoulou and with the assistance of Dr. Edward Chambers.

Chapter 6:

Dietary supplements were provided by Dr. Douglas Morrison (University of Glasgow). Radioimmunoassays were performed with the assistance of Dr. Edward Chambers and Dr. Katerina Petropoulou.

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Publications and conference presentations based on work presented in or related to this thesis are as follows:

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Nutrition Society Winter Meeting 4th-5th December 2018: A pilot study to evaluate the effect of increased colonic propionate on appetite homeostasis during a hypocaloric diet

Nutrition Society Summer Meeting 10th-12th July 2019: A pilot study to evaluate the effect of increased colonic propionate on glucose homeostasis during a hypocaloric diet

Published abstracts

Khatib, M., Chambers, E., Morrison D.J., and Frost. G. (2018) 'A pilot study to evaluate the effect of increased colonic propionate on appetite during a hypocaloric diet', *Proceedings of the Nutrition Society*, 77(OCE4), p. E119.

Khatib, M., Chambers, E., Morrison D.J., and Frost. G. (2019) 'A pilot study to evaluate the effect of increased colonic propionate on glucose homeostasis during a hypocaloric diet', *Proceedings of the Nutrition Society*, 78(OCE1), p. E10.

Manuscript in preparation

A manuscript is being prepared that include part of the detailed work presented in the current thesis by K. Petropoulou et al. "The influence of plant starch genetics on postprandial blood glucose: A complex relationship between plant structure and the intestinal environment."

Abbreviations

AgRP	Agouti-related peptide
AMP	Adenosine mono-phosphate
AP	Area postrema
ARC	Arcuate nucleus
ATP	Adenosine tri-phosphate
BBB	Blood-brain barrier
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BOLD	Blood-oxygen-level-dependent
BP	Blood pressure
CART	Cocaine- and amphetamine-regulated transcript
CCK	Cholecystokinin
CHO	Carbohydrate
CI	Confidence intervals
Cl ⁻	Chloride
CNS	Central nervous system
CO ₂	Carbon dioxide
CRF	Clinical Research Facility
CT	Computed tomography
CV	Coefficient of variation
CVD	Cardiovascular disease
CWs	Cell walls
DIT	Diet-induced thermogenesis
DMN	Dorsomedial nucleus
DMX	Dorsal motor nucleus of the vagus
DPP-4	Dipeptidyl-peptidase IV
DRV	Dietary reference value
DVC	Dorsal vagal complex

ECG	Electrocardiogram
EE	Energy expenditure
EEC	Enteroendocrine cells
EI	Energy intake
FFAR	Free fatty acids receptor
FFM	Fat-free mass
GE	Gastric emptying
GI	Gastrointestinal
GLP	Glucagon-Like Peptide
GLUT	Glucose transporter
GPCR	G-protein coupled receptor
H ⁺ -dependent	Hydrogen-dependent
HCO ₃ ⁻	Bicarbonate
HOMA	Homeostatic model assessment
iAUC	Incremental area under the curve
IGI	Insulinogenic index
IGN	Intestinal gluconeogenesis
IPE	Inulin-propionate ester
IR	Insulin resistant
IRMS	Isotope ratio mass spectrometry
IS	Internal standard
Kcal	Kilocalorie
KO	Knockout
LC	Liquid chromatography
LHA	Lateral hypothalamic area
MCT	Monocarboxylate transporters
mRNA	Messenger RNA
Na ⁺	Sodium
NADH	Nicotinamide Adenine Dinucleotide Hydrogen

NDC	Non-digestible carbohydrate
NDNS	National diet and nutrition survey
NHS	National Health Service
NPY	Neuropeptide Y
NTS	Nucleus tractus solitaries
OAT	Organic anion transporter
OFC	Orbitofrontal cortex
OXM	Oxyntomodulin
PAL	Physical activity level
PET	Positron emission tomography
PIS	Participant information sheet
POMC	Pro-opiomelanocortin
PP	Polypeptide
PPAR	Proliferator-activated receptors
PVN	Paraventricular nucleus
PYY	Polypeptide tyrosine tyrosine
RCF	Relative centrifugal force
RDS	Rapidly digested starch
REC	Research Ethics Committee
RMR	Resting metabolic rate
RS	Resistant starch
RS1	Type 1 resistant starch
RS2	Type 2 resistant starch
RS3	Type 3 resistant starch
RS4	Type 4 resistant starch
RS5	Type 5 resistant starch
RYGB	Roux-en-Y gastric bypass
SBE	Starch-branching enzyme
SCFAs	Short chain fatty acids

SDS	Slowly digested starch
SEM	Scanning electron microscope
SEM	Standard error of the mean
SGLT	Sodium-glucose transporter
SMCT	Sodium-dependent monocarboxylate transporters
SUERC	Scottish Universities Environmental Research Centre
$T_{1/2}$	Half-time
T2D	Type 2 diabetes
tAUC	Total area under the curve
TBW	Total body water
TEE	Total energy expenditure
T_{lag}	Lag phase
UCP	Uncoupling protein
UKN	UK Nutritional
VAS	Visual analogue scale
VMN	Ventromedial nucleus
VPDB	Vienna Pee Dee Belemnite
WHO	World Health Organisation
WT	Wild type

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Chapter 1

1. General Introduction

1.1 Obesity:

1.1.1 Definition, prevalence, and economic burden

The World Health Organisation (WHO) defines obesity as the accumulation of excess body fat, characterised by a disproportionately high weight for a given height (World Health Organisation, 2016). Weight status is classified based on body mass index (BMI), which is calculated as the ratio of a person's body weight to the square of their height. BMIs of $\geq 25\text{kg/m}^2$ and $\geq 30\text{kg/m}^2$ or above are defined as overweight and obesity, respectively (World Health Organisation, 2016).

Obesity is a global pandemic, affecting both adults and children in developing and developed countries (World Health Organisation, 2016). Between 1993 and 2014, the proportion of men categorised as obese increased from 13.2% to 24.3%, and the proportion of women increased from 16.4% to 26.8%. The rates are predicted to rise to 60% of men and 50% of women by 2050 (National Obesity Observatory, 2016). Obesity is a major driver in the development of serious non-communicable diseases, such as type 2 diabetes (T2D), various cancers, and cardiovascular disease (CVD) (Cani and Delzenne, 2009; World Health Organisation, 2016). The estimated financial burden on the National Health Service (NHS) related to treating obesity and its comorbidities increased from £479.3 million in 1998 to £4.2 billion in 2007 (Kopelman, Jebb and Butland, 2007), and it is projected to reach £9.7 billion by 2050 (National Obesity Observatory, 2016). These statistics reflect an obesity epidemic requiring immediate strategic attention at the population level.

1.1.2 Causes

From a nutritional point of view, the excessive consumption of highly palatable and energy-dense foods, which have disrupted food structures due to processing, together with a sedentary lifestyle, have resulted in metabolic-related disturbances and obesity. An imbalance between energy input (calories consumed) and energy output (calories burned) causes a 'surplus' energy balance, which is a major factor in the current obesity epidemic (Keesey and Hirvonen, 1997). Indeed, it is considered over the past few decades that most of the human population are living in an 'obesogenic' environment, as the current Western-style diet followed is characterised by high-energy, poor nutrient foods and drinks, increased reliance on fast and processed foods with

disrupted structures, and unbalanced dietary composition, which can easily lead to the overconsumption of energy (National Obesity Observatory, 2016). These ‘convenient’ foods and drinks are readily available at a relatively low cost (Popkin, Adair and Ng, 2012) and in increased portion sizes, both within and outside the home (Nielsen and Popkin, 2003). However, it is important to highlight that obesity can result from a steady and gradual increase in weight, which could be caused by a positive energy balance of as little as 50–100 kcal/day (Hill, 2006). It has been estimated that an average early- or middle-aged adult can gain 0.4-0.9 kg/year in this way, which can contribute to unhealthy weight gain over decades (Hill, 2006; Mozaffarian *et al.*, 2011).

This small positive energy balance can easily occur due to the composition of the current Western diet, which contains non-fibrous, processed carbohydrates with almost no structure (Cordain *et al.* 2005; Cordain *et al.* 2002; Eaton 2006). Indeed, numerous studies have found strong positive associations between refined grains and processed foods and weight gain (Mozaffarian *et al.*, 2011). These processed foods, which have almost no structure, are consumed quickly and easily, and are they are more energy dense than is appropriate based on their lower capability for satiety (i.e., they may be less satiating, thus increasing subsequent hunger signals as compared to less-processed intact-structure foods); hence, they inherently over-nourish those who consume them and lead to weight gain (Heaton, 1973; Cleave, 1974; Rolls *et al.*, 2005; Bornet *et al.*, 2007; Ello-Martin *et al.*, 2007; Ledikwe *et al.*, 2007). These processed foods in the Westernised diet are mainly carbohydrates from plant sources, which, due to the effect of processing, have little to no fibrous content and are thus highly digestible (Foster, 2011). In addition to deconstructing foods, industrial food processing uses various ingredients (mainly sugars and saturated fats) that have undergone thermal, mechanical, or chemical processing, resulting in foods with disrupted structures and higher energy densities, which are thus highly palatable (Foster, 2011). Epidemiological studies suggest that the processing of whole grain cereals and dietary fibre removal contribute to weight gain and the development of cardio-metabolic diseases (Reynolds *et al.*, 2019).

On the other hand, consuming quality carbohydrates (i.e. dietary fibre and resistant starches (will be discussed in more details under section 1.5), which are found in many foods, including whole cereals, whole fruits and vegetables, and legumes) is associated with healthy metabolic outcomes

(Reynolds *et al.*, 2019). Epidemiological studies have shown that there is a 9% reduction in CVD risk and coronary heart diseases for each 7 g/d increment in total fibre intake (Threapleton *et al.*, 2013). Likewise, there is a 9% lower risk of T2D with every additional 10 g/d total fibre consumed (InterAct Consortium, 2015). A multi-ethnic epidemiological pooled study of 159,683 humans over 25 years found that consuming 1 g of food high in dietary fibre per 200 kcal led to 18% and 23% lower risks of excess weight gain in men and women, respectively (Maskarinec *et al.*, 2006). In addition, a systematic review and meta-analysis demonstrated a positive dose-response curve between dietary fibre intake and body weight. Pooling the results of 1294 participants from 27 studies whose intake of fibre was 25-29 g/day, it was found that this fibre intake was associated with lowered bodyweight, with a pooled effect estimated mean difference of -0.37 kg (Reynolds *et al.*, 2019). Further, a 12 g/day increase in dietary fibre intake per 1000 kcal/day in 74,091 US females resulted in ~3.5kg less weight gain than controls (Liu *et al.*, 2003). Similarly, intervention studies, such as the Finnish Diabetes Prevention study, showed a sustained weight reduction (>5%) in 500 overweight middle-aged men and women with impaired glucose tolerance after three years of following a high-fibre diet compared with the control group (Lindström *et al.*, 2006). Interestingly, weight reduction was shown to be associated with fibre density in a dose-dependent manner, and individuals who ingested >15 g/1000 kcal of fibre per day had a mean weight loss of 3.0 kg after three years compared to 0.7 kg for those who consumed <10 g/1000 kcal per day (Lindström *et al.*, 2006).

The Scientific Advisory Committee on Nutrition's (SACN) published dietary fibre intake recommendation for the UK dietary reference value (DRV) is 30 g/day for adults (SACN, 2015). However, data from the National Diet and Nutrition Survey (NDNS) suggest that the intake of adults in the UK falls between 13.4 and 14.0 g/day (British Nutrition Foundation, 2016; PUBLIC HEALTH ENGLAND, 2016). This marked low intake is historically different from what the human gut adapted to over several millennia. Evidence has shown that hunter-gatherers consumed >100 g/day dietary fibre, mainly through indigestible plant materials, such as tubers, sedges, and grasses (Eaton, 2006). The low digestibility of Palaeolithic diets has led to the suggestion that microbial fermentation played a significant role in energy homeostasis by serving as a system for energy recovery (Slavin, 2005). However, this physiological adaptation has seemingly been

underutilised by humans in the Western world due to the lower intake of fibre and hence the increased consumption of refined digestible carbohydrates. This marked shift from a high-fibre, low-energy diet to the average Western low fibre, high-energy diet may be a significant contributor to the current obesity pandemic. Although there are multiple aspects to consider in this dietary shift, this thesis will focus on the role of food structure on starch digestibility in appetite regulation and energy homeostasis.

Accordingly, it has been recommended to substitute whole, structured foods for refined processed foods with no structure and ensure a daily intake of dietary fibre of at least 25–29 g (Reynolds *et al.*, 2019). However, current nutritional guidelines fail to focus on the beneficial effects of consuming whole-structure foods (NICE, 2015; British Nutrition Foundation, 2018). Moreover, there is a need to improve the understanding of the interplay between structural components of foods and how they are digested/absorbed or escape early digestion and become available for fermentation in the human GI tract, which results in energy homeostasis and appetite regulation. This information would make it possible to develop new ways to manipulate commonly consumed carbohydrate-based foods to regulate metabolic response and tackle obesity problem throughout life.

The rest of the introduction will discuss the following: neural and hormonal mechanisms regulating appetite; problems maintaining appetite; carbohydrate classification; the interplay between food structure, starch fermentation, and appetite regulation; identifying dietary tools to promote fermentation; and fermentation by-products.

1.2 Appetite regulation and energy homeostasis:

Energy homeostasis is a complex process that refers to controlling the energy balance of both intake and expenditure. On one side, energy intake is the sum of the energy of consumed nutrients (e.g. glucose, amino acids, and fatty acids). On the other side, energy expenditure is the amount of energy spent to maintain the resting metabolic rate (RMR), physical activity, and diet-induced thermogenesis (DIT). Energy intake is strongly driven by appetite, which is the desire to eat. Although appetite is affected by different elements, including cultural, economic, and social factors, it is regulated by a network of interactions between the psychological (cravings, hunger, hedonic sensation) and behavioural events (macronutrient/energy intake, meals), peripheral physiology and metabolic events, and neuronal activities in the brain (Blundell, 1999).

The GI tract senses the presence of food following consumption through different chemoreceptors and mechanoreceptors. These sensors are later transferred to the brain via the vagus nerve and stimulate the secretion of peptides and gut hormones from the periphery (e.g. the colon). Together, neural and peripheral signals regulate energy homeostasis by inhibiting or stimulating eating. All signals are later integrated via central circuits in the brain (Cooke and Bloom, 2006). Satiety describes the sum processes causing termination of meal consumption. In contrast, satiety is the fullness feeling that prevents the initiation of another eating event (Blundell, 1999).

The homeostatic and hedonic regulation of energy will be briefly summarised in the following section.

1.2.1 Homeostatic regulation of appetite

The homeostatic system regulates appetite through three main sites: the central nervous system (CNS), the GI tract and the abdominal viscera.

1.2.1.1 Appetite centres of the central nervous system

The CNS plays a major role in modulating energy homeostasis, adjusting both the energy expenditure and the drive to eat in response to nutritional and hormonal signals. This happens

in response to receiving signals from the periphery regarding energy status in the form of afferent vagus and spinal cord neuronal signals as well as signals from the circulatory system (Morton *et al.*, 2006). Several brain regions are involved in this process, including the hypothalamus, the caudal brainstem, and parts of the cortex. These brain regions together receive and integrate peripheral and hormonal signals as well as neuronal signals from other brain areas to alter feeding behaviour.

1.2.1.1.1 The hypothalamus

The hypothalamus is a crucial region of the CNS that is involved in the coordination of energy homeostasis and appetite regulation. It is located at the base of the brain below the thalamus. The hypothalamus is comprised of several distinct nuclei, which form a more complex and integrated neural network, including the arcuate nucleus (ARC), dorsomedial nucleus (DMN), lateral hypothalamic area (LHA), ventromedial nucleus (VMN), and paraventricular nucleus (PVN). It is thought that the ARC plays a critical role in modulating energy homeostasis as part of receiving input signals from the caudal brainstem and thus receiving neuronal signals from the GI tract. These crucial features are due to its anatomical location, as it is positioned in an area with an incomplete blood–brain barrier adjacent to the median eminence, allowing the ARC nuclei to directly sense the metabolic and endocrine circulatory signals (Broadwell and Brightman, 1976). To regulate appetite, these signals act on two functionally different populations of neurons in the ARC: one co-expresses anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) peptides, which inhibit food intake (Elias *et al.*, 1998), whereas the other co-express the orexigenic agouti-related peptide (AgRP) and neuropeptide Y (NPY), which trigger appetite and stimulate food intake (Broberger *et al.*, 1998). These neurons extensively communicate with other hypothalamic nuclei together with extrahypothalamic areas of the CNS to modulate energy homeostasis (Bagnol *et al.*, 1999).

1.2.1.1.2 The caudal brainstem

The brainstem also plays a key role in regulating energy balance. It is located in the hindbrain, comprised of three major areas involved in energy homeostasis: the nucleus tractus solitarius (NTS), the area postrema (AP), and the dorsal motor nucleus of the vagus (DMX) (the latter two

comprise the dorsal vagal complex (DVC)) (Schwartz, 2006). Appetite signals from the GI tract reach the brainstem and the NTS through the vagal afferents, which then transfer signals to the ARC and the hypothalamus (Grill and Kaplan, 2002). Similar to the median eminence of the hypothalamus, the AP has an incomplete blood–brain barrier, allowing circulatory signals to be sensed by the brainstem and later to be transferred to the hypothalamus (Gotow and Hashimoto, 1980).

1.2.1.2 Appetite-regulating hormones

The GI tract is the largest endocrine organ in the body and is known to secrete over a hundred bioactive peptide hormones (Ahlman and Nilsson, 2001). These hormones are secreted from specialised epithelial endocrine cells in the GI tract called enteroendocrine cells (EEC). EEC can, directly or indirectly, sense changes in nutrient content in the lumen, GI tract distension, and pH level and modulate a variety of GI functions, including motility, regulation of blood flow, and hormone secretion, all of which are essential functions in the digestion and absorption of nutrients (Sternini, Anselmi and Rozengurt, 2008). A wide range of gut hormones have been implicated in the regulation of appetite, each with a different mechanism of action; for example, ghrelin has an orexigenic effect while cholecystokinin (CCK) has an anorexigenic effect (Murphy, Dhillon and Bloom, 2006). However, only specific hormones that are relevant to the present work will be discussed below.

1.2.1.2.1 Polypeptide tyrosine tyrosine (PYY)

PYY is a gut hormone that belongs to the pancreatic polypeptide-fold family, which also includes the gut hormone polypeptide (PP) and NPY. PYY is a 36-amino acid anorexigenic peptide hormone that is produced in the enteroendocrine L-cells of the GI tract. PYY is secreted into the circulation following food intake and reaches a peak concentration 1–2 hours postprandial, remaining elevated for up to six hours (Adrian *et al.*, 1985). Two forms of PYY have been identified: PYY₁₋₃₆ and PYY₃₋₃₆. The biologically active form PYY₃₋₃₆ is produced from the cleavage of PYY₁₋₃₆ at the N-terminal by dipeptidyl-peptidase IV (DPP-4) (Grandt *et al.*, 1994). PYY₁₋₃₆ binds to the receptors

Y1, Y2, and Y5, whereas PYY₃₋₃₆ is a selective Y2 receptor agonist and is found along the GI tract (Walther, Mörl and Beck-Sicking, 2011).

Obese individuals have lower fasting PYY concentrations when compared to lean individuals (Batterham *et al.*, 2003). Interestingly, intravenous administration of PYY₃₋₃₆ was shown to increase intestinal transit time, inhibit gastric emptying, and reduces food intake (Savage *et al.*, 1987; Batterham *et al.*, 2002, 2003; Challis *et al.*, 2003; Pittner *et al.*, 2004; Chelikani, Haver and Reidelberger, 2005). Moreover, peripheral administration of PYY₃₋₃₆ decreased hypothalamic NPY messenger RNA (mRNA) and increased c-Fos expression, which is a marker of neuronal activity, in the ARC. It was also shown that the administration of PYY₃₋₃₆ directly into the ARC caused food intake inhibition (Batterham *et al.*, 2002). Nevertheless, the way in which PYY₃₋₃₆ induces its anorexigenic effects is not fully understood, although it has been suggested that it causes appetite suppression through binding to its Y2 receptor expressed on vagal afferent fibres and in the brainstem (Koda *et al.*, 2005), as the anorexigenic effect was abolished in vagotomised rodents (Abbott *et al.*, 2005).

1.2.1.2.2 Glucagon-Like Peptide-1 (GLP-1)

GLP-1 is an anorexigenic 30-amino acid peptide that is a product of the post-translational processing of proglucagon, a 160-amino acid polypeptide precursor. Proglucagon is synthesised by neurons within the NTS, pancreatic α -cells, and enteroendocrine L cells in the GI tract (Holst, 2007). Proglucagon can also be selectively cleaved to form other hormones, namely, glucagon, oxyntomodulin (OXM), and GLP-2 (Nelson and Tucker, 1996). There are two primary bioactive forms of circulatory GLP-1: GLP-1₇₋₃₇ and the main circulating form, GLP-1₇₋₃₆ amide. Both forms of GLP-1 bind to GLP-1R receptors, which are widely distributed in the pancreas, the GI tract, and the brain (Yamato *et al.*, 1997).

Postprandial GLP-1 levels elevate proportionally to the number of calories ingested and are low in fasted states (Herrmann *et al.*, 2009). Interestingly, fasting GLP-1 was found to be similar in obese and lean individuals (Batterham *et al.*, 2003). Both GLP-1 and PYY are co-expressed in the GI tract L-cells and are co-secreted (Eissele *et al.*, 1992). Peripheral administration of GLP-1 has been shown to increase c-Fos expression in the PVN and brainstem (Baggio *et al.*, 2004) and to reduce energy intake in both rodents and humans (Flint *et al.*, 1998; E Näslund *et al.*, 1999;

Abbott *et al.*, 2005). It was shown that the anorexigenic effects of GLP-1 were eliminated by blocking ARC neuronal signalling (Tang-Christensen, Vrang and Larsen, 1998). However, the way in which GLP-1 suppresses appetite is not yet fully understood. Currently, it is suggested that GLP-1 stimulates appetite suppression through the activation of GLP-1R on vagal afferents in the GI tract and possibly the hepatoportal region (Hansen *et al.*, 1999; Abbott *et al.*, 2005; Holst and Deacon, 2005; Vahl *et al.*, 2007; Williams, Baskin and Schwartz, 2009). Other physiological functions of GLP-1 include delaying gastric emptying (Tolessa *et al.*, 1998), suppressing glucagon secretion (Erik Näslund *et al.*, 1999), and acting as an incretin that stimulates glucose-dependent insulin secretion (Kreymann *et al.*, 1987).

1.2.1.2.3 Insulin

Insulin is a known peripheral adiposity peptide hormone, composed of 51-amino acid, which is secreted from β -cells of the pancreatic islets of Langerhans. Insulin is the most crucial glucose regulator in the human body. Its secretion is increased in the postprandial phase, which results in cellular uptake of the elevated glucose circulated in plasma. As insulin is considered to be an adiposity signal, the magnitude of insulin secretion is correlated with body weight and the level of adiposity, particularly visceral fat, with levels elevated during weight gain and reduced during weight loss (Bagdade, Bierman and Porte, 1967). However, numerous studies have confirmed the negative relationship between weight gain and insulin sensitivity, especially during the first-phase secretion, which is a well-known characteristic for developing type 2 diabetes.

The hypoglycaemic effect of insulin is paralleled with its impact on satiety, and insulin has been shown to be a potent anorexigenic stimulus within the CNS (Bruning *et al.*, 2000). Further, central administration of insulin has been shown to reduce energy intake and body weight in a dose-dependent manner (Porte and Woods, 1981). Insulin is able to cross the blood–brain barrier and exert its anorexigenic effect on central brain circuits through a saturable-receptor mechanism (Woods *et al.*, 2003). Insulin is known to exert its action through binding to its receptor, which is found in numerous hypothalamic regions, including the ARC (Marks *et al.*, 1990). Central administration of insulin increases the expression of POMC, decreases fasting-induced NPY release in the PVN, and decreases preproNPY expression in the ARC (Schwartz *et al.*, 1992). Other

evidence suggests that anorexigenic insulin effects may also reach other CNS areas involved in appetite regulation (Pardini *et al.*, 2006).

1.2.2 Hedonic (non-homeostatic) regulation of appetite

Clearly, the homeostatic regulatory mechanism is actively responsible for maintaining body weight; however, hedonic non-homeostatic pathways also exist (Berthoud, 2011). These hedonic responses involve a network of corticolimbic brain structures and are modulated by psychological and emotional factors (fear, anxiety, and stress) as well as perceived reward and sensory cues. In addition to the reward-based mechanism, socio-cultural factors can also affect the choice of foods, including the person's knowledge of food preparation and cooking, economic stability, food availability and cooking tradition, besides having inter-individual variability.

Several brain regions are involved in this corticolimbic reward system. It was shown that viewing pictures of high-calorie foods caused greater activation of these specific regions in individuals with obesity than in normal-weight individuals (Rothmund *et al.*, 2007; Stoeckel *et al.*, 2008). These regions include the striatum, amygdala, insula, and orbitofrontal cortex (OFC).

- Both the striatum and insula have been implicated in food reward processing, with activation in both regions shown in a fasted state in response to thoughts of food or food pictures (Hinton *et al.*, 2004; Pelchat *et al.*, 2004; Wang *et al.*, 2004; Holsen *et al.*, 2006; Porubská *et al.*, 2006; Uher *et al.*, 2006; Führer, Zysset and Stumvoll, 2008; Cornier *et al.*, 2009; Goldstone *et al.*, 2009). For example, the sight of chocolate caused greater activation in these brain areas associated with increased desire to consume it in cravers than non-cravers (Rolls and McCabe, 2007).
- The amygdala plays a role in reward, emotion, learning, motivation, attention, and memory (Murray, 2007). It has also been implicated in food reward processing, where greater activation in the amygdala has been reported in the fasted state in response to food stimuli (LaBar *et al.*, 2001; Hinton *et al.*, 2004; Holsen *et al.*, 2005; Goldstone *et al.*, 2009).
- The OFC is involved in decision-making and has also been implicated in food reward processing. In the fasted state, greater OFC activation has been noted in response to food

pictures (Goldstone *et al.*, 2009), with high-calorie foods resulting in significantly higher activation (Killgore *et al.*, 2003). Moreover, OFC activation is positively correlated with the subjective pleasantness of a specific food (Kringelbach *et al.*, 2003) and with an increased subjective preference toward high-calorie foods when in the fasted state (Wang *et al.*, 2004; Goldstone *et al.*, 2009). Similarly, greater activation in the OFC has been reported in response to the sight of chocolate in cravers compared with non-cravers (Rolls and McCabe, 2007) and in response to images of high-incentive foods (Hinton *et al.*, 2004). In addition to visual cues, food odours have been shown to cause OFC activation (O'Doherty *et al.*, 2000).

1.2.3 Interaction between homeostatic and hedonic pathways

There is evidence to suggest that homeostatic and hedonic pathways directly interact with one another. During caloric restriction, the hedonic responses increase and activate the reward-related regions of the brain in response to food stimuli (Goldstone *et al.*, 2009; Stice, Burger and Yokum, 2013). If these hedonic factors override homeostatic regulatory mechanisms, it might lead to weight gain if sustained over the long term. However, as peripheral signals such as gut hormones are involved in the homeostatic regulation of energy homeostasis, it has been suggested that these homeostatic signals may influence the hedonic responses and alter satiety (Batterham *et al.*, 2007; Malik *et al.*, 2008; Goldstone *et al.*, 2009; De Silva *et al.*, 2011; Dagher, 2012; Stice, Burger and Yokum, 2013). For instance, with the aid of functional magnetic resonance imaging (fMRI), Batterham *et al.* demonstrated that there was significant whole-brain blood-oxygen-level-dependent (BOLD) reward system brain activity within the orbitofrontal cortex (OFC) accompanied by a significant reduction in an *ad libitum* meal following the intravenous infusion of a physiologically relevant concentration of PYY in healthy volunteers when compared with a saline infusion, confirming the links between gut hormones and central hedonic pathways (Batterham *et al.*, 2007). This crosstalk happening between homeostatic and hedonic pathways can be advantageous in developing strategies to reduce the hedonic overconsumption of foods and thus regulating appetite and combating obesity in the long run.

1.3 Challenge in regulating appetite and energy homeostasis

Although the complex human body system mentioned above is continuously attempting to maintain an energy balance through a wide range of hormonal and neural regulatory mechanisms (Murphy and Bloom, 2004), obesity rates have continued to increase. Theoretically, obesity can result from any pathological dysfunction or lack of environmental adaptation that would influence the control system discussed above. The major aspects influencing obesity are inadequate sensing of ingested nutrients and overstimulation of sensory mechanisms (Berthoud and Morrison, 2008). Keeping that in mind, and given the relatively new obesogenic environment compared to our ancestors, the mismatch between the physiological mechanisms adapted to regulate energy balance is predictable (Yeo and Heisler, 2012). It seems that this natural energy conservation is an evolutionary mechanism that allows our bodies to adapt to periods of starvation and food shortages. In contrast, the effectiveness of this complex system in curbing excessive food intake seems to be low when surplus, energy-dense, palatable (mostly high in sugar and fats) refined foods are consumed, resulting in overeating and weight gain in the long run. Unfortunately, the mechanisms by which neural signals induce/inhibit intake based on the availability of highly energy dense and palatable foods are poorly understood and require research attention.

On the other hand, recent findings have shown that the structure of food plays a vital role in promoting satiety and that the concentrations of appetite-regulating hormones PYY and GLP-1 vary according to different food structures (Lundin, Golding and Wooster, 2008). This can explain the parallel increase in obesity and the shift in diet from 100 g of whole, structured, non-digestible carbohydrate consumption daily (the average amount of fibre our ancestors consumed), mainly from complex structured dietary components including whole cell wall plants (raw fruits and vegetables) (Cordain *et al.*, 2002, 2005; Eaton, 2006), to the refined destructured components of the Westernised diet. There is also growing evidence that postprandial metabolic responses are not governed solely by the macronutrient ingested; rather, additional factors, such as food structure, rheology, and breakdown, may also play a role in the digestive process and subsequent metabolic response (Holm and Bjorck, 1992; Traore *et al.*, 2008). Nevertheless, it is difficult to maintain satiety for hours due to the lack of structure and rapid digestion (Osterdahl

et al., 2008), which are typical features of current Westernised foods (Fiolet *et al.*, 2018). Thus, several factors should be considered when trying to achieve lower energy intake over a set time period, including increasing the satiating signals of foods and beverages, reducing the portions of individual consumption, and increasing the duration until the next meal is consumed (Blundell *et al.*, 2010; van Kleef *et al.*, 2012), which could be achieved by replacing processed foods with no structure with whole structured ones. Suggested mechanisms of action explaining the interplay between food structure and appetite regulation will be covered in the following sections. Carbohydrates are the major energy source for the human body and thus have a strong influence on these mechanisms. Accordingly, the following sections will discuss how different carbohydrate structures affect digestion and subsequent differential metabolic responses.

1.4 Nutrient sensing

Nutrient sensing is thought to play a role in maintaining energy balance. Gut cells and tissue can sense and interact with ingested energy molecules (i.e. amino acids, glucose, and fatty acids) (Lindsley and Rutter, 2004). Nutrient sensing is a known function of enteroendocrine cells, which can detect any fluctuation in the concentration of nutrients passing the luminal tract as well as any increase in the absorbed concentration of nutrients across the intestinal epithelium, with the aid of sensory receptors and transporters (Sternini, Anselmi and Rozengurt, 2008). Activating these nutrient sensors can trigger intracellular signalling responses, including membrane depolarisation, increased intracellular calcium concentration, and subsequent second messenger cascades, leading to gene expression alteration or gut hormone secretion (Tolhurst, Reimann and Gribble, 2012). An example of a nutrient sensing pathway within the EEC, G-protein-coupled receptors (GPCR) activation which causes the utilisation of membrane solute transporter and thus intracellular receptor binding or metabolism (Tolhurst, Reimann and Gribble, 2012).

Nutrient sensors are novel targets in treating obesity. Indeed, a large number of GPCR receptors have been identified as nutrient sensing. For example, some GPCR (GPCR41 and GPCR43; currently known as FFAR3 and FFAR2) are known to be activated by short-chain fatty acids, stimulating the release of gut hormones (Hirasawa *et al.*, 2005; Edfalk, Steneberg and Edlund, 2008). The connection between food structure and appetite modulation relies significantly on the activity of these gut hormones on gut–brain communication (Shen *et al.*, 2009). For instance, non-digestible carbohydrate consumption is associated with increased release of the anorexic gut hormones PYY and GLP-1 (Keenan *et al.*, 2006; Zhou *et al.*, 2006; Li *et al.*, 2017). These gut hormones are known candidates for anti-obesity drugs (Murphy, Dhillo and Bloom, 2006; Young, 2006), as they have shown to be associated with reducing the feeling of hunger, increasing the feeling of fullness, and reducing energy intake (Cani *et al.*, 2006; Parnell and Reimer, 2009; Pedersen *et al.*, 2013; Daud *et al.*, 2014). These two hormones affect energy balance by sending signals from the gut to the brain, directly or through the vagal nerve (Abbott *et al.*, 2005; Koda *et al.*, 2005; Osaka *et al.*, 2005), resulting in decreased NPY/AgRP mRNA expression and increased POMC mRNA expression in the hypothalamus (Challis *et al.*, 2003).

1.5 Carbohydrate and energy homeostasis

1.5.1 Carbohydrate classification

Carbohydrates constitute the largest portion of human daily energy intake, and thus the quality and quantity of carbohydrates chosen for consumption are important, especially when trying to control or lose weight. Carbohydrates are classified based on their chemical structure and molecular size (e.g. degree of polymerisation, individual monomers, and linkage bonds) into monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Considering these classification, it is also important to add these chemical properties to the nutritional and physiological properties in order to relate their effects on digestibility and health, which will be summarised below.

1.5.1.1 Monosaccharides and disaccharides

Monosaccharides (e.g. glucose) and disaccharides (e.g. sucrose) are available to be digested and absorbed in the small intestine for immediate energy supply (Frayn, 2010). The final absorbed single units of glucose are absorbed through an active ATP-driven transport system against gradient via GLUT2 and SGLT1 transporters in the gut lumen, while fructose is absorbed entirely through facilitated diffusion via the GLUT5 transporter (Frayn, 2010). Based on their digestibility in the GI tract, these compounds can be referred to as simple, non-fibrous, non-structural, or refined carbohydrates.

1.5.1.2 Polysaccharides

Polysaccharides are the most complex and extended structure of carbohydrates, characterised by the polymerisation of monomers subunits from 200 to 2500. They can be found in foods such as fruits and vegetables and as components of the plant cell wall (e.g.) cellulose and pectin (Cummings and Stephen, 2007; Frayn, 2010). The most abundant form of polysaccharides in foods is starch. The differences between polysaccharides depend on their chain length and the nature (α - or β -) and position (1-4 or 1-6) of the bonds between individual subunits. The fraction

of polysaccharides that include β -linkages are not digested in humans and thus pass through the GI tract mostly intact and largely unfermented (e.g. cellulose).

It is also important to add these chemical features of polysaccharides to the nutritional and physiological aspects in order to relate their effects on digestibility and health in the human GI tract, which will be covered in the next section together with the factors affecting their digestibility in the human GI tract.

1.5.2 Starch classification

Starch is the energy storage form in plants, and it is the most abundant carbohydrate found in plant seeds and tubers. It is the main energy source in our daily diets; indeed, 30% of the average UK diet consists of starches (Wang, Bogracheva and Hedley, 1998). Multiple factors can significantly affect starch breakdown during digestion, including the botanical source, industrial and domestic processing, and starch composition and granule morphology, as will be discussed in the following sections. Examples of the most commonly consumed starch-based foods include flour-based products, such as cakes and bread. If made of white flour, these can contain high amounts of rapidly digestible starches which will contribute to higher plasma glucose levels. Physiologically, starch can be classified as rapidly digested starch (RDS), slowly digested starch (SDS), and resistant starch (RS).

1.5.2.1 Rapidly and slowly digestible starches

RDS results in rapid breakdown and absorption of substrate, thus resulting in a high blood glucose concentration and high insulin response affecting tissue-specific glucose uptake, leading to low plasma glucose levels (Birt *et al.*, 2013). These cycles of high and low plasma glucose release can contribute to insulin resistance, T2D, and other metabolic defects (Birt *et al.*, 2013). On the other hand, SDS is characterised by more attenuated breakdown and thus slower digestion and lower absorption and plasma glucose release, which results in more sustained energy (Cummings and Stephen, 2007).

1.5.2.2 Resistant starches

RS is defined as any starch not digested in the small intestine (Lattimer and Haub, 2010). This type of starch is found in some fruits, such as green bananas, whole-meal cereals, and vegetables, such as legumes. RS has been classified into five basic types (Table 1.1) (Englyst, Kingman and Cummings, 1992; Nugent, 2005; Sajilata, Singhal and Kulkarni, 2006; Lunn and Buttriss, 2007; Sharma and Yadav, 2008; Fuentes-Zaragoza *et al.*, 2011; Raigond, Ezekiel and Raigond, 2015). Type 1 RS (RS1) is made up of grains or seeds that are physically inaccessible due to the presence of intact cell walls. Type 2 RS (RS2) occurs in native starch granules, such as raw potato and green banana. Type 3 RS (RS3) is retrograded starch formed during the cooling of gelatinised starch. Type 4 RS (RS4) is found in chemically modified starches by cross-linking to chemicals, etherisation, and/or esterisation, which does not occur in nature. Type 5 RS (RS5) arises from the formation of amylose–lipid complexes during the processing of high-amylose starches, which is highly dependent on the botanical source of the plant food.

Table 1.1 Types of RS, food sources, and factors affecting their resistance to digestion in the GI tract

	RS1	RS2	RS3	RS4	RS5
Description	Physically inaccessible	Starch granules with type B crystallinity	Retrograded starch	Chemically modified starches	Amylose–lipid complexes
Food sources	Whole grains, legumes, and seeds	Green bananas, some legumes, raw potatoes, and high-amylose corn	Cooked and cooled bread, cornflakes, and potatoes	Foods with modified starches (e.g. cakes, bread)	High-amylose foods
Reducing resistance by	Chewing, milling	Cooking and food processing	Food processing	Digestibility is reduced in vitro	Not digested by α -amylase
Small intestinal digestion	Slowly digested; totally digested if properly milled	Prolonged rate of digestion as being slowly hydrolysed by α -amylase	Slow rate of digestion; digestibility improved by reheating	Can resist hydrolysis	Can resist hydrolysis

Table adapted from (Raigond, Ezekiel and Raigond, 2015)

1.5.3 Dietary fibre

Dietary fibre is 'the proportion of food that is derived from the plant cell walls and is digested very poorly in humans' (Trowell, 1972). As defined, it cannot be digested by mammalian enzymes and passes relatively intact through the upper GI tract to be subjected to fermentation by the native intestinal microbiota (Eaton, 2006). However, to provide a physiological benefit, newer definitions have highlighted the need for a carbohydrate source in order for it to be classified as dietary fibre. Dietary fibres are constituted of three main components: non-starch polysaccharides and oligosaccharides (e.g. cellulose, inulin, and pectins), analogous carbohydrates (e.g. resistant starches), and lignin (e.g. phytate) (American Association of Cereal Chemists, 2001).

It is important to emphasise at this point the differences between dietary fibres. Despite the mentioned main components, shared properties of dietary fibres allowed for further categorisation based on the functional aspects. For instance, soluble and insoluble fibres refers to the ability of the ingested fibre to interact with water molecules to swell and forms gels; this will lead to certain effects that differ than non-soluble fibres. Also, in plant foods, dietary fibre would be mainly found as a component of plant cell wall (CW), and thus provide rigidity and stability for the plant structure. Thus, fibre-containing CW will be encapsulated and can act as a physical barrier against any destruction to the plant structure and CW matrix as in digestion. For the purpose of this thesis, the term 'dietary fibre' will refer to a broad category of fibres, encompassing dozens of different molecules that would be found as a component of the plant CW (e.g. cellulose) and matrix (e.g. pectin). Dietary fibre is complex, and the amount that is available in the GI tract is unknown, which makes it difficult to draw a solid conclusion regarding cause and the subsequent effect on postprandial metabolic responses. Meanwhile, RS refers to the structural components within a carbohydrate or starch source. Emphasising the structural differences within the carbohydrates in feeding studies would allow researchers to gain more mechanistic insight into the effects of food structure on health. Finally, the term non-digestible carbohydrates specifically refers to non-digestible fractions of carbohydrate foods ingested.

Given the major role of starch structure in generating the hypotheses of the work presented in this thesis, the remainder of this section will focus on the factors affecting starch digestibility, both on the molecular and cellular structural levels, along with the effects during processing.

1.5.4 Factors influencing the food structure of starch and its digestion

The hydrolysis of starches can vary greatly, depending on the morphology and crystallinity organisation of the starch granules as well as the intra granular amylose-to-amylopectin ratio (Tester, Karkalas and Qi, 2004a). Interaction with the granular surface porosity and the nature of the matrix (e.g. high lipid component) are factors that could prevent effective starch digestion and impact α -amylase diffusion and adsorption into the intra-granular substrate (Colonna, Leloup and Buléon, 1992).

1.5.4.1 Penetration into cellular content: the effect of cell walls

One of the critical factors in plant foods (e.g. legumes and nuts) that affects the release and/or availability of nutrients (i.e. starch and lipids) during mastication and digestion is the structure of the cell wall (Waldron, Parker and Smith, 2003; Ellis *et al.*, 2004; Berry *et al.*, 2008; Mandalari *et al.*, 2008; Lemmens *et al.*, 2010; Tydeman *et al.*, 2010; Butterworth *et al.*, 2012). Plant cell walls (CWs) are supramolecular structures that are composed of heterogeneous complexes of cellulose, hemicelluloses, lignin, pectins, protein, and water (Grundy *et al.*, 2016). These polysaccharides vary in their amount and relative proportions depending on the maturity and type of plant tissue. This variation explains the reason that CW matrices have different properties, including cell separation or rupture, porosity, viscosity, and fragmentation, in response to digestion (Grundy *et al.*, 2016). Thus, it can be suggested that the bioavailability of nutrients (i.e. the biological use of nutrients after digestion and absorption) is affected by these properties as well as by nutrient bioaccessibility (i.e. the proportion of a nutrient released from ingested foods), the rate of digestion and absorption by intestinal cells, and the amount of delivery of digested/undigested components to targeted body cells (Boland, Golding and Singh, 2014; Grundy *et al.*, 2016). In order for intracellular nutrients (e.g. saccharides) to be digested in the human GI tract, either the CW need to be ruptured to allow contact with digestive enzymes or amylase can be diffused to the separated cells that would still contain an intact CW. Availability of CWs together with other cell layers can act as physical barriers in some plants preventing enzyme penetration to the starch granules, leading to starch encapsulation and

resulting in a slower digestion rate and lower postprandial glucose levels (Edwards, Grundy, *et al.*, 2015; Grundy *et al.*, 2016). This behaviour can happen in case of cell separation. A recent study found that the consumption of bread containing coarse wheat particles that were encapsulated by intact CWs attenuated the rise of postprandial blood glucose when compared with an identical milled-wheat bread containing disrupted CWs, highlighting the importance of physical encapsulation by CWs and starch integrity in response to digestion (Edwards, Grundy, *et al.*, 2015).

In addition, the thickness of CWs can play an additive role in slowing nutrient release, as plants with thick CWs tend to be less susceptible to rupturing and less permeable (Grundy *et al.*, 2016). This behaviour has been shown in legumes, in which the thick CWs have been reported to act as a physical barrier against gelatinisation and swelling. Thus, under thermal processing or mechanical forces (e.g. mastication), legumes CWs have the tendency to separate since a significant proportion of them consist of pectin (Mishra, Hardacre and Monro, 2012). This can result in encapsulating starch, a reduced digestion rate, decreased postprandial glucose response when compared to other plant foods, and a significant proportion of ingested legumes can escape digestion to serve as substrate for fermentation in the lower gut (Venn and Mann, 2004). To support the role of legume CWs, an earlier study utilising ileal intubation found that RS of white beans was recovered as partially degraded undigested starch that were protected by the cell walls during their transit through the gut (Noah *et al.*, 1998).

Bio-accessibility and the availability of nutrients for digestion could serve as an advantage for people with obesity who are trying to reduce energy consumption by decreasing macronutrient digestion and absorption. Despite that, the current assessment of food healthiness is based on the nutrient composition of the food. For example, based on nutritional information, almonds contain an excessive amount of energy that comes from lipids, which has been associated with heart disease. However, faecal analysis showed that a significant amount of lipid remained enclosed inside the parenchyma cells of almond cotyledon following oral consumption (Ellis *et al.*, 2004). Furthermore, the faeces of individuals who consumed whole peanuts had a greater proportion of fat recovery than when they ate peanut flour, peanut oil, or peanut butter (Traoret *et al.*, 2008). This misconception about the health properties of a food is due to the limited

information on nutrient availability in most foods. This highlights the importance of studying the interplay between a food's structure and its consequent disassembly in the human GI tract.

1.5.4.2 Amylose-to-amylopectin ratio

Starch consists of two structural forms of glucose polymers: amylose and amylopectin (Figure 1.1). Amylose is comprised of long chains of glucose units joined by α -1,4 links, while amylopectin consists of linear chains of α -1,4 linked glucose units with α -1,6 linked branches, resulting in a larger highly branched molecule, very similar to glycogen (Frayn, 2010; Lovegrove *et al.*, 2017). Most carbohydrate foods have a higher amylopectin-to-amylose ratio (70% to 30%). These chains are deposited within specialised plastids (called amyloplasts) in highly organised and crystalline granules (Wu and Sarko, 1978; Tester, Karkalas and Qi, 2004a, 2004b). Native starch granules are semi-crystalline, with amorphous regions composed mainly of amylose and the branch points in amylopectin molecules as well as crystallites formed from long chains of amylopectin that twist into bonded double helices (Wu and Sarko, 1978). The difference in the ratio between amylose and amylopectin in seed plants is caused by variations in the activities of enzymes involved in the biosynthesis of linear and branched components. These enzyme activities are affected by genetics, the botanical source, and environmental factors of the planted starch.

The amorphous structure of the branched amylopectin is believed to be more digestible by α -amylases than the compact stabilised amylose. Thus, starchy plant-based foods with a high amylose-to-amylopectin ratio tend to be digested slowly in the small intestine, releasing less glucose in the circulation, while the rest of the undigested starch travels to the lower gut and acts as a substrate for fermentation by gut microbiota (Botham *et al.*, 1996). The nature of the compact and crystalline amylose might explain this lower digestibility feature, as the linear-long chains increase the stabilisation of the amylose structure (due to the high hydrogen bonding) and makes it hard for the α -amylase to degrade it when compared with the branched amylopectin (Thorne, Thompson and Jenkins, 1983; Hoover *et al.*, 2003). Amylopectin has a much larger surface area per molecule than amylose, making it a preferable substrate for amylolytic attack (Thorne, Thompson and Jenkins, 1983). Indeed, it was shown that raw legume starch with a high amylose content was less digestible than corn starch in rats, and the rate of hydrolysis of legume

starch in vitro was less than that of corn starch (Thorne, Thompson and Jenkins, 1983). This explains why legumes with high amylose-to-amylopectin starch ratios were hydrolysed more slowly in the small intestine, with the rest of the indigestible starch arriving intact in the lower gut.

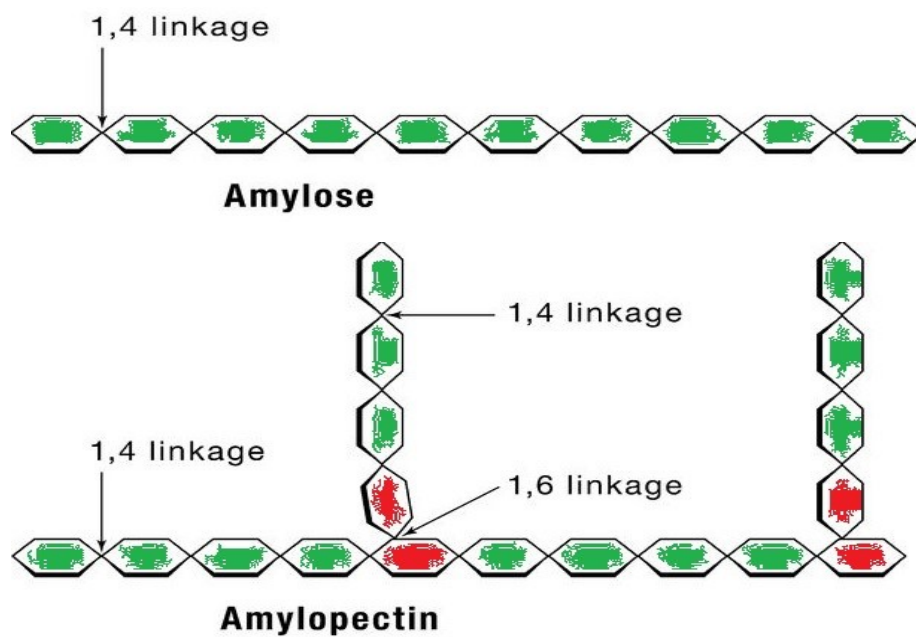


Figure 1.1 amylose and amylopectin chains

1.5.4.3 Starch granule structure

The arrangement of these polysaccharide chains within the granules and the length and density of chains are important factors in forming the crystallinity structure (A-, B-, or C- type) of these granules, which will contribute to their physical properties and chemical characteristics such as solubility (Wu and Sarko, 1978). These structures can play significant role in the inhibition of pancreatic amylase actions during digestion which will be reflected in postprandial metabolic responses as will be discussed in depth in the following section.

A-type polymorphs, such as cereal starches, are composed mainly of highly packed amylopectin with a chain length variation of 23 to 29 glucose units and are thus characterised by short double helices and interior crystallites (Sajilata, Singhal and Kulkarni, 2006). Foods containing these granules tend to be small and polycentric (Annison and Topping, 1994; Sajilata, Singhal and Kulkarni, 2006). The shorter double helices of the interior crystallites in A-type polymorph

starches (e.g. corn) tend to hydrolyse in an inside-out manner and hence have higher digestibility (Fannon, Hauber and Bemiller, 1992; Jane, Wong and McPherson, 1997; Benmoussa *et al.*, 2006). Acquiring the A-type polymorphs allows for more peripheral pores to be located on the surface area, leading to easier penetration of α -amylase through these channels (Fannon, Hauber and Bemiller, 1992; Benmoussa *et al.*, 2006). However, A-type starches have of the ability to withstand higher temperatures (Bogracheva *et al.*, 1998).

B-type polymorphs (e.g. raw potato and green banana) are composed of tightly packed double helical amylopectin with a chain length variation of 30 to 40 glucose units and are thus characterised by longer double helices and superior crystallites. Foods containing these granules tend to be large and spherical in shape (Annison and Topping, 1994; Sajilata, Singhal and Kulkarni, 2006). B-type starches (e.g. native potato) have lower digestibility characterised by exocorrosion hydrolysis in these amorphous starches with fewer pores (i.e. starting from the surface of the starch granule to inside) (Gallant, Bouchet and Baldwin, 1997). However, they can be disrupted and swell quickly in response to temperature (Bogracheva *et al.*, 1998). Figure 1.2 below illustrates these granular structures.

The crystalline structure of C-type starch granules (e.g. peas and other legumes) is a combination of both A and B polymorphs (Gernat *et al.*, 1990; Cairns *et al.*, 1997), with the B polymorphs being arranged centrally and the A polymorphs peripherally within each of the starch granules (Bogracheva *et al.*, 1998, 1999).

C-granules are composed mainly of amylopectin with a chain length variation of 26 to 29 glucose units (Annison and Topping, 1994; Sajilata, Singhal and Kulkarni, 2006). The combination of both of the types leads to the acquisition of the unique C-type unique properties, which differ from those of the A and B types. For example, in response to hydrothermal processing, the disruption of peas starts in the central parts, where B polymorphs are arranged, while the crystallinity of the outer parts of the A polymorphs of the granules are unaffected until the higher temperature is reached. However, the central parts continue to swell according to the capacity available and the flexibility of the surrounding crystalline parts. The disruption of A polymorphs occurs only at a temperature that is several degrees higher, after which the central crystallites are completely disrupted (Bogracheva *et al.*, 1998).

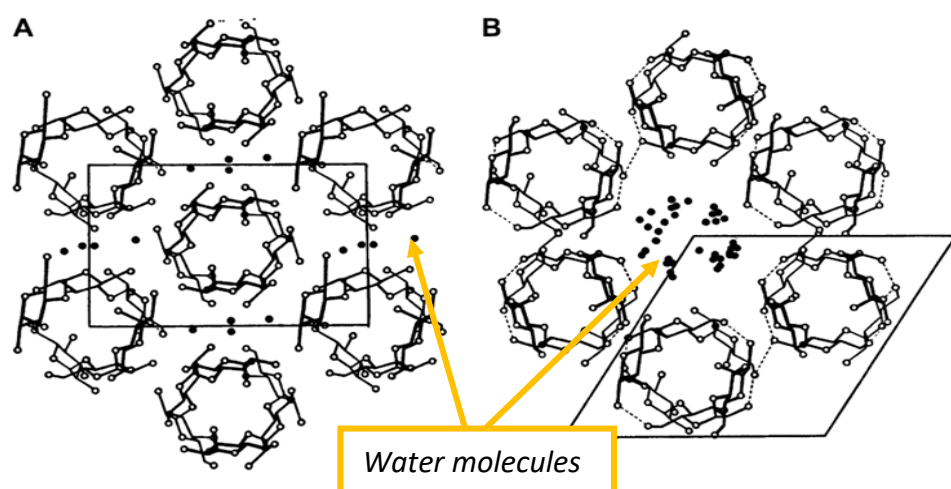


Figure 1.2 type A and type B starch crystallinity. Type C is a combination of both A and B.

Adapted from (Wu and Sarko, 1978)

1.5.4.4 Starch granule configuration and surface characteristics

The organisation of starch chains during plant growth and development and thus the morphological nature of the granule is also an essential factor when considering digestibility at the molecular level. Starch granules are structured as concentric rings of amorphous and pseudo-crystalline regions that have developed during granular starch growth, and they can be identified by the typical Maltese cross birefringence pattern in polarised light (Figure 1.3) (Donald, 2004; Mishra, Hardacre and Monro, 2012). The crystalline regions are made up of highly organised short chains of glucose subunits that are tightly packed. The amorphous regions are made up of branched points of the amylopectin that are also interspersed with amylose (Donald, 2004). The crystalline regions are characterised by a resistance to amylase digestion when compared to amorphous regions.

Moreover, a lipid and protein coating on the surface of starch granules can induce further resistance to digestion by acting as a barrier against amylase penetration (Oates, 1997; Donald, 2004). Additionally, it must be noted that most of starch granules contain pores that allow penetration of digestive enzymes to the core of the granules. It is essential to consider this property in the field of starch digestibility, as large open pores facilitate quicker enzyme penetration, resulting in glucose leaching through endocorrosion hydrolysis, as seen in cereal granules (Dreher, Dreher and Berry, 1984). Meanwhile, uncooked or native potato starch granules are more resistant to enzyme hydrolysis due to their smoother surface and the absence of any pits or pores (Langworthy and Deuel, 1920). In order for the raw potato starches to be digested, erosion of the entire granule surface or a section of it must occur first through exocorrosion hydrolysis (Tester, Qi and Karkalas, 2006).

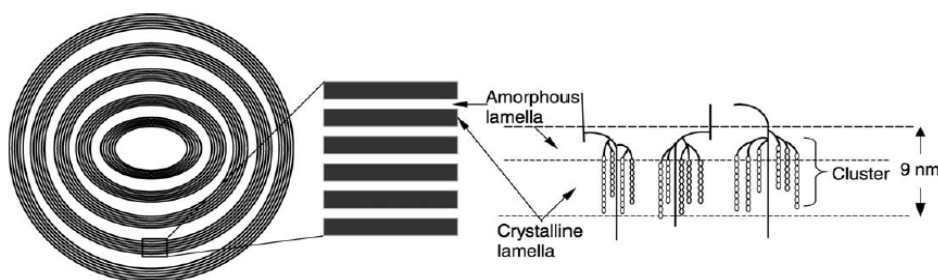


Figure 1.3: Morphology of starch granule.

Adapted from (Tester, Karkalas and Qi, 2004a)

1.5.4.5 Gelatinisation and retrogradation

Both gelatinisation and retrogradation effects can influence the rate of starch digestion. Gelatinisation occurs when starches are continuously heated in excessively aqueous conditions, resulting in swelling and loss of the starch granules' pseudo-crystallinity structure (Donald, 2004). Once cooled, the disrupted material can recrystallise into a form that is resistant to amylolysis effects. This material is known as retrograded starch (Najjar, Adra and Hwalla, 2004; Mishra, Hardacre and Monro, 2012).

1.5.4.5.1 Gelatinisation

The process of gelatinisation can cause changes in starch chains at the molecular level, where the amorphous regions tend to swell, causing the crystalline structure to disintegrate (Donald, 2004; Singh, Kaur and McCarthy, 2007). Both cooking at high temperature and excess water can facilitate water permeability and gelatinisation of starch, causing structural conformational changes and making it more susceptible to enzyme attack (Bravo, Siddhuraju and Saura-Calixto, 1998). This is because the water molecules link to the exposed hydroxyl groups of amylose and amylopectin through hydrogen bonds, leading to increased accessibility of hydrolytic enzymes to the glycosidic bonds as well as increased granular solubility and swelling. These effects were reported previously in gelatinised legumes when compared to freshly cooked ones (Bravo, Siddhuraju and Saura-Calixto, 1998). In this process, it is important to consider the distribution and number of pores found on the surface area of starch granules, which, as mentioned above, will facilitate the penetration of water into the core of the granules and thus affect their swelling and gelatinisation (Mishra, Hardacre and Monro, 2012). Another factor affecting gelatinisation is high amylose content of starch granules, as such starches tend to not gelatinise fully until exceeding 100°C—a behaviour that has been reported in *r* mutant peas (which will be covered later in the introduction) (Bogracheva *et al.*, 1999; Debet and Gidley, 2006; Tahir *et al.*, 2011). Such starches with high melting temperature would be less susceptible to amylase reactions (Bogracheva *et al.*, 1999; Tahir *et al.*, 2011). Moreover, availability of enough water is a parameter affecting gelatinisation. In case of insufficient water, the plant CW will not be able to gelatinise in response to heating or cooking, and starches will stay entrapped within the food matrix

without much room to swell and expand, which will prevent crystalline starch granules to be disrupted and this will lower starch digestibility. This phenomenon can be best seen in legume crops. If the CW remains intact in response to heating and the densely packed starches do not have enough space or water to swell and gelatinise, their digestibility will be affected (Tovar *et al.*, 1991; Tovar, Granfeldt and Björck, 1992; Edwards, Warren, *et al.*, 2015).

1.5.4.5.2 Retrogradation

After gelatinisation and cooling of the cooked starch, retrogradation of the starch chains is expected to occur and is known to affect starch digestibility. While amylopectin crystallises and aggregates at later stages during refrigerated storage, amylose aggregation and crystallisation is completed within the first few hours after cooking. The natural structures of amylose and amylopectin play an essential role in this process; the branched chain amylopectin delays re-crystallisation, whereas the linear chain amylose facilitates the cross-linkages through hydrogen bonds, thereby limiting enzymatic actions (Singh, McCarthy and Singh, 2006; Singh *et al.*, 2008). This explains the lower digestibility of high-amylose starchy foods, as they tend to form a firm gel in response to cooking and cooling and high amounts of retrograded starches subsequently (Hamaker, Zhang and Venkatachalam, 2007). Meanwhile, retrograded amylopectin from potato and pasta tend to occur at refrigerated temperatures, leading to a reduction in their digestibility but to a lesser extent (Frei, Siddhuraju and Becker, 2003; Hu *et al.*, 2004). This phenomenon was reported previously in cooked and cooled potatoes kept at refrigerated temperatures for up to two days (Mishra, Hardacre and Monroe, 2012).

1.5.4.6 Starch structural modifications and starch complexes

Any chemical modification that results in structural changes of starch granules (e.g. cross-linking or addition of substituted groups, depolymerisation by acid or enzymes, altering gelatinisation or retrogradation properties) can delay starch digestion and absorption and induce changes to its rheological properties (Sitohy and Ramadan, 2001; Mishra, Hardacre and Monro, 2012). For instance, the interaction of amylose with lipid to form the amylose–lipid complex further hinders the susceptibility of digestive enzymes by limiting water uptake and the swelling capacity (Sitohy and Ramadan, 2001). It was confirmed that any hydrophobic complex could bond with the amylose helix through van der Waals forces with the adjacent C-hydrogen of amylose (Godet *et al.*, 1993; Zhou *et al.*, 2007). Indeed, it was reported that around 60% of amylose was hydrolysed into glucose in one hour, reaching up to 90% after six hours. Alternatively, when a source of lipids was added, the hydrolysis of amylose decreased by 35% (Crowe, Seligman and Copeland, 2000). The formation of amylose–lipid complexes can occur rapidly under reasonable physiological conditions and contributes to the formation of RS, resulting in behavioural changes in the starch and leading to decreased solubility, increased gelatinisation temperature, and delayed retrogradation and resistance against digestion (Crowe, Seligman and Copeland, 2000). However, under the same physiological conditions, the addition of lipids was not found to affect amylopectin hydrolysis (Crowe, Seligman and Copeland, 2000). The rate of amylose–lipid complex structure digestion has been found to be higher than that of retrograded amylose starches but less than amylose alone (Holm *et al.*, 1983).

1.5.4.7 Food structure, processing, and starch digestion

The concept of food processing has emerged as a protective step to remove harmful bacteria from food substances or to make them suitable for use and consumption. In general, starch is consumed after processing to give it a finer texture and/or longer shelf life. Several mechanical processing methods can alter the structural integrity of the plant CW, which could later affect the digestion rate and extent. In plants, these structural changes can affect starch granule morphology, CWs, and the barrier created by proteins and lipids (Mishra, Hardacre and Monro, 2012). The resultant secondary by-product will acquire new features and characteristics that will determine the food's digestion profile, which will differ from the original food. The effect of physical processing on legumes has been examined, revealing that starches are well protected from the α -amylase unless they have been physically altered (Golay *et al.*, 1986; Berg *et al.*, 2012). This can explain the different metabolic responses after the consumption of the same food in intact or processed forms (Mourao *et al.*, 2007), which may subsequently reduce the period of satiety (Ellis *et al.*, 2004; Mourao *et al.*, 2007; Zhu, Hsu and Hollis, 2013).

Many types of physical processing, including chopping, milling, and dehulling, can be applied in both industrial and domestic environments. Each type of processing can result in different types of damage (Ziegler, Greer and Pomeranz, 1971). Dehulling, for instance, results in removal of the hull material that limits water uptake and enzyme penetration, leading to a rapidly digestible starchy food (Donald, 2004; Mishra, Hardacre and Monro, 2012). Moreover, milling, in which shearing forces are applied, breaks down both the seed coat and the endosperm, leading to decreased particle size and increased surface area, which in turn decreases resistant starches and rapidly increases digestible starches (Hallfrisch, Facn and Behall, 2000; Anguita *et al.*, 2006). Such processing can also lead to an increased number of pores on the surface area, depending on the nature of the food, which can facilitate digestive enzyme penetration (Mishra, Hardacre and Monro, 2012). On the other hand, cutting or chopping results in less tissue damage (Mishra, Hardacre and Monro, 2012), and the intracellular content can still be encapsulated and thus serve as a barrier against enzyme penetration (Mandalari *et al.*, 2008). This has been demonstrated in the case of almond seeds, where thick CWs acted as a barrier against GI digestion for up to 12

hours post ingestion (Mandalari *et al.*, 2008; Palafox-Carlos, Ayala-Zavala and González-Aguilar, 2011).

The loss of polyphenols, phytic acid, and tannins is one of the factors contributing to decreased starch digestibility, as these substances are naturally available and normally inhibit the activity of α -amylase. This phenomenon can occur during soaking, germination, and/or dehulling. The elimination of phytic acid and tannins creates a large space within the food matrix, increasing the susceptibility to enzymatic attack and thus increasing digestibility (Rehman and Shah, 2005). Other kinds of processing, such as roasting, flaking, and popping, have also been reported to increase starch digestibility and the consequent glycaemic index (Capriles *et al.*, 2008). An increase in the sheeting cycle of pasta dough has been also shown to increase starch digestibility in vitro (Kim *et al.*, 2008). The structural change of the compact pasta structure due to pulling proteins away from the starch granules reduces the cohesiveness of the dough and allows for the penetration of α -amylase, leading to greater digestibility (Petitot, Abecassis and Micard, 2009).

1.6 The interplay between food structure, starch fermentation and appetite regulation

So far, the introduction has focused on the different structural factors influencing starch digestion of plant foods at the cellular and molecular levels as well as the alteration resulting from processing. The impact of these factors on digestion results in more encapsulated, complex, and resistant starches to the ileum, caecum, and ascending colon, where fermentation of these substrates occurs. This physiological process refers to the anaerobic energy yielding of unmetabolised molecules, where organic molecules are reduced to provide energy to microorganisms, promote growth and cell turnover, and allow the rescue of up to 10% of the daily energy requirement (McNeil, 1984; Frayn, 2010). It mainly happens in the lower GI tract due to the limited oxygen supply and the availability of undigested food remnants (Macfarlane and Macfarlane, 2003)

Among other products, the process of fermentation generates short chain fatty acids (SCFAs), which can be absorbed by colonic cells, used as an energy source by the microbiota, transported to the liver for further metabolism, or eliminated from the body in faeces. A direct proportional relationship was found between the number of non-digestible carbohydrates (NDCs) consumed and the amount of SCFAs produced (Levrat, Rémésy and Demigné, 1991; Berggren *et al.*, 1993), with the highest SCFA proportion found following high plant-based food consumption (e.g. kidney beans and oats) (McBurney *et al.*, 1988). However, other factors were also found to affect the ratio, rate, and extent of SCFAs, including the gut transient rate, substrate availability, and the functional activity and diversity of the microbiome (Wisker, Maltz and Feldheim, 1988; Macfarlane and Macfarlane, 2003; Brinkworth *et al.*, 2009; Murphy *et al.*, 2010; Schwiertz *et al.*, 2010; Peng *et al.*, 2013).

The human gut microbiota accounts for >1kg of an adult's body weight and is composed of 10^{13} – 10^{14} microorganisms (Neish, 2009; Bäckhed, 2012). The density of the microbiome generally increases as we travel along the gut, with the lowest concentration of around 10^5 microbes per ml found in the stomach and the highest concentration found in the colon (up to 10^{12} microbes per ml) (Mowat and Agace, 2014). While the composition of the gut microbiota varies between individuals, it was confirmed that a common 'core microbiome' profile exists among humans (Turnbaugh and Gordon, 2009). Variations were shown to be related to genetic background, diet

composition, age, and antibiotic use (Hasan and Yang, 2019). More than 90% of all bacteria phylotypes belong to Bacteroidetes (gram-negative bacteria) and Firmicutes (gram-positive bacteria), two of the seventy known phyla (Turnbaugh *et al.*, 2007).

A growing body of evidence also suggests the beneficial role of colonic fermentation and SCFA in regulating appetite and glucose and energy homeostasis as will be discussed under sections 1.6.2 and 6.1.2.

1.6.1 SCFAs

1.6.1.1 SCFAs production

SCFAs are carboxylic acids with a straight or branched chain configuration that contain 1–6 carbon atoms. More than 95% of the SCFAs produced from NDC are acetate, propionate, and butyrate, whereas other SCFAs (formate, valerate, and caproate) are produced in smaller concentrations (Cummings *et al.*, 1987). It was demonstrated that SCFA production is profoundly affected by NDC intake (Cummings *et al.*, 1987; Jenkins *et al.*, 1998). Moreover, the current Westernised diet, which contains a higher ratio of de-structured foods, processed, and simple carbohydrates, can yield approximately 600 mmol of SCFAs per day (McNeil, 1984; Bergman, 1990), with an approximate molar ratio of the principal SCFAs of 60:20:20 for acetate, propionate, and butyrate (Cummings *et al.*, 1979; Bergman, 1990; Topping and Clifton, 2001). The breakdown of NDCs into their simpler sugars (pentoses and hexoses) occurs in preparation for their fermentation. The degrading effect of NDC to SCFAs by gut microbiota occurs through two distinct pathways: pentoses containing substrates are metabolised through the pentose phosphate pathway, while hexose substrates follow the Embden–Myerhoff–Parnas glycolytic pathway or can be converted to 6-phosphate-gluconate before their breakdown through the pentose phosphate pathway. As a result of both of these pathways, pyruvate is generated, which is an essential intra-cellular metabolite for producing SCFAs through a series of further reactions. Acetate is formed by oxidative decarboxylation of pyruvate, and butyrate is produced by acetoacetate reduction. Moreover, pyruvate is converted to propionate via three known pathways: the succinate decarboxylation pathway, in which succinate results from the fixation of CO₂ to pyruvate, leading to the decarboxylation of succinate to generate propionate; the acrylate

cycle, in which propionate is formed from acrylate (with lactate being the precursor); and the propanediol pathway (Cummings, 1981; Wong *et al.*, 2006; Chmelarova, 2007; Al-Lahham, Peppelenbosch, *et al.*, 2010). High interconversion was shown to happen from acetate to butyrate, while there was minimal interconversion from acetate or butyrate to propionate (den Besten, Lange, *et al.*, 2013).

1.6.1.2 SCFAs absorption and transport

The absorption of SCFAs into the intestinal epithelium occurs through the apical and basolateral membranes of the colonic epithelium via two proposed mechanisms: passive diffusion of protonated SCFA (the acid form) and, the major pathway, active transport of anionic SCFAs mediated by a number of different transporters (den Besten, van Eunen, *et al.*, 2013). There are three mechanisms for actively transporting SCFAs anions: anion exchange, where SCFA anions are coupled to HCO_3^- and absorption occurs through an unidentified transporter; transporting SCFA in an H^+ -dependent electroneutral manner through monocarboxylate transporters (MCT) 1 and 4; and electrogenic coupling of SCFA anions to Na^+ and stimulation of Cl^- and water absorption through sodium-dependent monocarboxylate transporters (SMCT) 1 and 2. While MCT1 is expressed both in the apical and basolateral membranes, MCT4 is expressed in the basolateral membrane, and both SMCT 1 and 2 are expressed in the apical membrane; MCT4 is specifically expressed in the basolateral membrane (Sivaprakasam *et al.*, 2018).

Limited is known about the transporters required for SCFA uptake from the peripheral circulation into tissues. However, it was reported that organic anion transporter 7 (OAT7), expressed in the liver, and OAT2, expressed in the kidney and liver, are transporters for butyrate and propionate, respectively, across the sinusoidal membrane of hepatocytes (Shin *et al.*, 2007; Islam *et al.*, 2008). It was also reported that transportation of SCFAs to the portal vein and liver occurs through the mesenteric and inferior mesenteric veins (Bloemen *et al.*, 2009, 2010; Bailey *et al.*, 2012). Additionally, SCFAs have also been found to pass the portal vein and liver through the pelvic plexus into the inferior vena cava and the systemic circulation (Bailey *et al.*, 2012).

1.6.1.3 SCFAs metabolism

While SCFAs pass through the portal vein to the peripheral circulation, it has been proposed that the approximate molar ratio of acetate, propionate, and butyrate changes from 60:20:20 in the GI lumen to 70:20:10 in the portal vein due to the preferential use of butyrate by colonocytes (Cummings *et al.*, 1987; Bloemen *et al.*, 2009), and to 90:5:5 in the peripheral circulation (Cummings *et al.*, 1987). These changes in the ratio indicate liver extraction of propionate and, to a lesser extent, of butyrate. Indeed, it was estimated previously that more than 90% of propionate is metabolised by the liver (Cummings *et al.*, 1987), suggesting that propionate is the major SCFA affecting hepatic structure and function. Alternatively, acetate is not significantly taken up; rather, it is mostly excreted by the liver (Bloemen *et al.*, 2009), and thus it is the main SCFA found in the peripheral circulation, as previously mentioned. This suggests that acetate is the major SCFA affecting physiological function of the peripheral tissues, such as adipose tissues and skeletal muscle. The estimated concentration of total SCFAs in the proximal colonic lumen is between 70 and 130 mmol/l (Cummings *et al.*, 1979; Mortensen and Clausen, 1996). Notably, the available SCFA concentrations measured in the human portal and hepatic veins are from surgical and sudden death patients due to the invasive applicability in sampling these sites (Bloemen *et al.*, 2009, 2010). A summary of SCFA concentrations in the portal and hepatic veins and the periphery are highlighted in Table 1.2 below (Cummings *et al.*, 1987; Bloemen *et al.*, 2009, 2010).

Of the total amount of SCFA produced in the colon, <10% is excreted into the faeces (Boets *et al.*, 2015). However, weight plays a role in that proportion, as overweight and obese individuals have higher total faecal SCFA concentrations than lean individuals, with a significant proportion of propionate (Schwiertz *et al.*, 2010), suggesting more SCFA production due to more microbial energy harvesting in obesity (Tremaroli and Bäckhed, 2012). However, this variation in SCFA production between lean and obese individuals is debatable, and some research has suggested that no differences exist (Bloemen *et al.*, 2009). Importantly, faecal SCFAs may not serve as a good indicator of colonic SCFA production and absorption, as faecal acetate was inversely related to SCFA absorption (Vogt *et al.*, 2006).

Table 1.2: Previously reported SCFA concentrations in portal, hepatic and peripheral circulation, μM .

	Portal	Hepatic	Peripheral
Total SCFA	375	148	79
Acetate	177.5-262.8	115-219.5	70
Propionate	15.6-88	5.6-21	5
Butyrate	19.5-30.1	4.8-12.0	4

Adapted from (Cummings et al., 1987; Bloemen et al., 2009, 2010)

1.6.1.4 SCFAs receptors

In 2003, it was discovered that SCFAs are ligands for FFAR 2 and 3 (previously known as orphaned GPR41 and GPR43, respectively) (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). Acetate and propionate were found to be the most potent activators of FFAR2, while FFAR3 has an affinity order of propionate > butyrate $\geq$ acetate (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003).

GPCR receptors are attached to heterotrimeric G-proteins, which are attached to the cytoplasmic side of the receptors. These receptors are activated by an external signal that stimulates the individual G-protein, leading to the activation of specific secondary messenger cascades to drive different cellular responses. G-protein signalling differs among these receptors. FFAR2 (GPCR 43) can signal via both the pertussis toxin-sensitive $G_{i/o}$ and the pertussis toxin-insensitive $G_{q/11}$ pathways, while FFAR3 (GPCR 41) can signal only via the pertussis toxin-sensitive $G_{i/o}$ pathway (Brown *et al.*, 2003; Le Poul *et al.*, 2003).

Both FFAR2 and FFAR3 receptors are expressed not only in the gut epithelium, where the highest concentration of SCFAs are produced (Brown *et al.*, 2003; Karaki *et al.*, 2008; Nøhr *et al.*, 2013; Li *et al.*, 2014), but also in areas other than the GI tract, such as in adipose tissue (Hong *et al.*, 2005; Kimura *et al.*, 2013), skeletal muscle (Nilsson *et al.*, 2003), the pancreas, and immune cells (Brown *et al.*, 2003). The widespread distribution of these receptors suggests that SCFAs likely play beneficial systematic roles.

1.6.2 SCFAs, appetite regulation, and energy homeostasis

1.6.2.1 The role of SCFAs in gut–brain signalling

It was first suggested that SCFAs play a significant role in appetite regulation as signalling molecules in the gut–brain neural circuits, with broad expression of FFAR2 and FFAR3 found in the enteric neurons, such as the vagal nerve (Karaki *et al.*, 2008; Sleeth *et al.*, 2010; Kaji *et al.*, 2011; Kimura *et al.*, 2011; Nøhr *et al.*, 2015). Indeed, elevated colonic propionate production was shown to induce vagal signalling in the gut or portal vein via FFAR3 (De Vadder *et al.*, 2014). Similarly, a decrease in food intake was found following intragastric administration of butyrate in mice, which was blocked after vagotomy (Li *et al.*, 2017). There was a significant increase in energy intake relative to the body weight of FFAR2-KO mice fed a high-fat diet when compared with wild mice fed standard chow (Bjursell *et al.*, 2011; Kimura *et al.*, 2013). In addition, there was a significant increase in energy intake in FFAR3-KO mice fed a high-fat diet when compared with controls (Lin *et al.*, 2012). Together, these results suggest that these receptors are involved in decreasing energy intake.

Nevertheless, it is also suggested that SCFAs have a direct effect on appetite regulation through the brain. It was shown that SCFAs could cross the blood–brain barrier (BBB) via MCT (Vijay and Morris, 2014), and this has led to the suggestion that SCFAs may affect BBB permeability (Braniste *et al.*, 2014). Specifically, acetate was found to be an energy source for astrocytes (Muir, Berl and Clarke, 1986) and has been detected in cerebrospinal fluid at concentrations of 30–40 μM (Nagashima *et al.*, 2010). Moreover, acetate has been shown to be involved in central appetite regulation in mice (Frost *et al.*, 2014). The latter study investigated the effect of intravenous and colonic infusions of acetate using positron emission tomography (PET)-computed tomography (CT) scanning and confirmed that 3% of acetate crossed the BBB and was taken up by the brain. Subsequently, acetate was shown to activate hypothalamic POMC neurons and reduce AgRP expression in the brain following interperitoneal administration, further suggesting the anorexigenic signalling effect of acetate itself.

1.6.2.2 The effect of SCFAs on anorectic gut hormones

Recent evidence regarding the possible effects of SCFAs in regulating appetite emerged with the discovery that FFAR2 and FFAR3 are coexpressed in GLP-1 and PYY releasing enteroendocrine L-cells (Karaki *et al.*, 2008; Tazoe *et al.*, 2009). The highest density of L-cells was found to be on the gut epithelium (Eissele *et al.*, 1992), where they secrete PYY and GLP-1 following food intake to stimulate fullness (Murphy and Bloom, 2006; Sternini, Anselmi and Rozengurt, 2008). One of the critical factors for sustained weight loss in bariatric surgeries, especially gastric bypass, is elevated postprandial levels of PYY and GLP-1 (le Roux *et al.*, 2007). However, postprandial levels of anorectic hormones are higher in lean people than in those who are obese (Bueter and le Roux, 2011), suggesting that the altered regulation of appetite hormones can affect energy homeostasis. Moreover, falling concentrations of GLP-1 and PYY have been reported for individuals following the hypo-calorific diet, leading to an increased hunger sensation, which might be an essential factor in the failure to manage obesity through diet (Sumithran *et al.*, 2011). It was recently shown that the gut hormones PYY and GLP-1 can stimulate thermogenesis in brown adipose tissue by activating uncoupling protein (UCP). This was followed by secretin secretion, which enhanced satiety through POMC expression and inhibition of AgRP neurons—effects that were absent in UCP^{-/-} (Li *et al.*, 2018).

Nevertheless, all the main SCFAs are known to be produced endogenously from fat oxidation in the liver and carbohydrate fermentation. However, evidence has shown that these amounts are not sufficient to induce the release of gut hormones. Specifically, it was shown that while PYY stimulation occurs at 300 mM of SCFAs, the mean colonic production was 100 mM (Cherbut *et al.*, 1998). Thus, increasing colonic SCFAs levels to stimulate gut hormone release is a highly encouraging method to combat obesity at the population level, which could be achieved through higher intake of SCFA-producing sources, such as NDCs, or through targeted delivery of high amounts of SCFAs to the colon through supplementation.

Several studies have proposed hypotheses to test the effect of stimulating the secretion of GLP-1 and PYY by SCFAs, using FFAR2 and FFAR3 knockout (KO) rodents (Karaki *et al.*, 2006, 2008; Tolhurst *et al.*, 2012). Meanwhile, an in vitro study confirmed the direct effect of SCFAs on the

secretion of GLP-1 from L-cells, and the same effect was abolished when tested on FFAR2^{-/-} mice (Tolhurst *et al.*, 2012). Specifically, while propionate was shown to stimulate the release of GLP-1 and PYY in humans (Chambers *et al.*, 2014) and wild type (WT) primary murine colonic crypt cultures (Psichas *et al.*, 2015), there was a significant reduction in the secretion of both gut hormones in response to propionate in FFAR2 KO mice cultures. Another in vivo study reported a direct proportional increase in both GLP-1 and PYY in response to intra-colonic propionate infusions, while the same effect was abolished in FFAR2^{-/-} mice (Psichas *et al.*, 2015). Moreover, it was recently shown that inulin, an NDC, is associated with an increase in circulating PYY concentrations through a FFAR2-dependent mechanism that stimulates an increase in colonic L-cell density, leading to energy intake reduction and diet-induced obesity prevention (Brooks *et al.*, 2017). All these data support the role of NDC in appetite regulation by the secretion of gut hormones through FFAR2 and FFAR3 of the colonic L-cells as an SCFA-mediated mechanism of action.

1.6.2.3 The effect of SCFAs on energy expenditure

With regard to the other side of the equation, SCFAs were shown to influence energy expenditure and thus to protect against diet-induced obesity. This occurs as a consequence of fat oxidation. For instance, dietary butyrate supplementation was shown to enhance adaptive thermogenesis and fat oxidation in high-fat-diet rodents, promoting energy expenditure and preventing body weight gain (Gao *et al.*, 2009). Another study of healthy overweight and obese individuals demonstrated that distal acetate infusion increased fat oxidation (van der Beek *et al.*, 2016). Moreover, when all three were rectally infused in humans, SCFAs increased fasting fat oxidation, whereas propionate and acetate alone resulted in elevated resting energy expenditure (Canfora *et al.*, 2017).

There is evidence to suggest that FFAR3 activation by SCFAs increases energy expenditure (EE). One of the proposed mechanisms is related to the activation of the sympathetic nervous system. In support of this pathway, propionate was shown to cause an increase in heart rate and oxygen consumption through direct activation of FFAR3 in the sympathetic ganglion in mice (Kimura *et al.*, 2011). Another mechanism proposes the activation of intestinal gluconeogenesis (IGN).

Gluconeogenic propionate has been shown to induce the expression of IGN-related genes through binding to FFAR3 in the periportal afferent system (De Vadder *et al.*, 2014). Despite similar energy intake, it was shown that IGN resulted in improvements in body composition, suggesting these beneficial effects might result from increasing EE. However, the exact mechanism of how IGN elevates EE remains to be elucidated. Moreover, it has been suggested that FFAR2 is another pathway for enhancing EE. Despite a similar physical activity level, obese FFAR2 KO mice on a normal diet exhibited a reduction in EE when compared with WT mice (Kimura *et al.*, 2013). Moreover, FFAR2 adipose-specific over-expression resulted in an increase of EE in mice, which remained lean on a high-fat diet (Kimura *et al.*, 2013).

1.6.2.4 The effect of SCFA on glucose homeostasis and gluconeogenesis

There are direct and indirect effects of SCFAs on improving glucose homeostasis. SCFAs were shown to stimulate the release of GLP-1, which is a known incretin that promotes insulin secretion or sensitises beta cells to glucose-stimulated insulin production. The direct effects of SCFAs on beta cell function were suggested after detecting the expression of FFAR2 and FFAR3 in human pancreatic islets, which are known receptors for SCFAs. A recent theory suggests an improvement in beta cell function in humans in response to increasing colonic propionate production (Pingitore *et al.*, 2017). The same study also suggested the enhancement of glucose-stimulated insulin secretion in human islets in vitro using a methodology that provides a sensitive dynamic profile of insulin secretion called perfusions (Pingitore *et al.*, 2017).

IGN is considered to be a beneficial regulator of glucose and energy homeostasis (Mithieux *et al.*, 2005; Troy *et al.*, 2008). Of the main SCFAs, propionate was shown to be a substrate for IGN, and butyrate was shown to directly activate IGN-related gene expression (De Vadder *et al.*, 2014). A study in mice demonstrated a reduction in hepatic glucose production and improvements in glucose control and body composition in SCFA-supplemented animals. These beneficial effects were absent in intestinal glucose-6-phosphatase KO mice—the catalytic subunit of a key enzyme involved in IGN—despite a similar shift in gut microbiota composition. It was proposed that IGN causes activation of portal glucose sensors, which signal the brain to mediate these beneficial effects (Delaere *et al.*, 2013). Moreover, portal vein infusion of propionate significantly decreased

energy intake in sheep when compared with controls (Anil and Forbes, 1980). This effect vanished following total hepatic innervation or hepatic vagotomy (Anil and Forbes, 1988), suggesting the ability of hepatic glucose production to be sensed centrally, consequently altering feeding intake and behaviour. These data suggest the vital role of IGN in mediating a number of the beneficial effects reported following the consumption of NDCs.

1.6.2.5 Other impacts of SCFAs relevant to energy homeostasis

Acetate, together with the other two principal SCFAs, has been shown to down-regulate PPAR- γ activity and increase mitochondrial UCP-2 expression while raising the AMP/ATP ratio, thereby promoting a switch to lipid oxidation in the liver instead of lipid synthesis (Den Besten *et al.*, 2015). Positive effects of SCFAs have also been proposed on brown adipose tissue activity, where acetate was shown to stimulate mitochondrial biogenesis via the up-regulation of UCP-1 expression in brown adipocytes and PPAR- γ (Hu *et al.*, 2016). In addition, acetate was shown to increase the expression of genes related to lipolysis and reduce lipid accumulation in brown adipose tissue (BAT) (Yamashita *et al.*, 2009) as well as to increase the 'browning' effect of white adipose tissue, increasing its thermogenic capacity (Sahuri-Arisoylu *et al.*, 2016). Furthermore, administration of butyrate has also been shown to increase the expression of thermogenesis-related genes in BAT and to increase adaptive thermogenesis (Gao *et al.*, 2009).

1.6.2.6 Beneficial effects associated with SCFA independent of food intake

Faecal transplantation studies have proposed an association between energy metabolism effects and SCFAs independent of food intake. This was based on transplantation of the faecal microbiota of twins discordant for obesity to germ-free mice, which led to the development of a similar phenotype in the recipient lean mice, which had significantly increased caecal butyrate and propionate concentrations compared with their obese counterparts (Ridaura *et al.*, 2013). Evidence is still limited to elucidate the contribution of SCFA production on inhibiting the fat accumulation associated with obesity. Similarly, the microbiota faecal transplantation of mice undergoing Roux-en-Y gastric bypass (RYGB) surgery into germ-free mice resulted in replicating the phenotype, leading to reduced fat mass and weight loss in the recipient mice (Liou *et al.*,

2013). Interestingly, the RYGB-recipient mice had lower acetate production and higher propionate production compared with germ-free mice, which received microbiota from sham-treated mice. These data suggest an active role of colonic SCFA production in beneficial host health.

1.7 Dietary tools to increase SCFAs levels in the human lower GI tract

The literature has supported the primary role of RS and dietary fibre consumption and their fermentation by-products, SCFAs, on appetite regulation and energy homeostasis. However, most of these reports have been based on animal studies. One of the most substantial issues preventing using humans as the model is the intolerability of large amounts of NDCs, leading to the small amount used in comparison with animal studies. There is an essential need to conclude from human findings. However, as the consumption of NDCs in general has remained below the recommended level in Western societies, as the UK, where the averaged habitual NDCs intake (in the form of fibre) of an adult is around 15g/day, which can result in lower SCFAs production. Thus, manipulating the RS content of the diet to deliver more undigested starch to the microbiota to enhance SCFA production and targeting the delivery of SCFAs to the colon are attractive areas of research.

1.7.1 Alteration of starch structure to increase resistant starch content and influence digestion profile

Substituting refined processed foods that have lost their structure with whole structured ones while maintaining palatability to attract consumers is challenging. At the same time, the increasing reliance on processed foods is positively related to increasing obesity rates. Identifying dietary tools that will increase the quality of carbohydrates of commonly consumed foods to deliver more substrate for microbiota fermentation is an attractive strategy for providing small but positive energy intake on daily basis, which would help to address overweight and obesity through long-term consumption at the population level.

Peas (*Pisum sativum*) were selected as a 'food model' for studying starch digestibility and glucose release due to their identified mutational backgrounds as well as their widespread consumption in the UK (Food and Agriculture Organization of the United Nations., 2012). In the UK, pea

consumption is limited to whole, boiled, mature or immature seeds. They are principally eaten as a side dish or mixed with the main course meal. An effective idea to expand peas consumption is to incorporate peas into foods in different forms using pea flour. In order to maintain the functional properties of the peas that are incorporated into different foods, we need to understand the key factors affecting their digestibility.

Peas are an edible, non-oilseed, leguminous crop similar to lentils, chickpeas, and beans. They all belong to the Leguminosae family and have green dicotyledons that are dry and smooth (Hoover and Sosulski, 1991; Li *et al.*, 2014). During embryo development, the cotyledons absorb the food reserves from the endosperm, which is mostly degraded in the mature seed. Pea seeds are classified as non-endospermic seeds, in which the dicotyledons serve as sole food storage organs. Both the cotyledons and the embryo are enclosed by the seed coat testa (Marinos, 1970; Hardham, 1976). *Pisum sativum* is a naturally self-pollinating species that requires little heat and water to produce healthy homozygous specimens 5 mm in size with a round shape and smooth surface (Hedley, Bogracheva and Wang, 2002).

1.7.1.1 The interplay between pea seed natural mutation, starch structure, and digestion

It has been over 150 years since Gregor Mendel established the law of inheritance, through which the fundamental principles of genetics were established by careful studies using pea as a substrate. Today, the molecular basis for the differences in most of the traits studied and observed by Mendel in peas is identified and well understood. As part of plant hybridisation studies, Mendel worked with seven characteristics of pea plants: plant height, flower position and colour, pod shape and colour, and seed coat shape and colour. Some of the resulting peas with new phenotypes have been important for human health and nutrition (Bhattacharyya *et al.*, 1990; Ellis *et al.*, 2011).

Of these, a mutation in the gene coding for pea seed coat shape, known as the *r* genetic locus, results in pleiotropic phenotypes, including a wrinkled rather than the wild round shape. It has been confirmed that this mutation was caused by a transposon-like insertion in a gene encoding the starch-branching enzyme (SBE) isoform (Bhattacharyya *et al.*, 1990; Ellis *et al.*, 2011). The enzyme SBE is one of the major enzymes responsible for elongating and generating branches of

starch polymers in peas but and other plant crops. SBE acts by hydrolysing α -1,4-glycosidic linkages in their place and forms α -1,6-bonds to glucose polymers, thus forming branches of amylopectin. The resulting mutant pea seeds have a higher proportion of amylose to amylopectin (around 70% amylose), which is more than double in wild pea seeds. This change in starch structure will vastly affect starch behaviour during digestion. The linear starch structure of the higher amylose SBE-deficient peas results in a crystalline matrix with limited room for granules to expand and swell in response to soaking as well as a higher gelatinisation temperature in response to hydrothermal processing (Ridout *et al.*, 2003). This has resulted in lower water uptake compared to the wild pea seeds, and thus is known for its negative osmotic potential (Wang *et al.*, 1987). The limited solubilisation of the crystalline starches with higher amylose ratios makes it difficult for the enzyme to function as usual to allow amylose leaching (Tahir *et al.*, 2011).

As starch is found in the cotyledon cells and the seed coat of pea seeds, having different ratios of amylose to amylopectin in response to SBE mutation can result in changes in outer shape, explaining the acquired wrinkled shape of *rr* pea seeds (Dahl, Foster and Tyler, 2012). A significant proportion of the seed coat (also known as the hull) consists of the non-starch polysaccharides cellulose, whereas cellulose, hemicellulose, and pectin are all major components of cotyledon cells (Reichert and MacKenzie, 1982; Dahl, Foster and Tyler, 2012). In addition, the resulting mutant peas have also been found to have different starch granule morphology due to the differences in starch composition (i.e. higher amylose-to-amylopectin ratio) (Wang and Hedley, 1993; Ratnayake, Hoover and Warkentin, 2002). In addition, the SBE mutation in peas also results in higher sucrose content, which, in excess, is used by *r* pea seeds to synthesise raffinose oligosaccharides, the digestion of which does not occur in the human GI tract because it lacks the α -galactosidase enzyme responsible for their hydrolysis (French, 1954; Lloyd, Wang and Hedley, 1996).

It is worth noting that the effect of such mutations has been studied in plant crops other than pea seeds, such as maize, wheat, and barley, and the consumption of high-amylose starches in these mutants was reported to decrease postprandial glucose levels and increase microbiota fermentation (Garwood, 1973; Regina *et al.*, 2006; Keogh *et al.*, 2007). Moreover, the effect of

different pea genotypes, including the higher amylose content, on starch digestibility was studied in vitro. The results of these studies showed higher RS content characterised by fine structural differences in response to the amylose-to-amylopectin ratio of the starch granules as well as a higher degree of gelatinisation with extensive retro-gradation after cooking, which reduced digestion rates in vitro (Skrabanja *et al.*, 1999; Ridout *et al.*, 2003; Parker, Kirby and Morris, 2008; Edwards *et al.*, 2018). SBE-deficient peas can, therefore, result in significant metabolic outcomes, given the possible delivery of a higher amount of RS to the lower GI tract. However, these metabolic effects are not known and have not been studied in humans. Taken together, and as discussed earlier, these new characteristics of these pea seeds with a mutation in the *r* locus can significantly affect the resistance behaviour of starch during digestion and consequent nutrient bioaccessibility. It can be concluded that these structure-related factors of starch can serve as tools to influence digestibility through the breeding and selection of non-digestible characteristics that will behave as RS in the gut. However, the impact of the interplay between the food structure of starch and regulation of energy homeostasis and appetite regulation remains unclear.

1.7.2 Inulin-propionate esters

SCFA production rates following NDC fermentation vary depending on molecular weight, suggesting that some molecular weights may be more favourable for SCFA production. Moreover, the binding affinity between FFAR and SCFA appears to be adapted for the relatively high SCFA concentrations found in the colonic environment. Thus, alternative ways of optimising colonic SCFA production are needed. Orally consuming purely SCFAs instead of large quantities of NDCs is challenging. This is due to the short circulating half-life and the fast absorption of SCFAs in the proximal intestine before even reaching the assigned place of action in the lower GI tract to target large intestinal FFAR2, as happens after gut fermentation of NDC, as well as SCFAs poor organoleptic properties (Berggren *et al.*, 1996; Frost *et al.*, 2003). For instance, propionate has been suggested to induce satiety after its incorporation into different food products for oral intake in humans (Liljeberg, Lönner and Björck, 1995; Liljeberg and Björck, 1996; Ruijschop, Boelrijk and te Giffel, 2008) through delayed gastric emptying (Liljeberg and Björck, 1996). Other

mechanisms have been proposed subsequently, by which propionate can cause satiety not only based on gastric emptying but also due to its effect on increased plasma GLP-1 release and lowered postprandial glucose levels (Frost *et al.*, 2003). However, evidence has confirmed the unpleasant taste of propionate together with the high supplementation level of propionate can induce nausea, which can be a confounding factor in reducing energy intake.

As an alternative way to ingest highly propiogenic carbohydrates, the direct delivery of propionate to the colon is being investigated by our research group. Of the main SCFAs, propionate has the highest affinity for FFAR2 (Layden *et al.*, 2013). It is a bacterial fermentation end-product of NDCs that will not undergo further conversion to other SCFAs, unlike the exchangeable acetate-butyrate (Al-Lahham, Roelofsen, *et al.*, 2010). In humans, dietary supplementation of propionate in the form of an IPE, a novel way to increase colonic propionate enrichment (as will be discussed later in Chapter 6), has significantly increased postprandial PYY and GLP-1 levels and reduced ad libitum energy intake acutely in the intervention group when compared to controls (Chambers *et al.*, 2014). Over the long term, the elevation of colonic propionate has protected against weight gain in overweight adults. However, these long-term effects resulted independently from the gut hormones, the levels of which did not change significantly. This study suggests that the long-term beneficial effects of elevated colonic propionate are not achieved through gut hormones stimulation. The exact mechanism remains unclear.

A novel system has been developed by Dr. Douglas Morrison, Scottish Universities Environmental Research Centre (SUERC). An inulin (β -2,1-linked polymer of fructose) carrier with a propionate ester was demonstrated to reliably and reproducibly increase colonic propionate (Chambers *et al.*, 2015). Inulin was used as the prodrug delivery system for the SCFA to the colon based on the fact that inulin is water soluble and its gastrointestinal handling had been well characterised. Nevertheless, to optimise colonic propionate delivery in humans, the degree of inulin–propionate esterification was studied. IPE with a degree of esterification of 0.8 (this equates to a propionate loading of 27 of weight%) was shown to be the variant with the highest degree of

esterification that remains water soluble through the downstream processing (Polyviou *et al.*, 2016). Chapter 6 will discuss the use of this supplement further in the field of appetite regulation.

1.8 Research summary

This thesis aimed to explain the effect of food structure on the starch digestion profile and the major effects of lower GI fermentation and the by-products generated on energy homeostasis and appetite regulation, using wild and *rr* pea seeds. In addition, to increase consumer consumption while expanding the range of products containing RS, the same experiments were performed using wild and *rr* pea flours. In addition, as our research group has suggested from recent human studies that propionate may be an essential SCFA contributing to appetite regulation (Chambers *et al.*, 2014; Byrne *et al.*, 2016), we were interested to investigate whether delivering a higher amount of that SCFA to the colon could regulate appetite while following a weight loss diet. This project will provide an opportunity to assess the beneficial physiological effects of decreasing the energy density of foods by decreasing their bio-accessibility and digestibility profiles so that they are subject to fermentation. This information can then be used to tackle nutrition- and metabolic-related diseases worldwide.

1.9 Research hypotheses

The following hypotheses were made:

- The structural characteristics of *rr* pea seeds and flour can reduce starch digestibility and regulate energy homeostasis, leading to higher fermentation and SCFA production when compared to that of *RR* pea seeds and flour.
- Promoting colonic propionate enrichment could regulate appetite in humans.

1.10 Research aims and objectives

The aims of this project were as follows:

- To investigate the digestion and fermentation profiles of *rr* pea seeds and flour during their passage through the GI tract and the effect on energy homeostasis and appetite regulation through:
 - Measuring gastric emptying and acute glycaemia.
 - Assessing the extent of starch digestion in the small intestine and its fermentation in the lower GI tract.
- To understand the impact of the SCFA propionate on long-term appetite regulation.

Chapter 2

2. General Methods

2.1 Introduction

The work presented in this chapter is based on methods that are commonly used throughout the thesis. The methods that relate to a single chapter are reported in the relevant chapter.

2.2 Overview

The work in this thesis consists of four investigations. The investigations included in Chapters 3, 4, and 5 are all acute, randomised, controlled, double-blind, cross-over trials involving four study visits. Cross-over trials were used to reduce inter-person variability and to allow a smaller sample size. The investigation included in Chapter 3 seeks to determine whether different pea interventions have an effect on gastric emptying. The investigation included in Chapter 4 aims to understand the digestibility of different starch structures related to glucose homeostasis. The investigation included in Chapter 5 examines the influence of starch delivery on the lower gut. Finally, the investigation included in Chapter 6 is a long-term, randomised, controlled, paralleled, double-blind trial examining the effect of propionate ester supplementation on appetite while following a weight loss plan.

2.3 Food materials

The food materials used during the pea experiments are listed below:

1. *RR* pea seeds (BC 1/19 *RR* line), used as a control group, which will be referred to as wild whole pea seeds
2. *rr* pea seeds (BC 1/19 *rr* line), used as the treatment group, which will be referred to as SBE-deficient whole pea seeds
3. *RR* pea seed flour (BC 1/19 *RR* line), used as a control group, which will be referred to as wild pea flour
4. *rr* pea flour (BC 1/19 *rr* Line), used as the treatment group, which will be referred to as SBE-deficient pea flour

The near-isogenic lines of pea (BC1/19RR, BC1/19rr) are available from the John Innes Centre Germplasm Resources Unit, an independent, international centre of excellence in plant science,

research, genetics, and microbiology, as accessions (J13316 = RR, round-seeded, J13317 = rr, wrinkled-seeded; <https://www.seedstor.ac.uk>). Bulk seed stocks were generated by growing plants on wire in field plots over successive seasons (March – July). The resulting seed stocks were used for the in vivo studies and supplied to Campden BRI to produce the pea derived products (whole pea seeds and pea flour) and to the University of Glasgow for ^{13}C labelling by Professor Tom Preston to produce ^{13}C incorporated pea seeds. Nutritional analyses of pea seeds from different genotypes were performed by the Eurofins testing laboratory group. Table 2.1 below presents the nutritional composition of whole pea seeds from both lines, with the results reported as g/50 g. Flour was made from the exact same amount and type of whole pea seeds, so their nutritional compositions are estimated to be the same. Pea seeds were milled into a flour using an electric coffee grinder (Krupps, Berkshire, UK), and were passed through a 1mm test sieve (Cole-Palmer, St. Neots, UK). All chemicals, reagents and enzymes were supplied by Sigma Aldrich (Dorset, UK) unless stated otherwise. Approximately 5 g dried pea seeds were weighed and soaked overnight in 100 mL ultrapure water at room temperature. After soaking, the water content of the peas was approximately 60 % for the *rr* peas and 45 % for the RR peas.

Table 2.1 Nutrition composition of different pea lines used in experimental chapters 3, 4 and 5

Whole pea or flour lines	Wild	SBE-deficient
Carbohydrate^b (g/100g dry weight of peas)	32.3	28.0
Of which total sugars (g/100g dry weight of peas)	3.2	5.5
Galactose (g/100g dry weight of peas)	0.1	0.3
Fructose (g/100g dry weight of peas)	<0.1	<0.1
Maltose (g/100g dry weight of peas)	<0.1	<0.1
Lactose (g/100g dry weight of peas)	0.8	1.2
Sucrose (g/100g dry weight of peas)	2.3	4.0
Fibre^a (g/100g dry weight of peas)	36.0	36.3
Protein (g/100g dry weight of peas)	21.3	23.8
Fat (g/100g dry weight of peas)	<1.0	1.7
Moisture (g/100g dry weight of peas)	7.7	7.2
Energy value (Kcal/100g dry weight of peas)	286	295

^a total dietary fibre as defined by the AOAC

^b detailed carbohydrate analysis is not available

2.4 Sample collection, processing and analysis

2.4.1 Breath hydrogen excretion levels

One of the end-products produced by the intestinal microbiota fermentation of NDC is hydrogen gas (Levitt, 1969). The breath hydrogen test was used as a marker of bacterial fermentation activity, which occurs upon the arrival of NDCs to the lower GI tract (Byrne *et al.*, 2018). This assessment involved a portable hand-held breath hydrogen meter (Gastrolyzer, Bedfont Scientific Ltd. Kent, UK), and the hydrogen was measured in parts per million (ppm). The meters were calibrated prior to use with the calibration gas (200 ppm hydrogen). Volunteers were instructed to inhale as deeply as possible, hold their breath for 15 seconds, and exhale through a mouthpiece attached to the hydrogen meter. The first sustained peak level of the breath H₂ expired was then recorded (henceforth referred to as breath H₂ excretion time).

During each study visit of the investigations included in Chapters 3 and 5, breath hydrogen concentrations were taken in duplicate (at -10 and 0 minutes) prior to breakfast and every 60 minutes postprandial until the end of the study day.

2.4.2 Subjective appetite scales: visual analogue scores

Appetite is defined as the sum of all processes that influence eating. Hunger is a conscious feeling and a physiological drive to eat that is followed by physical sensations in different body parts. Fullness is a conscious feeling of repletion, mainly sensed in the stomach. Satiation is the process of signalling from the stomach and upper intestine, taste, and reward to cause meal termination, which affects meal size. Satiety is the postprandial inhibition of eating and hunger (Blundell and Halford, 1994).

Previous studies have shown the reliability, validity, and reproducibility of visual analogue scales (VAS) as an appetite measure in terms of predicting feeding behaviour and its sensitivity to experimental manipulation (Flint *et al.*, 2000; Stubbs *et al.*, 2000). VAS was used in the current investigations to assess subjective appetite sensation during each study visit and during constant time intervals. With extremes of 'not at all' and 'extremely' at either end of the scale, the VAS questionnaire assessed hunger, fullness, nausea, desire to eat, and appetite for a prospective

meal using questions such as 'how hungry do you feel' and 'how full do you feel' (Flint *et al.*, 2000). Volunteers were asked to draw a small vertical line on each of the VAS 100 mm scale questions to indicate their feelings. A composite appetite score was then calculated based on the following formula (Woodend and Anderson, 2001; Anderson *et al.*, 2002; Abou-Samra *et al.*, 2011):

$$[\text{Hunger} + (100 - \text{Fullness}) + \text{Desire to Eat} + \text{Appetite for Meal}] / 4$$

Subjective appetite scores were measured using the 100 mm VAS. The scores were collected in duplicate (at -10 and 0 minutes) prior to breakfast and hourly postprandial until the end of the study day. The same questionnaire was also used to monitor gastrointestinal side effects, such as nausea, bloating, stomach discomfort, diarrhoea, and flatulence.

2.4.3 Collection of blood samples

Blood samples were withdrawn through peripheral intravenous cannula from one forearm to measure metabolites and gastrointestinal hormones. Fasting blood samples were collected in duplicate (at -10 and 0 minutes) prior to breakfast. Postprandial blood collection points for investigation in Chapter 3 were at 15, 30, 60, 90, 120, 180, 240, and 300 minutes. A total of 110 ml (11 ml X 10 samples) of blood was collected during each study visit. Postprandial blood collection points for investigation in Chapter 4 were at 15, 30, 60, 90, 120, and 180 minutes. A total of 80 ml (10 ml X 8 samples) of blood was collected during each study visit. Postprandial blood collection points for investigation in Chapter 5 were at 15, 30, 60, 90, 120, 180, 240, 300, 360, 420, and 480 minutes. A total of 143 ml (11 ml X 13 samples) of blood was collected during each study visit. Postprandial blood collection points for investigation in Chapter 6 were at 15, 30, 60, 90, 120, 180, and 240 minutes. A total of 72 ml (8 ml X 9 samples) of blood was collected during each study visit. Cannula were removed at the end of each study day, and volunteers were discharged from the clinical research facility (CRF).

Blood collected for measuring gut hormones was transferred to a BD Lithium Heparin Vacutainer, and 20 µl of Aprotinin was added per 1 ml of blood (pancreatic protease inhibitor to prevent the enzyme degradation of gut hormones) (Nordic Pharma UK Ltd, Reading, UK). Blood collected for measuring glucose was transferred to a BD Fluoride Ethylenediaminetetraacetic acid (EDTA)

Vacutainer. Blood collected for measuring insulin was transferred to a BD Serum SST Vacutainer and allowed to clot before centrifugation at 2500 relative centrifugal force (RCF) at 4°C for 10 minutes. The separated plasma and serum were aliquoted into Eppendorf tubes and stored in the -80°C freezer until the assaying process.

The analysis and assay protocols for different biological parameters are described below. To ensure results accuracy, all of the assays done in Imperial College laboratories were performed in duplicate. Variability within volunteer samples was minimised by including all visits in the same analysis. Relative variability was measured using the coefficient of variation (CV), calculated using the means and standard deviations of the measures.

2.4.4 Colorimetric assay

2.4.4.1 Principle of assay

Colorimetric assays are widely used in medical research and are designed to quantitate metabolites of biological samples. The principle behind this type of assay is based on a chemical reaction between the metabolite in question and the metabolite-specific enzyme that will be later added to the sample. The concentration of the yielded by-product can be measured using a spectrophotometer, a device that measures the specific absorbance of by-product wavelength. Then, the by-product concentration is extrapolated to the concentration of the metabolite of interest (Housecroft and Constable, 2010). In the current work, a colorimetric assay was used to quantify plasma glucose concentrations in Chapters 4 and 6.

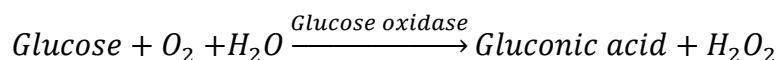
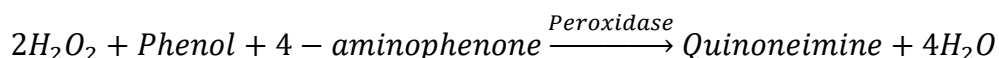
2.4.4.2 Glucose analysis

Two type of glucose analysis were used in these experimental studies:

- Plasma glucose analysis in the investigations included in Chapters 3 and 5 was performed at the Department of Clinical Biochemistry, Hammersmith Hospital. The glucose assays were analysed using an Abbott Architect ci8200 analyser (Abbott Diagnostics, USA). This is an enzymatic reaction assay performed using glucose-6-phosphate dehydrogenase and hexokinase as reagents. Briefly, glucose is phosphorylated by hexokinase, where one

micromole of nicotinamide adenine dinucleotide hydrogen (NADH) is produced for every micromole of glucose consumed. NADH absorbs light at 340 nm, and absorbance can be detected by spectrophotometry. The glucose concentration of the sample is then calculated based on the absorbance rate.

- For the investigations included in Chapters 4 and 6, blood glucose levels were measured following the glucose oxidase enzymatic method, using commercially purchased Randox Glucose (GLUC/PAP) kits supplied by Randox. This is a colorimetric assay that is based on glucose being oxidised into hydrogen peroxide in the presence of glucose oxidase. Hydrogen peroxide then reacts with phenol and 4-aminophenone to form quinoneimine dye, acting as an indicator coloured red-violet. The quinoneimine colour is measured using a colorimetry device, and the absorbance positively correlates with the glucose concentration in the unknown sample (Barham and Trinder, 1972).



A total of 20 µl of plasma samples were mixed with 1000 µl of glucose oxidase solution. Serial dilutions for standard preparation were performed, and the samples were incubated for 10 minutes at 37°C. A volume of 200 µl of the sample was then transferred to the microplate, and absorbance was measured for the standards and samples against the reagent blank using a colorimeter device at 540 nm. A CV below 5% was accepted for this assay.

2.4.5 Radioimmunoassays

2.4.5.1 Principals of radioimmunoassay

The principle of radioimmunoassay (RIA) analysis is to determine the percentage of unlabelled antibody-bound antigens. In this assay, a fixed concentration of a radioactive labelled antigen is incubated with a constant level of antibody and a variable concentration of unlabelled peptide

from serum. An unlabelled ligand and a radioactively labelled one competed for the antibody binding sites. As the unlabelled antigen level increases, the binding of labelled ligands to the antibodies decreases. The final volume of each tube was 700 μ l. After centrifugation, the radioactivity level of the supernatant containing the free tracer and the pellet containing the antibody bound tracer were measured using gamma counters. The amount of unlabelled antigen in the sample was calculated from interpolation of the standard curve. To achieve a standard curve, multiple standards were set up by incubating an increased concentrations of unlabelled peptides (standards) with the same level of antibody. This allowed determination of known levels of unlabelled antigens from the samples through interpolation from the standard curve.

To assess non-specific binding tubes (NSB), tubes that contained no antibody were added at the beginning of the assay. To assess the quality of the label, tubes containing half or twice the volume of the radio-labelled peptide were added. To monitor baseline drifts, tubes with no samples (zero tubes) were placed throughout the assay. To avoid any inter-assay variation, samples were analysed in one assay and in duplicate to increase the accuracy of the results.

2.4.5.1.1 Insulin analysis

Serum insulin was analysed using a Human Insulin Specific RIA kit (Millipore Corporation, Bellerica, USA). The assay did not cross-react with proinsulin; therefore, true insulin levels were measured. This analysis included ^{125} I-labelled human insulin and a human insulin antibody. Separation of the free from the antibody-bound label with a precipitating reagent was done after incubating the assay for 20–24 hours at room temperature. The tubes were decanted, and the precipitated pellets were counted using gamma counters (NE 1600, Thermo Electron Corporation). The limit of detection sensitivity was 2.7 μ U/ml. A CV below 10% was accepted for this assay.

2.4.5.1.2 GLP-1 analysis

Plasma GLP-1 was analysed using a previously established in-house sensitive radioimmunoassay using iodine-125 (Kreymann *et al.*, 1987). The antibody cross-reacted 100% with all GLP-1 amidated forms but not with glycine extended forms (GLP-11-37 and GLP-17-37) or any other

known gastrointestinal or pancreatic peptides. After incubating the assay for four days at 4°C, antibody-bound peptide was separated from the unbound peptide through charcoal adsorption (GLP-1). Both the charcoal-free and supernatant-bound fractions were counted using gamma counters (NE 1600, Thermo Electron Corporation). The limit of detection sensitivity was 7.5 pmol/l. A CV below 10% was accepted for this assay.

2.4.5.1.3 PYY analysis

Plasma PYY was analysed with a previously established in-house sensitive radioimmunoassay using iodine-125 (Adrian *et al.*, 1985). With this technique, total PYY is measured, which consists of the full-length PYY1-36 and the truncated fragment PYY3-36, both of which are biologically active. The antibody cross-reacted fully with the biological active forms of PYY but not with neuropeptide Y, pancreatic polypeptide, or other known gut hormones. After incubating the assay for three days at 4°C, antibody-bound peptide was separated from the unbound peptide using the primary/secondary antibody complex (PYY). Both the supernatant-free and antibody-bound fractions were counted using gamma counters (NE 1600, Thermo Electron Corporation). The limit of detection sensitivity was 2.5 pmol/l. A CV below 10% was accepted for this assay.

The following chapters include different studies, as briefly overviewed earlier in section 2.2.

Chapter 3

3. The impact of food structure on gastric emptying and postprandial glycaemia

3.1 Introduction

The GI tract is characterised by several ‘brake-points’, which serve as controllers of the rate at which the food moves through the GI tract. These brake-points include the stomach, where gastric emptying (GE) occurs. The process of GE involves muscular contractions/release of the pylorus sphincter, located at the end of the stomach, which is responsible for passing the chyme into the small intestine (Hellström, Grybäck and Jacobsson, 2006; Thomas, 2006; Boland, Golding and Singh, 2014). The initial lag phase of this process (T_{lag}) is the duration between food ingestion and the beginning of GE (Müller, Canfora and Blaak, 2018), and the half-time of GE ($T_{1/2}$) refers to the time required for half of the meal to leave the stomach (Müller, Canfora and Blaak, 2018). Thus, GE is considered a rate-limiting step in passing the chyme from the gastric to small intestinal areas, and delays in GE may prolong the amount of time needed for the food to come in contact with mechano- and chemo-receptors, thus extending meal digestion time (Hellström and Näslund, 2001). The regulation of GE involves coordination of complex neural and hormonal systems (Müller, Canfora and Blaak, 2018). Evidence in the literature supports the role of a number of enteroendocrine gut hormones, such as GLP-1, PYY, ghrelin, and cholecystikinin (CCK), in promoting or inhibiting GE (Hellström *et al.*, 2008; Field, Chaudhri and Bloom, 2010; Steinert *et al.*, 2017), thereby controlling the transit of food through the upper GI tract as well as the digestion and absorption of nutrients.

Many factors are known to affect GE, including the nature of foods (solid vs. liquid), availability of dietary fibre, and macronutrient composition (Bornhorst and Paul Singh, 2014). As such, highly viscous polysaccharides (e.g. guar gum) have been reported to delay GE when added to test meals (Torsdottir *et al.*, 1989, 1991). This could consequently influence postprandial glycaemia and other metabolic responses by reducing the rate of appearance and clearance of upper GI glucose concentrations, the main site of glucose absorption, which could significantly affect postprandial glycaemic response (O’Donovan *et al.*, 2004; Janssen *et al.*, 2011; Marathe *et al.*, 2013). Thirty-five per cent of postprandial glucose variability has been attributed to the rate of GE in response to a meal containing carbohydrates (Horowitz *et al.*, 1993; Pilichiewicz *et al.*, 2007; Ma *et al.*, 2012; Marathe *et al.*, 2013). Moreover, consuming simple carbohydrates has been

shown to induce higher and more rapid glucose and insulin responses than NDCs, including dietary fibre (Crapo, Reaven and Olefsky, 1976). This was shown to be related to differences in gastric digestion and GE rather than absorption. Both forms of dietary fibre (i.e. soluble, as in pectin and guar gum, and insoluble, as in cellulose and RS) can delay GE (Qi, Al-Ghazzewi and Tester, 2018). Known for their high level of fluid absorption during digestion, these starches can increase the bulkiness of food and causes the granules to swell and form gels that delay the transportation of nutrients to the small intestine (Cameron-Smith, Collier and O'Dea, 1994; Dikeman, Murphy and Fahey, 2006; Juvonen *et al.*, 2009). The effect of viscosity can have a major effect on overall digestion, leading to limited nutrient availability and decreasing the mixing effect of peristalsis with hydrolysing enzymes (Judd and Ellis, 2005; Takahashi, 2011), causing a delay in GE and reduced glycaemic response (Geliebter, Westreich and Gage, 1988; Bergmann *et al.*, 1992; Sturm *et al.*, 2004; Oesch *et al.*, 2006). Other plausible mechanisms affecting postprandial glycaemia include competitive bonding of different nutrients to enzymes during digestion. When other nutrients of the CW (e.g. cellulose) are present, they can interfere with gastric enzyme binding and thus delay chyme digestion and postprandial glycaemia. Moreover, NDCs and starches have been shown to increase gut hormone secretion, which can delay GE (Cuche, Cuber and Malbert, 2000; Malbert, G. Cuche, 2002). Collectively, it can be concluded that foods high in NDCs can have distinctive influences on our health due not only to their nutritional composition but also through their rate and extent of digestion and the subsequent metabolic responses.

3.1.1 SBE-deficient peas and the effect on glycaemic response

Both wild and SBE-deficient pea seeds contain approximately the same amount of dietary fibre (around 36 g/100 g). The main difference between SBE-deficient and wild pea seeds is that, as a result of having a starch assembly mutation, SBE-deficient pea seeds have a higher amylose-to-amylopectin ratio (around 70% amylose) than wild pea seeds (around 30% amylose) (Ridout *et al.*, 2003). The compact nature of the crystalline amylose results in a different digestion profile, as the long linear chains increase the stability of the amylose structure (due to the high hydrogen bonding) and makes it difficult for α -amylase to degrade it when compared with the branched

amylopectin (Thorne, Thompson and Jenkins, 1983; Hoover *et al.*, 2003). In contrast, amylopectin has a much larger surface area per molecule than amylose, making it a preferable substrate for amylolytic attack (Thorne, Thompson and Jenkins, 1983). This feature can cause SBE-deficient peas to acquire different properties in response to hydrothermal processing, gelatinisation, and retrogradation, all leading to decreased digestibility when compared to WT peas. Thus, it is plausible to assume that SBE-deficient pea seeds can lower postprandial glycaemia due to the effect on the starch digestion rate and extent. However, it is important to understand whether a starch assembly mutation in peas can lower postprandial glycaemia, given their structural properties (and whether this effect is retained after physically processing the structure to a flour form), or whether these glycaemic effects are attributed to changes in the rate of GE. In the current study, 50 g of both WT and SBE-deficient pea seeds were tested in two physical forms (whole pea seeds and pea flour) to understand whether a starch assembly mutation in peas can lower postprandial glycaemia due to impacts on GE.

3.2 Purpose of the study

3.2.1 Hypothesis

The study hypothesised that differences in postprandial glycaemia in response to consumption of starch assembly mutation-containing pea seeds in both whole and flour forms are not attributed to the rate of GE and gut hormone release.

3.2.2 Aim

This study aimed to investigate whether limiting starch availability due to the effect of a mutation in the starch structure of peas has an influence on postprandial glycaemia, if the same effect would be retained in the flour form, and whether these outcomes are attributed to an effect on GE.

3.2.3 Outcome measures

The co-primary outcome were as follows:

- GE $t_{1/2}$ rate
- Plasma glucose levels

Secondary outcomes were:

- Serum insulin and plasma gut hormones; GLP-1 and PYY levels
- Visual analogue scales to assess subjective appetite
- Breath hydrogen excretion levels

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Peas

Please refer to Chapter 2.

3.3.1.2 Randomisation

Participants were randomised using an independent telephone and internet company (sealedenvelope.com). Both the researcher and volunteers were blinded as to the type of dietary intervention consumed on each study visit and/or during the study duration.

3.3.1.3 Sample size and power calculation

This was a descriptive exploratory study in a new area, and thus a power calculation was not possible. Ten volunteers participated in this study.

3.3.1.4 Ethical approval

This study was approved by the South East-Coast Surrey Research Ethics Committee (REC project reference number 15/LO/0184) and the Imperial College Healthcare NHS Trust Joint Research Office. This study was performed in accordance with the Declaration of Helsinki.

3.3.1.5 Recruitment

The same recruitment strategy was used for all investigations. Volunteers were recruited through the National Institute for Health Research (NIHR)/Wellcome Trust Imperial Clinical Research Facility (CRF) at Hammersmith Hospital healthy volunteers' panel database as well as through poster advertisement. A participant information sheet (PIS) was provided to participants who expressed an interest in taking part in the study (Appendix 1). During the first stage, volunteer eligibility was determined via a brief phone interview. Before volunteers were able to participate in the study, they had to attend a screening visit at the NIHR/Imperial CRF at Hammersmith

Hospital to further assess their eligibility. During the screening visit, the study protocol was explained in full, and the participant provided full written informed consent before commencing any assessment (Appendix 2). The following information was collected: height, weight, blood pressure (BP), electrocardiogram (ECG), a standard screening blood sample, and a pregnancy test for all women of childbearing age. A research doctor had to check all of the volunteers' results before enrolling. Participants were then contacted with the results of their screening and given a start date for the study. Participants were asked not to start any intensive exercise regimen or new diet during the study period, as they might affect the results.

3.3.1.6 Volunteer inclusion and exclusion criteria

Healthy male and female volunteers between the ages of 18 and 65 years with a BMI of 20–35 kg/m² were invited to take part in this study. The exclusion criteria were as follows: any gastrointestinal disease or syndrome; fluctuation in weight of ≥ 3 kg in the preceding two months; diabetes; pregnancy; excess alcohol intake; current smoking; history of substance abuse; cancer, cardiovascular, kidney, or liver diseases; pancreatitis; and use of medications likely to interfere with hormonal balance, appetite regulation, and energy metabolism, including antibiotics, thyroid hormones, anti-inflammatory drugs or steroids, erythromycin, phenytoin, or androgens.

3.3.2 Method

3.3.2.1 Study day preparation and protocol

All participants were required to refrain from alcohol and strenuous exercise and to fast for 12 hours overnight. Participants were asked to consume the same standard meal of their choice the evening before the different visits to insure consistency between study visits.

Following overnight fasting from 09:00 p.m., volunteers were asked to attend the CRF at 09:00 a.m. (Figure 3.1). Before consuming the test breakfast, participants were asked to empty their bladder in order to measure their weight and body composition using bioelectrical impedance analysis (BIA). Participants were asked to stand barefoot on a Tanita segmental body composition analyser (Tanita MC-780MA analyser; Tanita Corporation, Japan). The BIA scale was used to

evaluate total body water (TBW) content with an electrical current. Then, TBW was used to estimate fat-free mass (FFM), and the fat mass was calculated by subtracting FFM from total body weight. Height was measured using a stadiometer. BMI was used to identify eligibility in order to recruit a homogenous cohort.

3.3.2.2 Study design

This was an acute, randomised, controlled, double-blind, cross-over trial involving four study visits in the CRF. A seven-day washout period was proposed between each study visit.

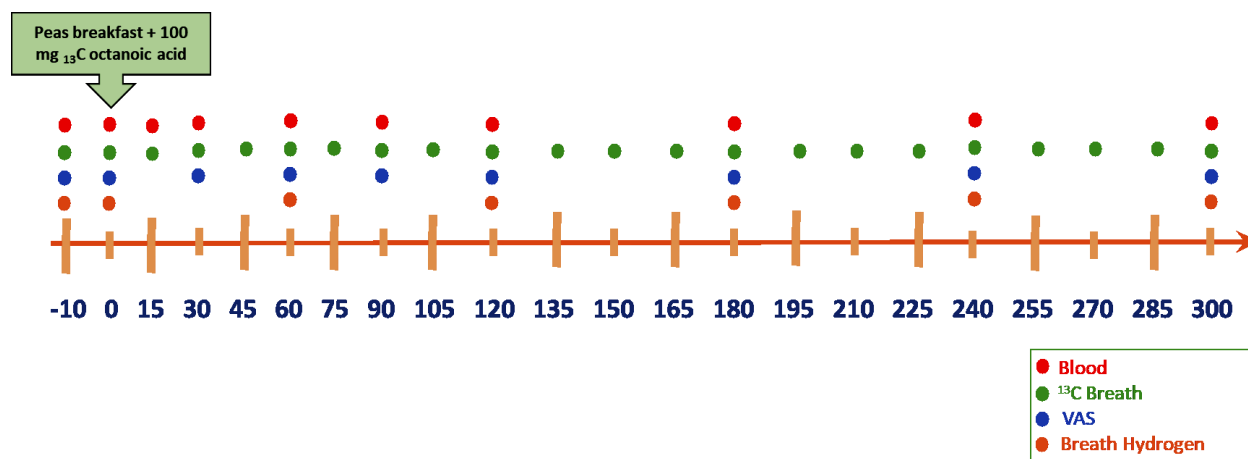


Figure 3.1: Study visit overview.

Overview of timings of blood sampling, VAS ratings and breath tests. Abbreviations: H₂, hydrogen; VAS, visual analogue scale.

3.3.2.3 Study meal

A standardised breakfast was served at 0 minutes. In random order, the nutritionally matched test meals were freshly prepared to differ only in containing 50 g dry weight of each pea material. The John Innes Center kindly provided the whole pea seeds, and Campden BRI kindly produced the flour from the same pea seeds (for more information about the food material, please refer to Chapter 2). Professor Tom Preston, University of Glasgow, provided the [¹³C] octanoic acid that was used in assessing GE.

The moisture content of the pea seeds was approximated to be 7.4 g/100 g. The moisture content of the pea flour was approximated to be the same, as it was produced from dried pea seeds;

however, it might be lower/higher following the loss of the seed coat. Before cooking, the whole pea seeds were soaked for 24 hours in 200 ml of water. Next, they were cooked for 1 hour at medium temperature on the morning of the study visit. Flour was prepared differently from the peas, and 50 g of dry weight flour was added to the tomato soup and microwaved for 2 minutes. The pea flour mixed test meal contained the same food products as whole pea seed meals, which are listed in Table 3.1. A total of 100 mg [¹³C] octanoic acid was injected into the egg yolk, and the egg was microwaved for 1 minute. Whole pea seed or flour were mixed with the egg and tomato soup. Test meals were served and consumed immediately after cooking. Thus, any observed metabolic effects could be attributed to the starch properties or food structure of the test meals during GI digestive transit. Table 3.1 below presents the nutrition composition of the study meal used in this experiment.

Table 3.1: Nutrition information of the current test meals provided from different pea lines

Food Products	Unit (g)	Kcal	CHO^a (g)	Fat (g)	Protein (g)	Fibre (g)
Heinz tomato soup	280	167	20	8.4	1.96	1.68
Microwaved Egg	1 (egg)	78	0.6	5	6	0
Hovis soft white bread	80	93.0	17.9	0.7	3.5	1.0
Flora butter	10	40	0.5	4.5	0.5	0
Wild (peas & flour)	50	143	17.6	<0.5	10.6	18
Total (with wild pea seeds & flour)	-	521	56.6	19.1	22.5	20.68
SBE-deficient (peas and flour)	50	147	14	0.85	11.6	18.1
Total (with SBE-deficient peas & flour)	-	523	54	19.9	23.5	20.69

^adetailed carbohydrate analysis is not available

3.3.2.4 Determination of gastric emptying by breath CO₂

Using γ-scintigraphic for measuring GE is considered to be the standard method, as it visualises real-time images about food transit through the stomach and the rest of the gut (Urbain *et al.*, 1990). However, due to the burden of γ radiation penetration and the high cost–benefit ratio,

researchers have attempted to discover a less invasive technique that has high sensitivity and specificity and is more convenient for research purposes.

The [^{13}C] octanoic acid has been validated against the scintigraphy technique and is an accurate test for measuring solid meal GE (Ghoos *et al.*, 1993; Maes *et al.*, 1994). It is also a more pragmatic method to be used in the current experiment, as it does not involve any radiation burden on the healthy volunteers. Its concept depends on the solubility of [^{13}C] octanoic acid in egg yolk, which is served as an omelette with a standard breakfast. [^{13}C] octanoic acid contains a carboxyl-carbon labelled with the non-radioactive stable isotope ^{13}C . [^{13}C] octanoic acid, a medium-chain fatty acid, passes the stomach unabsorbed, reaching the small intestine, where it is rapidly absorbed from the duodenum and metabolised in the liver (Clark and Holt, 1969; Bach and Babayan, 1982; Ghoos *et al.*, 1993). Following oxidation, $^{13}\text{CO}_2$ is expired into breath at a level that can be easily detected and measured by isotope ratio mass spectrometry (IRMS) (Lacroix *et al.*, 1973). A curve-fitting technique was used to analyse breath data, as previously described (Ghoos *et al.*, 1993), and $t_{1/2}$ is the parameter defined from the fitted curve as a marker of the GE rate. The limiting step in this process is the rate at which the solid meal empties from the stomach. ^{13}C enrichment in expelled air can, thus, be used as a marker of the GE rate

To trace the ^{13}C octanoic acid inside the body, ^{13}C octanoic acid exhaled breath samples were taken by having the volunteers blow into an Exetainer (Labco Ltd, Lampeter, Ceredigion, United Kingdom) through a straw until vapour condensed at the bottom of the tube (Morrison *et al.*, 2003) every 15 minutes for 300 minutes of the study visit. Breath samples analysis for this study was performed by the project collaborators at the university of Glasgow via IRMS (Preston and McMillan, 1988), which measures the relative abundance of isotopes in any given sample (Morrison *et al.*, 2003). Briefly, helium gas was flushed into a portion of breath. Before the introduction into the mass spectrometer, water and CO_2 were separated from other gasses using gas chromatography (AP2003, GV Instruments, Manchester, UK). An isotope ratio of $^{13}\text{C}:^{12}\text{C}$ was calculated from the ion abundance of m/z 44, 45, and 46 with a laboratory reference CO_2 value, calibrated against Vienne Pee Dee Belemnite (VPDB), with correction of the small contribution of $^{12}\text{C}^{16}\text{O}^{17}\text{O}$ at m/z 45, known as the Craig correction (Craig, 1957). For each time point, breath ^{13}C

enrichment (‰) over baseline was calculated. Finally, to compute GE $T_{1/2}$ times, the envelope of breath ^{13}C excretion was analysed using a modified version of the curve-fitting techniques (Ghoos *et al.*, 1993).

3.3.2.5 Breath hydrogen concentration

Please refer to Chapter 2.

3.3.2.6 Subjective appetite scores

Please refer to Chapter 2.

3.3.2.7 Blood sampling, collection, and analysis

Please refer to Chapter 2 regarding the sampling and collecting methods.

3.3.2.7.1 Glucose analysis

For the analysis protocols, please refer to Chapter 2.

3.3.2.7.2 Insulin analysis

For the analysis protocols, please refer to Chapter 2. The % CV of this assay was 3.75%.

3.3.2.7.3 Glucagon-like peptide-1 analysis

For the analysis protocols, please refer to Chapter 2. The % CV of this assay was 4.4%

3.3.2.7.4 Peptide tyrosine tyrosine analysis

For the analysis protocols, please refer to Chapter 2. The % CV for this assay was 3.68%

3.3.2.7.5 Statistical analysis

Data were checked for normality using a Shapiro–Wilk test (defined as $P \geq 0.01$). In the case of non-normal distribution, data were normalised through log-transformation (the function LN or LN+1 where applicable). If the data were still not normally distributed, non-parametric tests were used. All figures are presented as raw data. Results are expressed as mean difference with $\pm$

standard error of the mean (SEM). The average pre and post data points were calculated and used to replace any missing data value. The two baseline samples for blood, VAS, and breath hydrogen data were averaged and used as the 0 min measurement. Postprandial results are presented as total area under the curve (tAUC) for breath hydrogen and VAS, and as the incremental area under the curve (iAUC) for all blood parameters. tAUC and iAUC were calculated using the trapezoidal rule. iAUC data were the mean values, while tAUC mean data were divided by the study visit time frame. AUC mean values were compared to, first, look at the effect of starch branching enzyme mutation parameters (whole–whole and flour–flour) and, second, to look at the effect of cellular structure (wild whole pea seed flour and SBE-deficient whole pea seed flour) using a paired t-test or Wilcoxon signed-rank test where applicable. Repeated measures of analysis of variance (ANOVA) were used with time and meal as ‘within’ factors to determine the effect of the test meals on postprandial metabolic responses. Student’s paired t-tests were used to identify time points with significantly different values between the two meals, and the Fisher LSD method was used to correct for multiple comparisons. The acute postprandial glucose response to foods was evaluated by the incremental area of blood glucose concentration during a period of 2 hours post-ingestion (iAUC₀₋₁₂₀), which is the most dynamic period to measure metabolic response of a specific amount of food (usually 50 g of carbohydrates) compared to the response of a reference food (Jenkins *et al.*, 1982, 2002; American Diabetes Association *et al.*, 2008); thus, it was calculated for all blood parameters. Significance was considered when the p-value was ≤ 0.05 , with the significance level indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Statistical analysis was performed using IBM SPSS version 23. Graphs were prepared using GraphPad Prism version 7.04.

3.4 Results

3.4.1 Participant characteristics

Ten adult volunteers were randomised to take part in this study. The baseline characteristics of these participants are described in Table 3.2.

Table 3.2: Demographic characteristics of participants recruited and completed the study (n=10)

Characteristics	Visit 1	Visit 2	Visit 3	Visit 4	<i>P Value</i>
<i>Male, female (n)</i>			3,7		
<i>Age (Y)</i>			49.3 ±8.5		
<i>Weight (Kg)</i>	67.3±4.1	67.5±4.2	68.0±4.1	68.0±3.9	0.26
<i>BMI (Kg/m²)</i>	23.8 ±0.1	23.9 ±0.1	24.3 ±0.1	24.2 ±0.2	0.38
<i>Body Fat (%)</i>	27.4 ±1.7	27.7 ±2.0	27.5 ±1.6	27.6 ±1.6	0.44
<i>Data are means ± SEM or (n)</i>					

3.4.2 Breath ^{13}C octanoic acid recovery for measuring gastric emptying:

Figure 3.2 shows $^{13}\text{CO}_2$ recovery profile data for GE following the consumption of 100 mg of ^{13}C -octanoic acid in pea mixed meal, comparing the effects of mutation between whole pea seeds and between pea flours as well as the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

The results indicated no effect when comparing GE half-times between wild and SBE-deficient peas ($p=0.49$).

Pea flours:

The results indicated no effect when comparing GE half-times between wild and SBE-deficient flour ($p=0.56$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds and flour:

The results indicated a borderline increase in GE half-times following wild flour consumption (65.5 ± 3.3 min) when compared to wild pea consumption (57.9 ± 4.2 min). However, this difference did not reach a statistic significant level ($p=0.09$).

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing GE half-times between SBE-deficient peas and SBE-deficient flour ($p=0.28$).

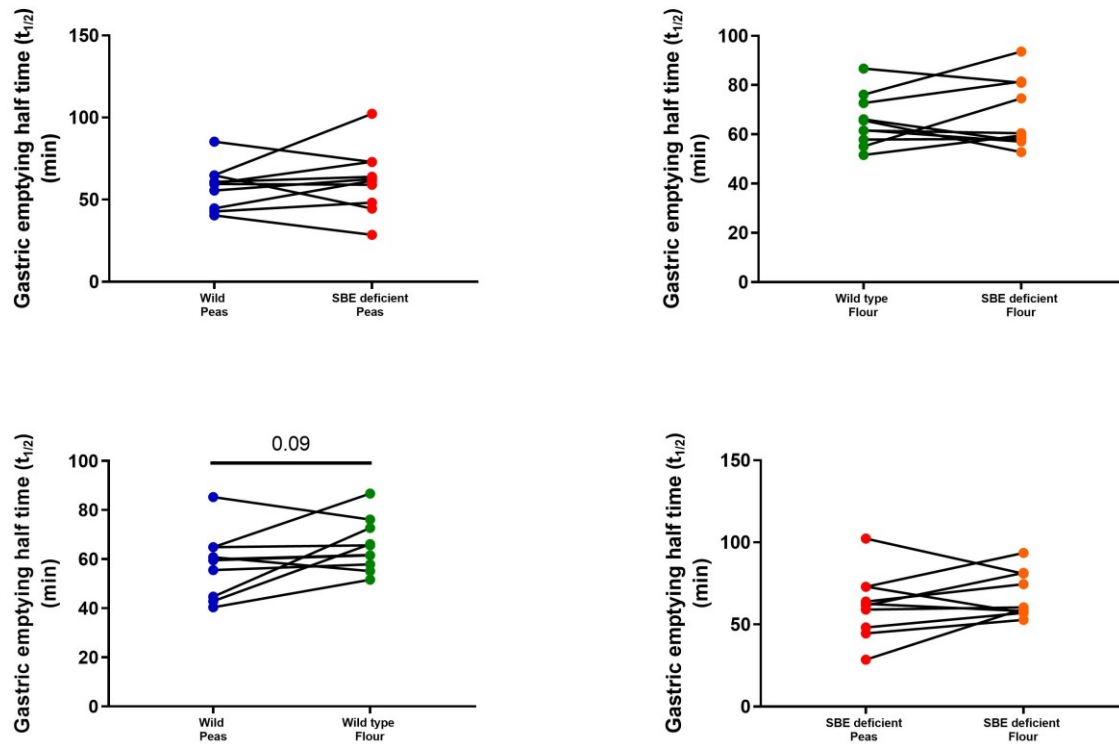


Figure 3.2: Postprandial gastric emptying $t_{1/2}$ in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) $t_{1/2}$ of wild peas (●) and SBE-deficient peas (●), (B) $t_{1/2}$ of wild flour (●) and SBE-deficient flour (●), (C) $t_{1/2}$ of wild peas (●) and wild flour (●), (D) $t_{1/2}$ of SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: $t_{1/2}$, half time.

3.4.3 Plasma glucose:

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 3.3 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for plasma glucose concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated a significant reduction in plasma glucose $iAUC_{0-300}$ following SBE-deficient pea consumption (44.5 ± 39.9 mmol/L x min) when compared to wild pea consumption (127.0 ± 39.3 mmol/L x min) ($p < 0.01$). The same effect was shown in plasma glucose $iAUC_{0-120}$ following SBE-deficient pea consumption (49.3 ± 24.9 mmol/L x min) when compared to wild pea consumption (99.5 ± 26.3 mmol/L x min) ($p < 0.01$).

No main effect of peas was found ($p = 0.24$). However, a significant effect of pea X time interaction was found ($p = 0.02$). A significant plasma glucose peak was found at 30 minutes ($p = 0.01$) followed by a decline at 60 minutes ($p = 0.01$) after wild pea consumption when compared with SBE-deficient pea consumption.

Pea flours:

The results indicated a borderline reduction in plasma glucose $iAUC_{0-300}$ following SBE-deficient flour consumption (94.3 ± 34.7 mmol/L x min) when compared to wild flour consumption (146.7 ± 40.7 mmol/L x min). However, this difference did not reach a statistic significant level ($p = 0.08$). There was also a significant reduction in plasma glucose $iAUC_{0-120}$ following SBE-deficient flour consumption (60.3 ± 17.0 mmol/L x min) when compared to wild flour consumption (105.2 ± 27.8 mmol/L x min) ($p = 0.02$).

No main effect of flour was found ($p = 0.16$). However, a significant effect of flour X time interaction was found ($p = 0.04$). A significant plasma glucose difference was found at 90 minutes ($p = 0.05$) after SBE-deficient flour consumption when compared to wild flour consumption.

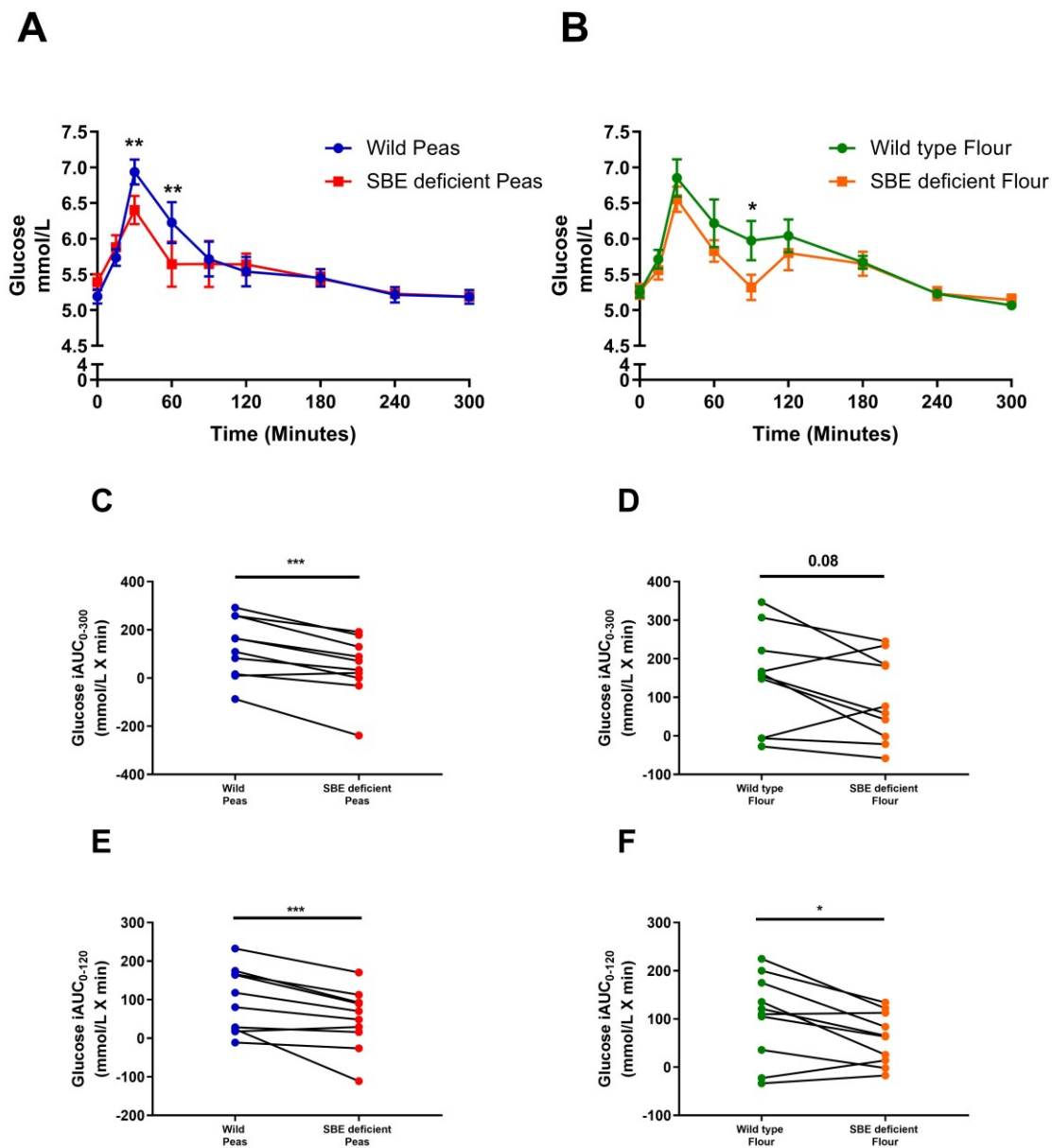


Figure 3.3 Postprandial glucose in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 3.4 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for plasma glucose concentrations, comparing the effects of structure between wild pea seed and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma glucose $iAUC_{0-300}$ between wild peas and wild flour ($p=0.46$). The same effect was shown in plasma glucose $iAUC_{0-120}$ between wild peas and wild flour ($p=0.74$).

No main effects of meal ($p=0.15$) and meal X time interaction ($p=0.12$) were found.

SBE-deficient pea seeds and flour:

The results indicated a borderline reduction in plasma glucose $iAUC_{0-300}$ following SBE-deficient pea consumption (44.5 ± 39.9 mmol/L x min) when compared to SBE-deficient flour consumption (94.3 ± 34.7 mmol/L x min). However, this difference did not reach a statistically significant level ($p=0.06$). The same difference was not shown in plasma glucose $iAUC_{0-120}$ between SBE-deficient peas and SBE-deficient flour ($p=0.50$).

No main effects of meal ($p=0.84$) or meal X time interaction ($p=0.20$) were found.

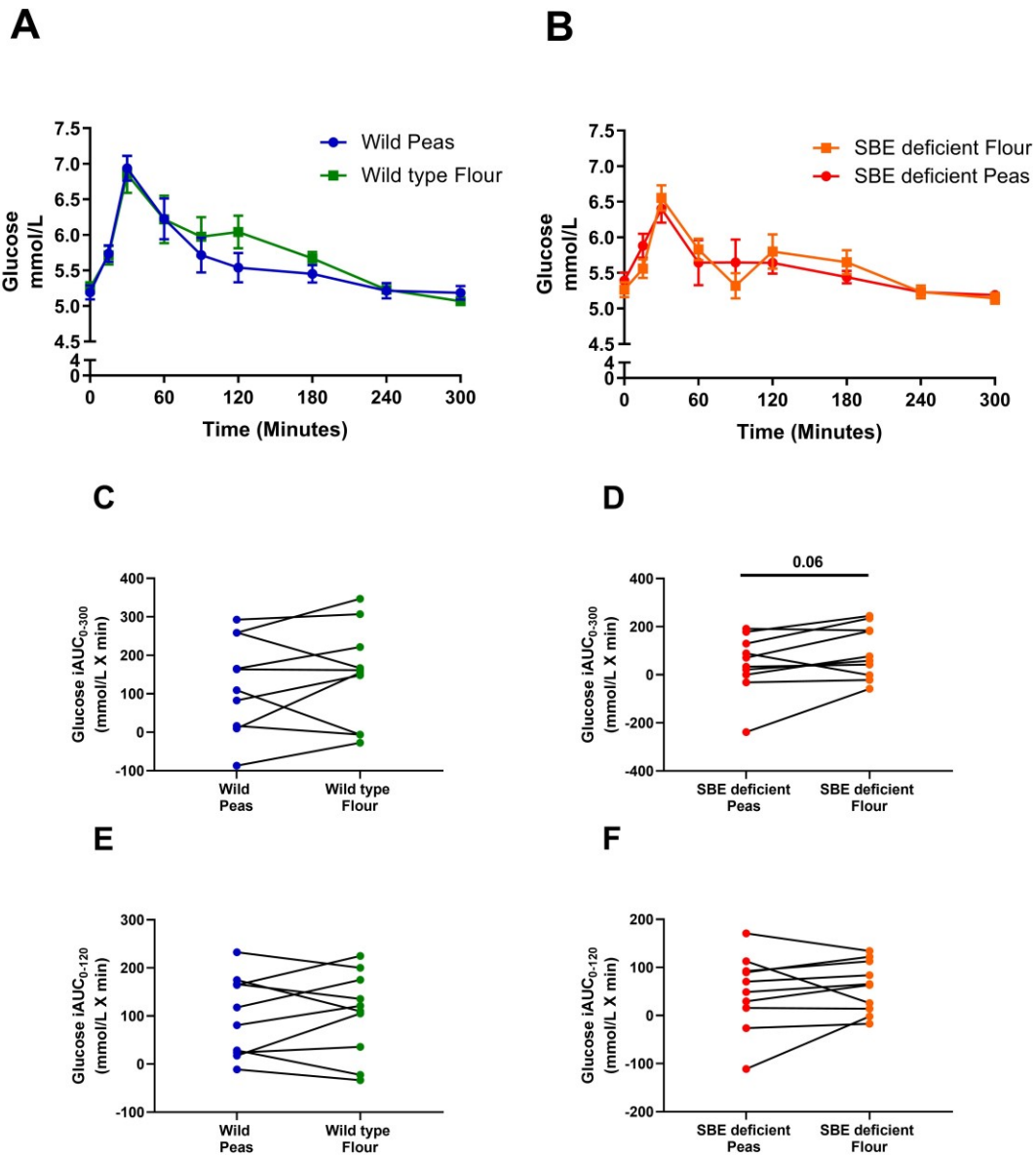


Figure 3.4 Postprandial glucose in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and iAUC₀₋₃₀₀ for wild flour (●), (D) SBE-deficient peas (●) and SBE-deficient flour (●), (E) wild peas (●) and iAUC₀₋₁₂₀ for wild flour (●), (F) SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

3.4.4 Serum insulin

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 3.5 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for serum insulin concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing serum insulin $iAUC_{0-300}$ between wild and SBE-deficient peas ($p=0.51$). The same lack of effect was observed in serum insulin $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.45$)

No main effects of peas ($p=0.17$) or pea X time interaction ($p=0.86$) were found.

Pea flour:

The results indicated a borderline reduction when comparing serum insulin $iAUC_{0-300}$ following SBE-deficient flour consumption ($4006.3 \pm 1112.9 \mu\text{U/ml} \times \text{min}$) with that following wild flour consumption ($5211.3 \pm 1647.9 \mu\text{U/ml} \times \text{min}$). However, this difference did not reach a statistic significant level ($p=0.09$). The same difference was not observed in serum insulin $iAUC_{0-120}$ between wild flour and SBE-deficient flour ($p=0.20$).

No main effects of flour ($p=0.25$) or flour X time interaction ($p=0.54$) were found.

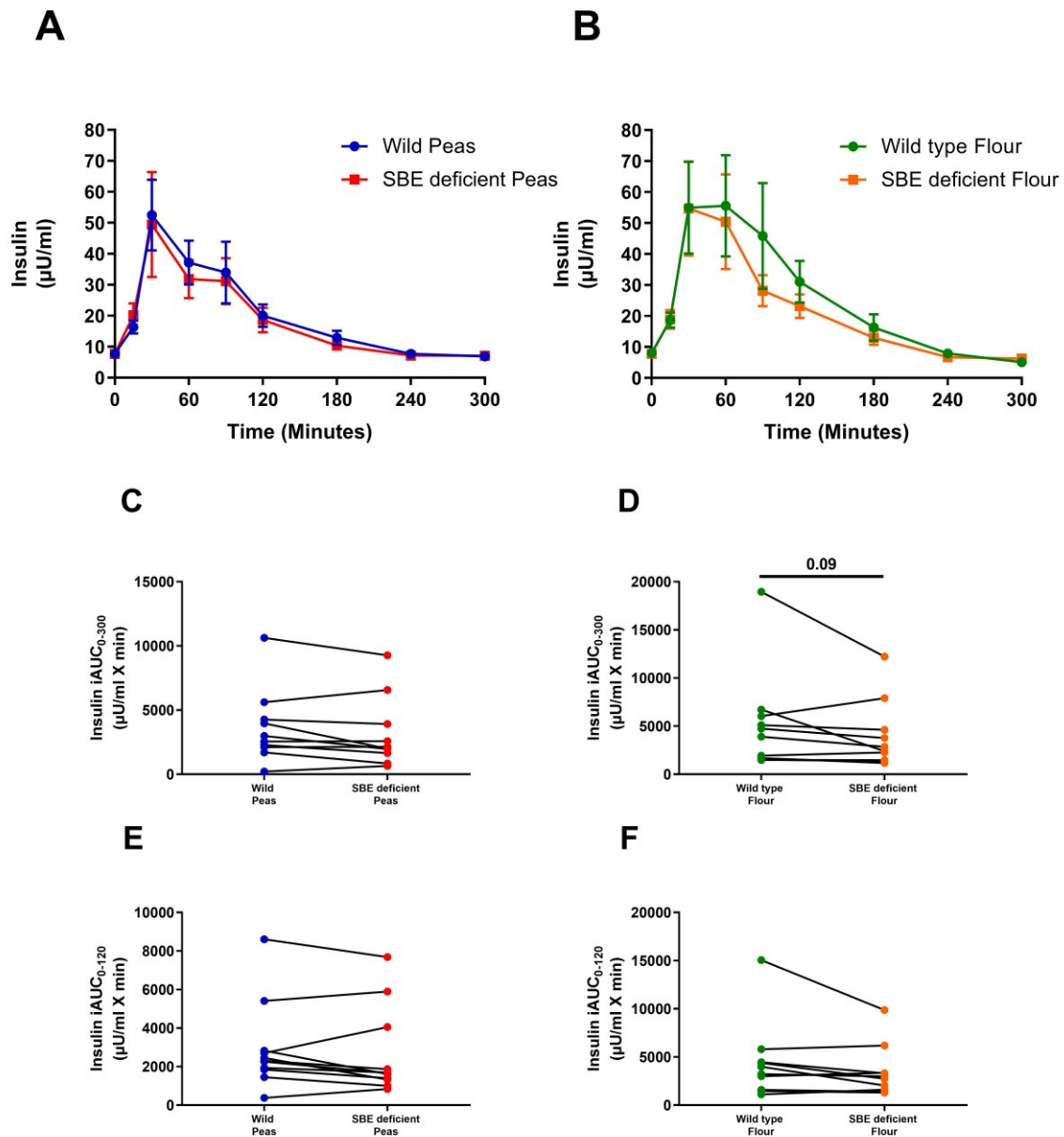


Figure 3.5 Postprandial insulin in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 3.6 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for serum insulin concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect on serum insulin $iAUC_{0-300}$ between wild peas and wild flour ($p=0.13$). The same lack of effect was observed in serum insulin $iAUC_{0-120}$ between wild peas and wild flour ($p=0.17$).

No main effect of meal ($p=0.23$) was found. However, a borderline meal X time interaction ($p=0.06$) was found. A significant increase in serum insulin was found at 60 minutes ($p=0.05$) following wild flour consumption when compared to wild pea consumption.

SBE-deficient pea seeds and flour:

The results indicated a significant reduction in serum insulin $iAUC_{0-300}$ following SBE-deficient pea consumption ($3160.9 \pm 866.7 \mu\text{U/ml} \times \text{min}$) when compared to SBE-deficient flour consumption ($4006.3 \pm 1112.9 \mu\text{U/ml} \times \text{min}$) ($p=0.02$). The same reduction was observed in serum insulin $iAUC_{0-120}$ between SBE-deficient peas ($2718.7 \pm 745.7 \mu\text{U/ml} \times \text{min}$) when compared to SBE-deficient flour ($3344.6 \pm 864.9 \mu\text{U/ml} \times \text{min}$) ($p=0.04$).

No main effects of meal ($p=0.11$) or meal X time interaction ($p=0.37$) were found.

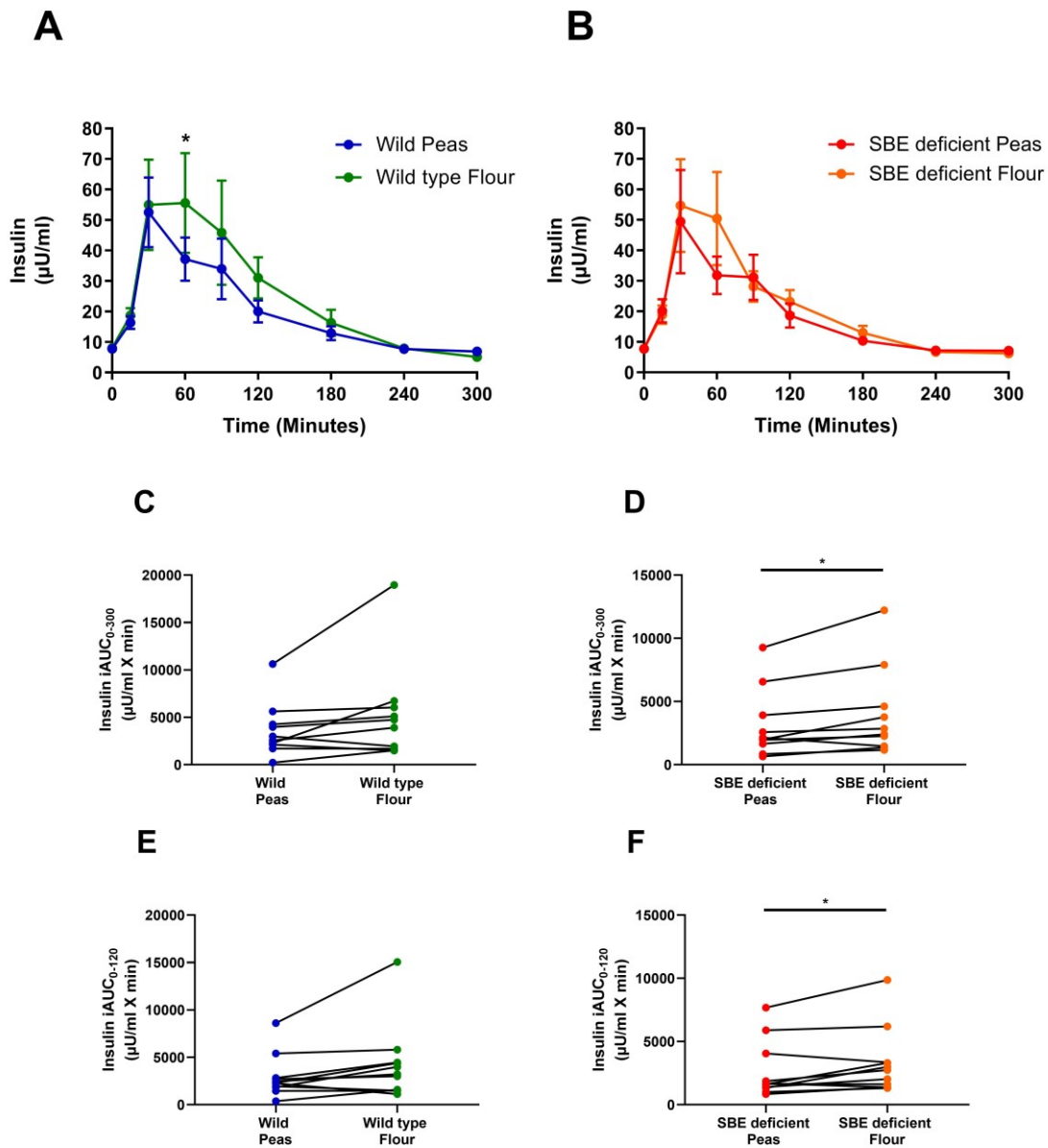


Figure 3.6: Postprandial insulin in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and iAUC₀₋₃₀₀ for wild flour (●), (D) SBE-deficient peas (●) and SBE-deficient flour (●), (E) wild peas (●) and iAUC₀₋₁₂₀ for wild flour (●), (F) SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

3.4.5 Plasma GLP-1

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 3.7 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for plasma GLP-1 concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-300}$ between wild and SBE-deficient peas ($p=0.88$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.92$).

No main effects of peas ($p=0.62$) or pea X time interaction ($p=0.62$) were found.

Pea flours:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-300}$ between wild and SBE-deficient flour ($p=0.77$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.42$).

A borderline effect of flour was found ($p=0.09$). However, no flour X time interaction ($p=0.62$) was found. A significant plasma GLP-1 peak was found at 90 minutes ($p=0.01$) following wild flour consumption when compared to SBE-deficient flour consumption.

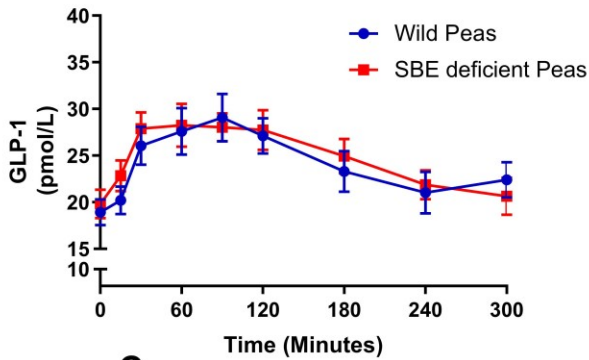
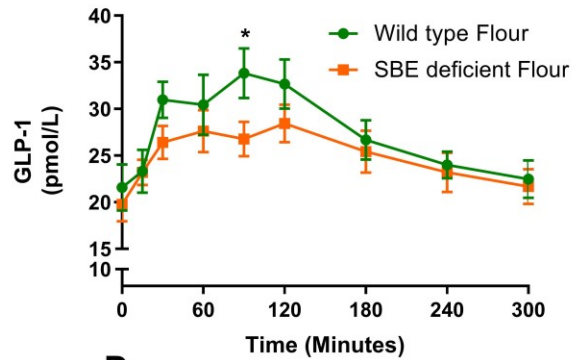
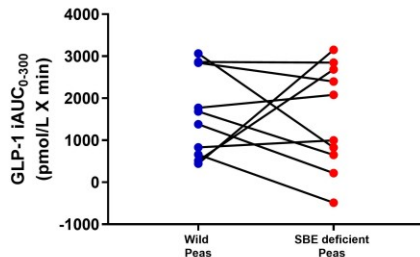
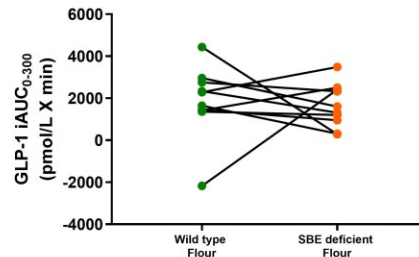
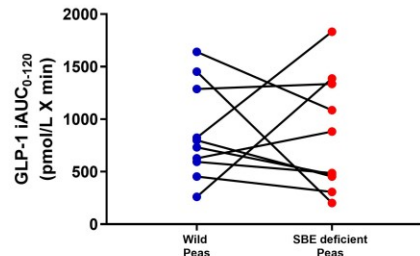
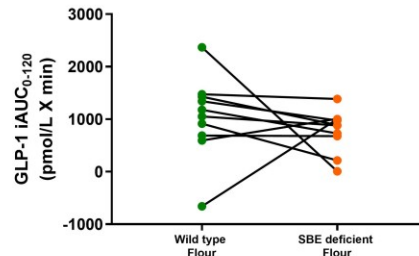
A**B****C****D****E****F**

Figure 3.7: Postprandial GLP-1 in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 3.8 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for plasma GLP-1 concentrations, comparing the effects of structure between wild pea seed and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-300}$ between wild peas and wild flour ($p=0.72$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between wild peas and wild flour ($p=0.51$).

A borderline effect of meal was found ($p=0.06$). However, no meal X time interaction ($p=0.55$) was found. Significant plasma GLP-1 peaks at 30 minutes ($p=0.04$) and 120 minutes ($p=0.02$) were found following wild flour consumption when compared to wild pea consumption.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-300}$ between SBE-deficient peas and SBE-deficient flour ($p=0.80$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between SBE-deficient peas and SBE-deficient flour ($p=0.76$).

No main effects of meal ($p=0.96$) or meal X time interaction ($p=0.91$) were found.

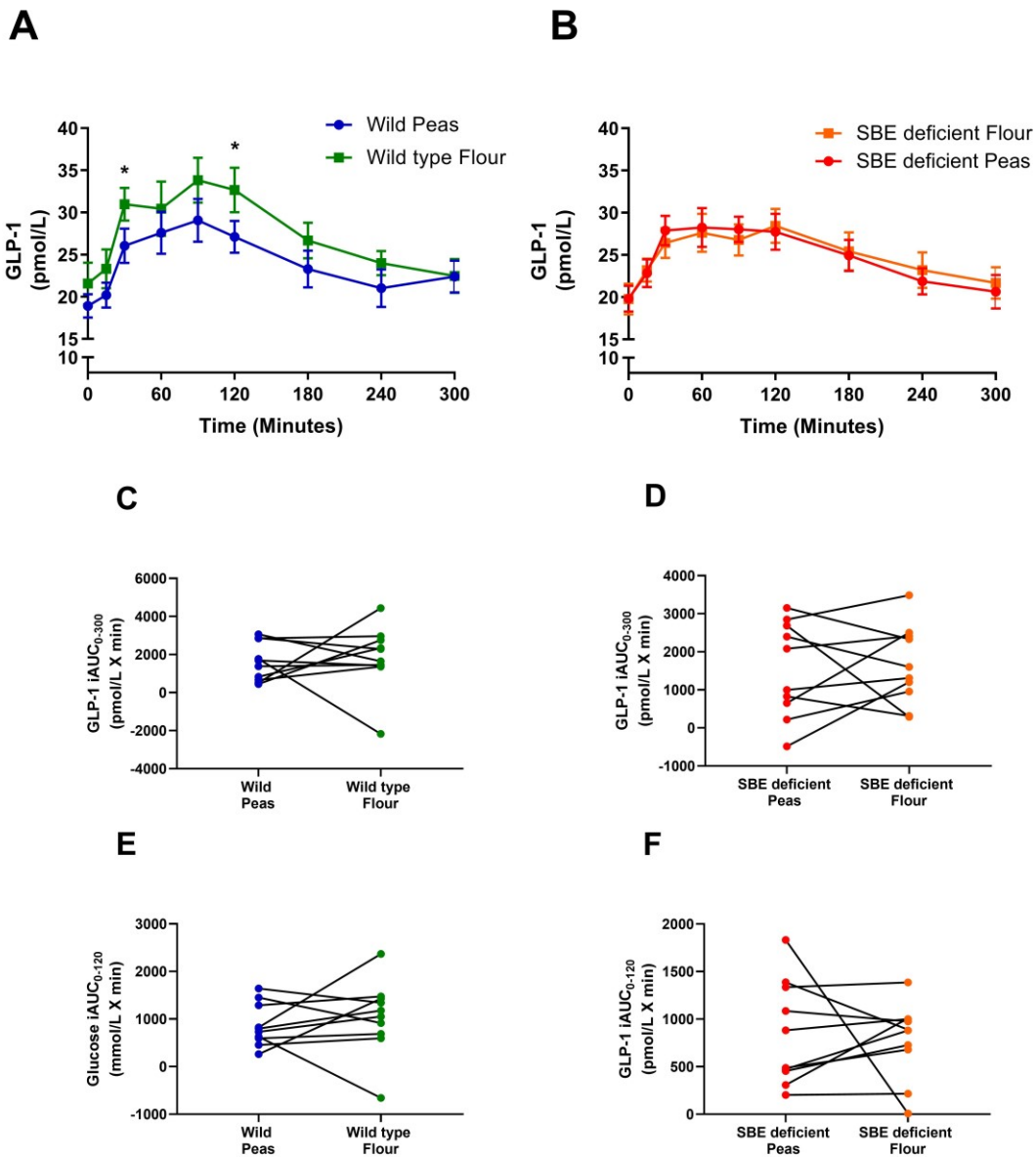


Figure 3.8: Postprandial GLP-1 in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and iAUC₀₋₃₀₀ for wild flour (●), (D) SBE-deficient peas (●) and SBE-deficient flour (●), (E) wild peas (●) and iAUC₀₋₁₂₀ for wild flour (●), (F) SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

3.4.6 Plasma PYY

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 3.9 shows time course, $iAUC_{0-300}$, and $iAUC_{0-120}$ data for plasma PYY concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma PYY $iAUC_{0-300}$ between wild and SBE-deficient peas ($p=0.11$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.32$).

No main effects of peas ($p=0.43$) or pea X time interaction ($p=0.36$) were found.

Pea flours:

The results indicated no effect when comparing plasma PYY between wild and SBE-deficient flour ($p=0.37$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.62$).

No main effects of flour ($p=0.47$) or flour X time interaction ($p=0.31$) were found.

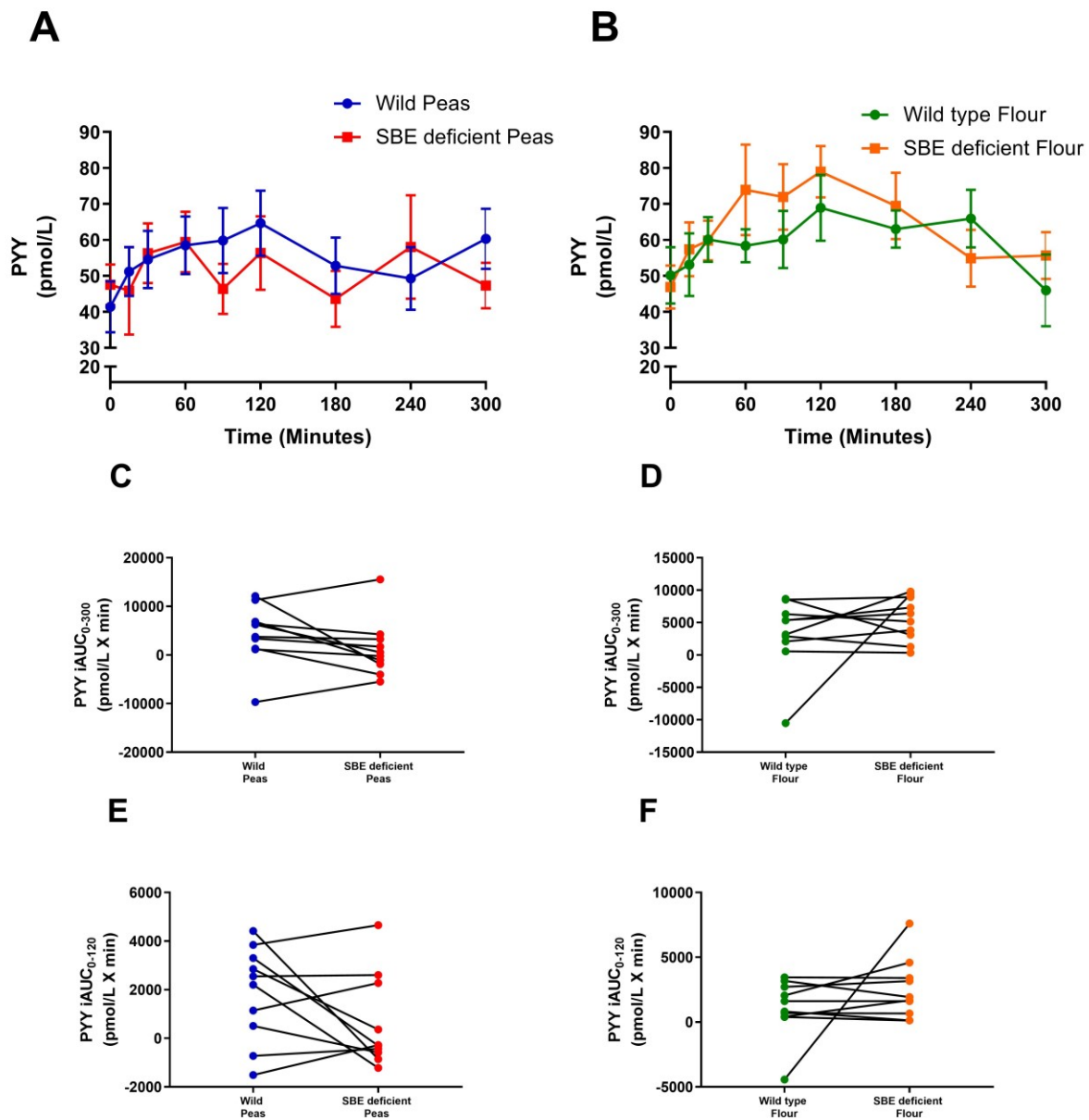


Figure 3.9: Postprandial PYY in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 3.10 shows time course, iAUC₀₋₃₀₀, and iAUC₀₋₁₂₀ data for plasma PYY concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma PYY iAUC₀₋₃₀₀ between wild peas and wild flour ($p=0.27$). The same lack of effect was observed when comparing plasma PYY iAUC₀₋₁₂₀ between wild peas and wild flour ($p=0.16$).

No main effects of meal ($p=0.58$) or meal X time interaction ($p=0.12$) were found.

SBE-deficient pea seeds and flour:

The results indicated a significant increase in plasma PYY iAUC₀₋₃₀₀ following SBE-deficient flour consumption (5546.4 ± 1066.8 pmol/L x min) when compared to SBE-deficient pea consumption (1265.5 ± 1850.2 pmol/L x min) ($p=0.04$). A similar borderline effect was observed when comparing plasma PYY iAUC₀₋₁₂₀ following SBE-deficient flour consumption (2491.8 ± 730.8 pmol/L x min) when compared to SBE-deficient pea consumption (623.1 ± 603.5 pmol/L x min). However, this difference did not reach a statistically significant level ($p=0.08$).

A main effect of meal ($p=0.05$) was found. However, no meal X time interaction ($p=0.18$) was found. A significant increase in plasma PYY was found at 90 minutes ($p<0.01$), 120 minutes ($p<0.01$), and 300 minutes ($p=0.02$) following SBE-deficient flour consumption when compared to SBE-deficient pea consumption.

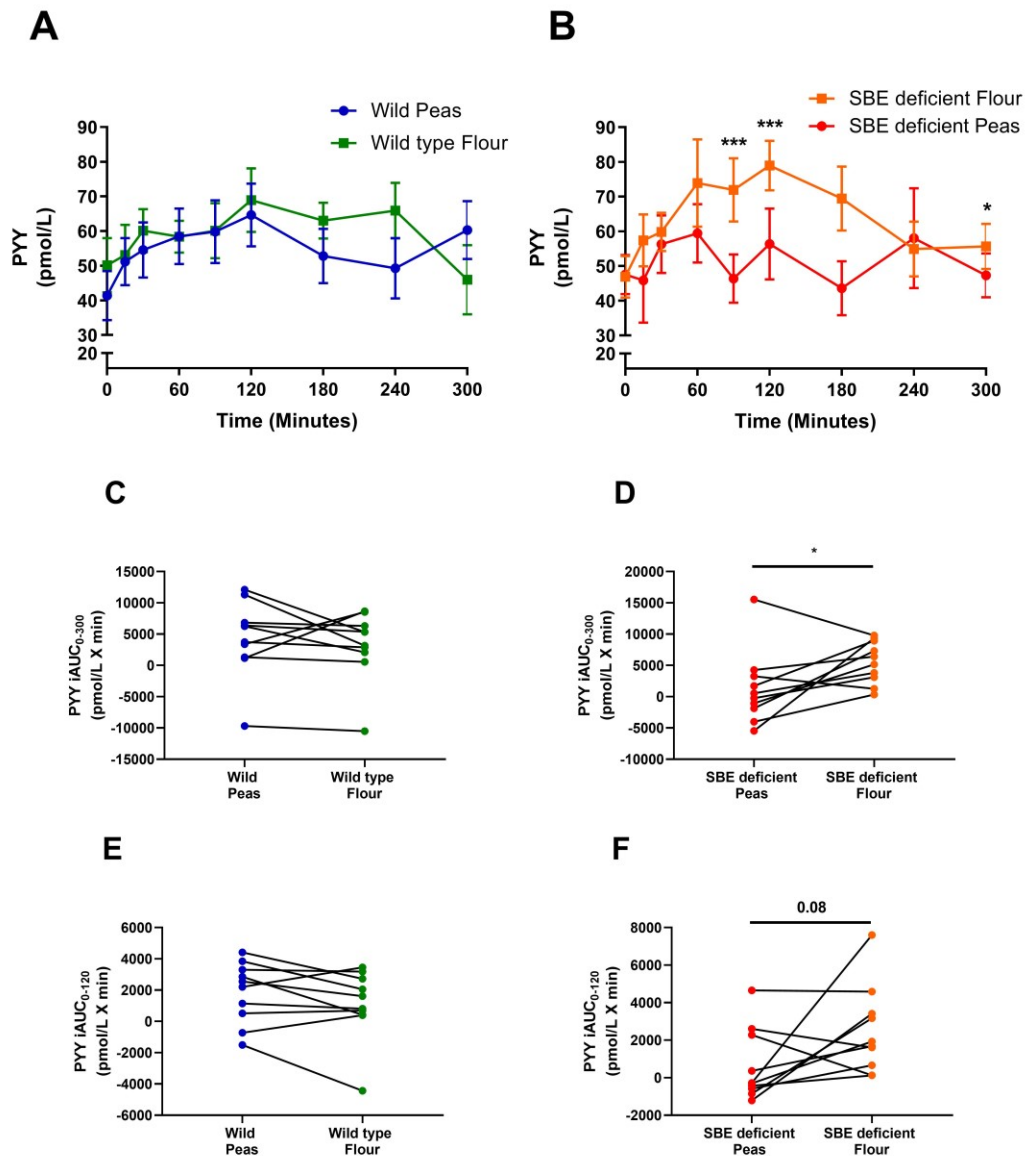


Figure 3.10 Postprandial PYY in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₃₀₀ for wild peas (●) and iAUC₀₋₃₀₀ for wild flour (●), (D) SBE-deficient peas (●) and SBE-deficient flour (●), (E) wild peas (●) and iAUC₀₋₁₂₀ for wild flour (●), (F) SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

3.4.7 Breath hydrogen

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 3.11 shows time course and tAUC data for breath hydrogen excretion readings, comparing the effects of mutation between whole pea seeds and between pea flours.

There were no differences in baseline values.

Whole pea seeds:

The results indicated no effect when comparing breath hydrogen excretion between wild and SBE-deficient peas ($p=0.95$).

No main effects of peas ($p=0.96$) or pea X time interaction ($p=0.51$) were found.

Pea flours:

The results indicated no effect when comparing breath hydrogen excretion between wild and SBE-deficient flour ($p=0.78$).

No main effects of flour ($p=0.94$) or flour X time interaction ($p=0.19$) were found.

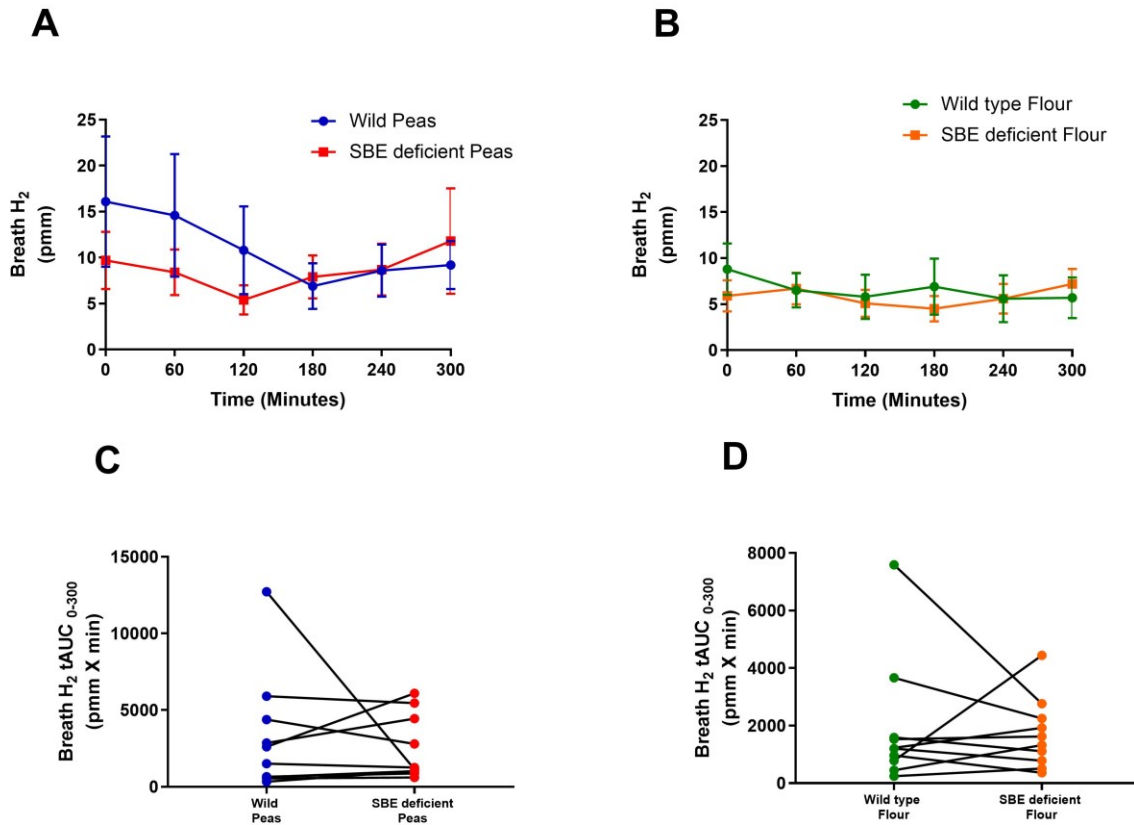


Figure 3.11: Breath hydrogen excretion in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for tAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 3.12 shows time course and tAUC data for breath hydrogen excretion readings, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

There were no differences in baseline values.

Wild pea seeds and flour:

The results indicated no effect when comparing breath hydrogen excretion between wild peas and wild flour ($p=0.43$).

No main effects of meal ($p=0.51$) or meal X time interaction ($p=0.36$) were found.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing breath hydrogen excretion between SBE-deficient peas and SBE-deficient flour ($p=0.25$).

No main effects of meal ($p=0.29$) or meal X time interaction ($p=0.52$) were found.

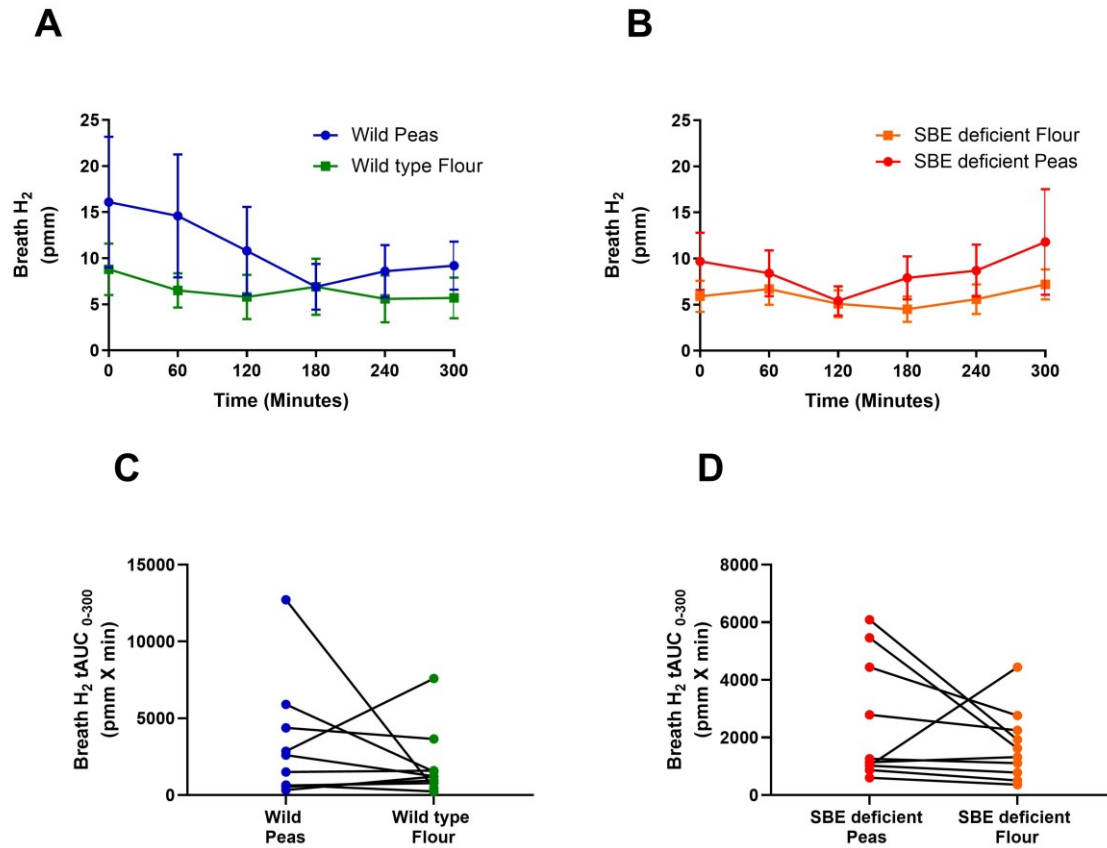


Figure 3.12: Breath hydrogen excretion in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). (C) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (D) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for tAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.4.8 Visual analogue scale

3.4.8.1 Hunger

Figure 3.13 presents time course and tAUC data on subjective hunger, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective hunger scores between wild and SBE-deficient pea consumption ($p=0.14$).

Pea flours:

There were no differences in subjective hunger scores between wild and SBE-deficient flour consumption ($p=0.37$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds and flour:

There were no differences in subjective hunger score between wild peas and wild flour consumption ($p=0.69$)

SBE-deficient pea seeds and flour:

There were no differences in subjective hunger scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.11$).

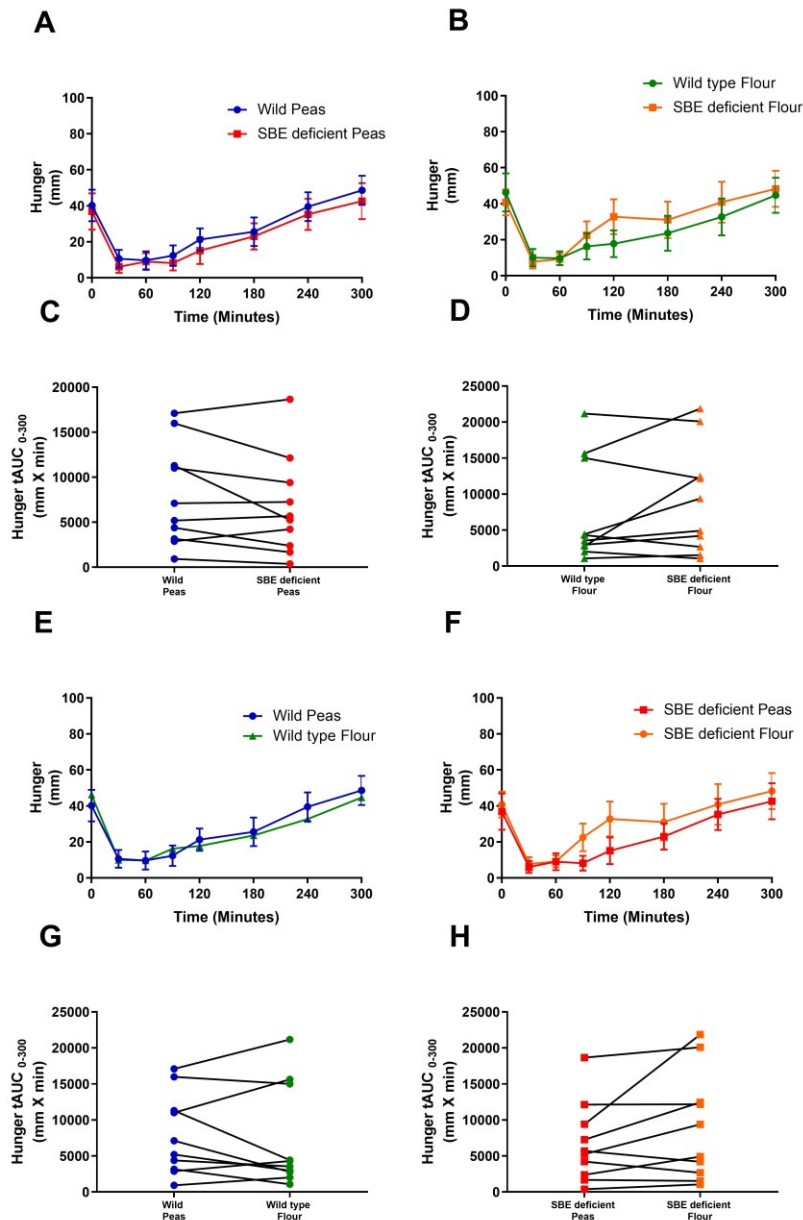


Figure 3.13 Subjective hunger score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve.

3.4.8.2 Fullness

Figure 3.14 shows time course and tAUC data on subjective fullness, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective fullness scores between wild and SBE-deficient peas consumption ($p=0.32$).

Pea flours:

There were no differences in subjective fullness scores between wild and SBE-deficient flour consumption ($p=0.26$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective fullness scores between wild peas and wild flour consumption ($p=0.47$).

Mutant pea seeds:

There were no differences in subjective fullness scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.33$).

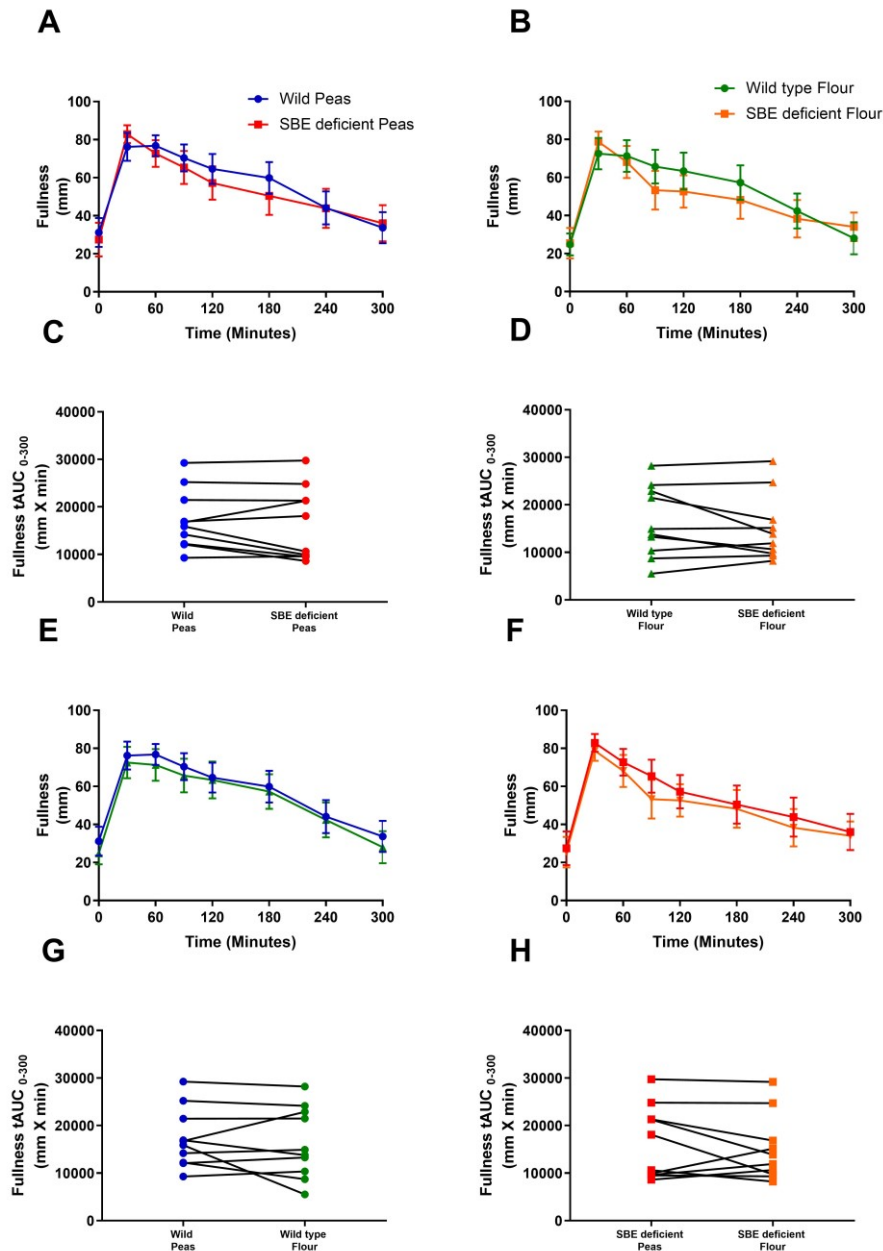


Figure 3.14 Subjective fullness score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.4.8.3 Desire to eat

Figure 3.15 presents time course and tAUC data on subjective desire to eat scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient peas consumption ($p=0.14$).

Flour pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient flour consumption ($p=0.33$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective desire to eat scores between wild peas and wild flour consumption ($p=0.93$).

Mutant pea seeds:

There were no differences in subjective desire to eat scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.40$).

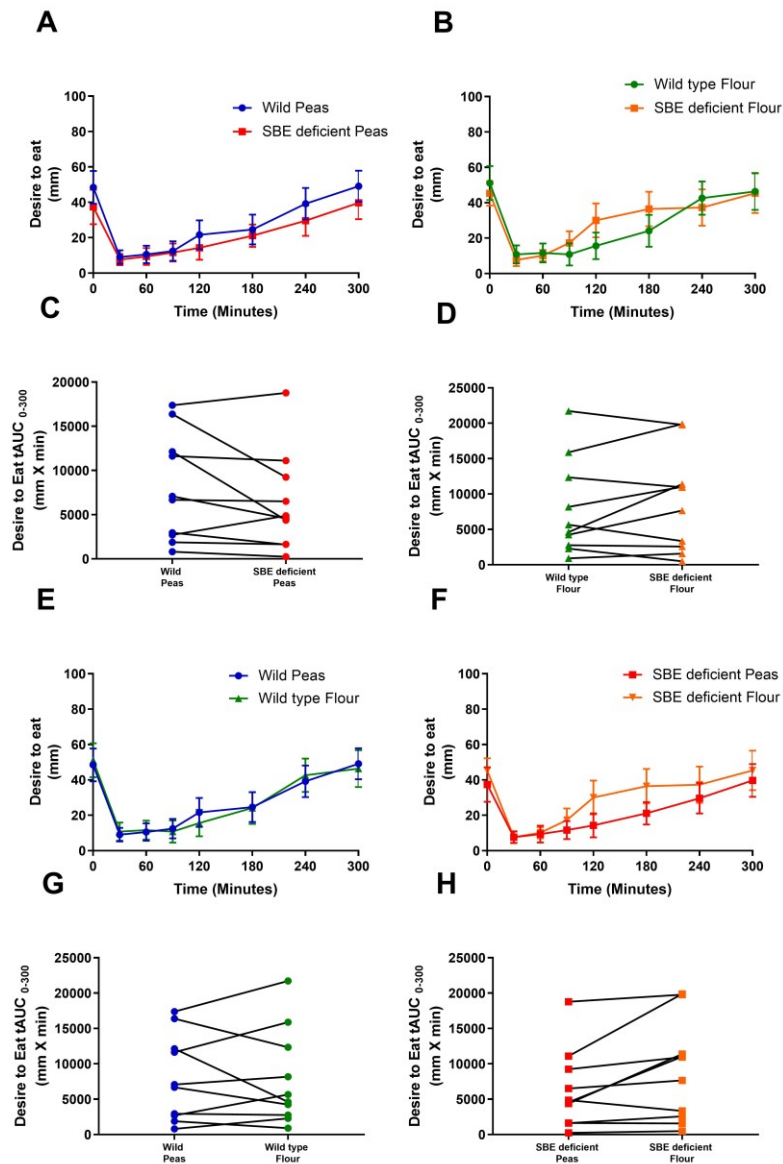


Figure 3.15 Subjective desire to eat score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.4.8.4 Appetite for a meal

Figure 3.16 shows time course and tAUC data on subjective appetite for a meal scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective appetite for a meal scores between wild and SBE-deficient peas consumption ($p=0.22$).

Flour pea seeds:

There were no differences in subjective appetite for a meal scores between wild and SBE-deficient flour consumption ($p=0.14$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective appetite for a meal scores between wild peas and wild flour consumption ($p=0.91$)

Mutant pea seeds:

There were no differences in subjective appetite for a meal scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.40$).

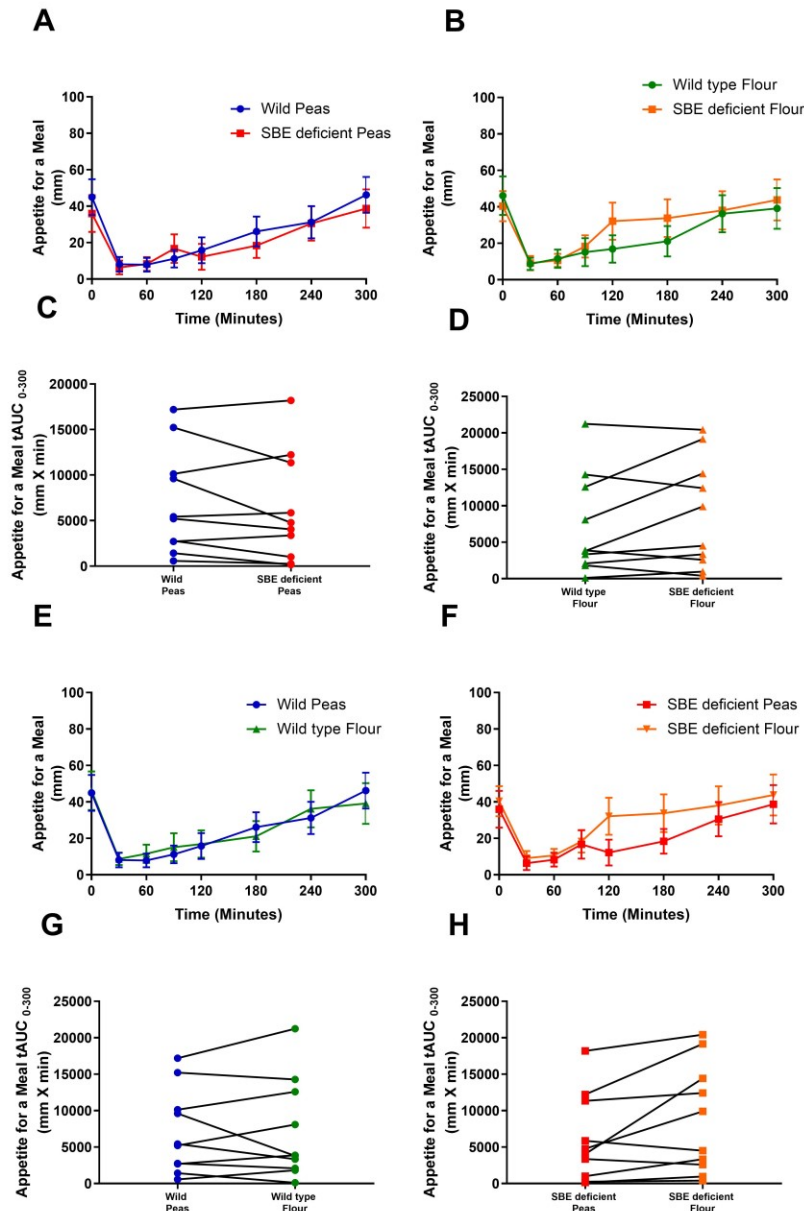


Figure 3.16 Subjective appetite for a meal score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.4.8.5 Composite appetite score

Figure 3.17 shows time course and tAUC data on composite appetite scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective composite appetite scores between wild and SBE-deficient peas ($p=0.37$).

Flour pea seeds:

There were no differences in subjective composite appetite scores between wild and SBE-deficient flour ($p=0.19$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective composite appetite scores between wild peas and wild flour ($p=0.92$).

Mutant pea seeds:

There were no differences in subjective composite appetite scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.08$).

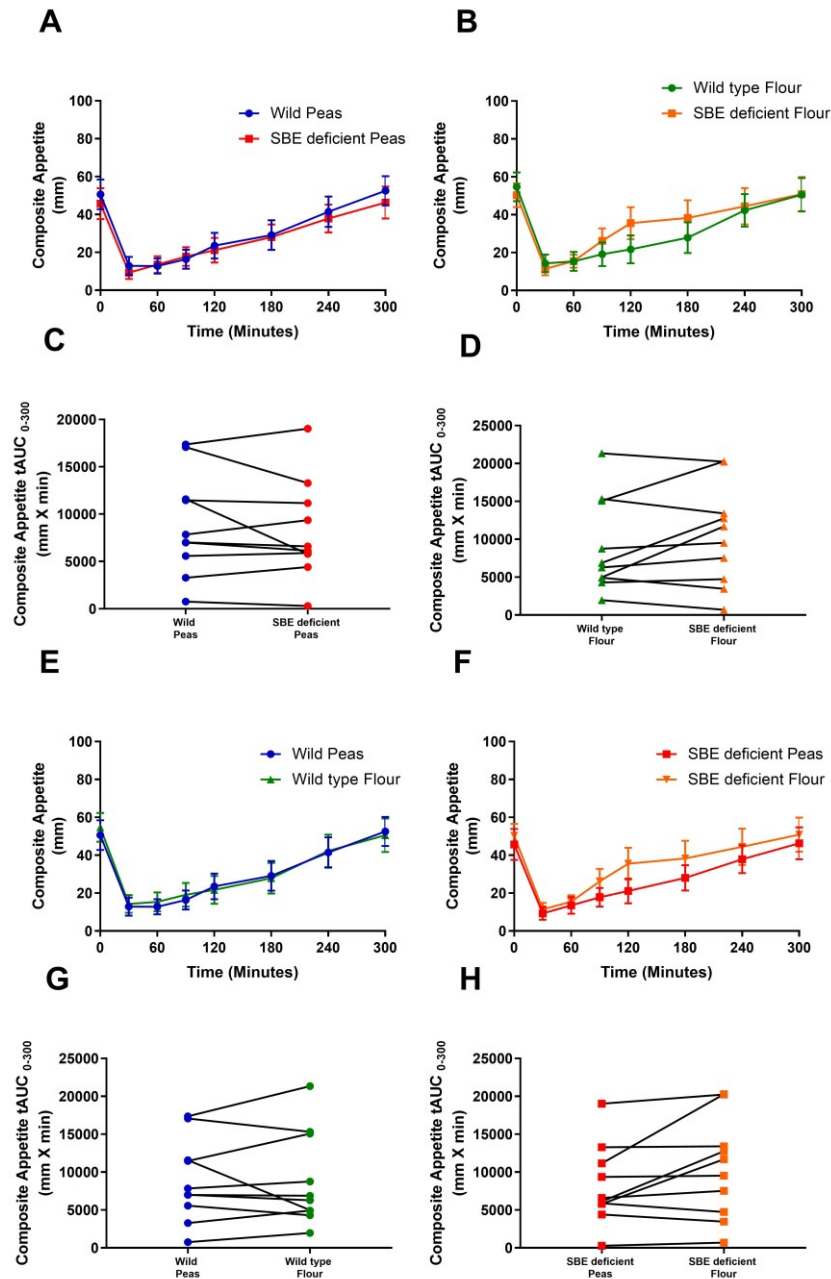


Figure 3.17 Subjective composite appetite score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.4.8.6 Nausea

Figure 3.18 presents time course and tAUC data on nausea scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient peas ($p=0.98$).

Flour pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient flour ($p=0.96$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective nausea scores between wild peas and wild flour ($p=0.12$)

Mutant pea seeds:

There were no differences in subjective nausea scores between SBE-deficient peas and SBE-deficient flour ($p=0.35$).

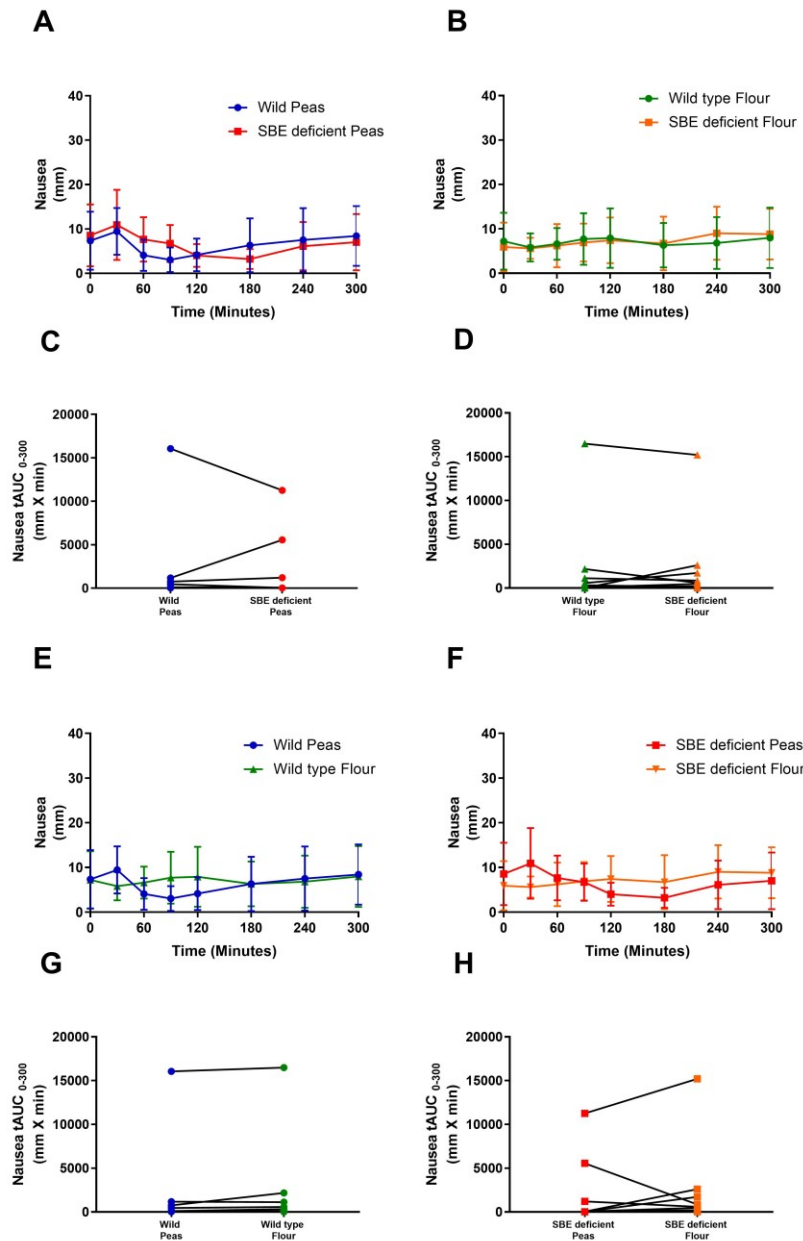


Figure 3.18 Subjective nausea score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₃₀₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₃₀₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₃₀₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₃₀₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

3.5 Discussion

3.5.1 Summary of findings

Primary findings looking at the effect of starch branching enzyme mutation

- GE half-times did not differ between SBE-deficient or wild pea seeds. The same lack of effect was shown in the case of pea flours.
- Plasma glucose was reduced following SBE-deficient consumption compared to wild pea consumption. The same effect was shown in the case of pea flours.
- Serum insulin was not affected differently by SBE-deficient or wild peas. The same lack of effect was shown in the case of pea flours.
- Plasma GLP-1 was not affected differently by SBE-deficient or wild peas. The same effect was not shown in the case of pea flours, as plasma GLP-1 was increased following wild flour consumption when compared with SBE-deficient flour consumption.
- Plasma PYY was not affected differently by SBE-deficient or wild peas. The same lack of effect was shown in the case of pea flours.
- Breath hydrogen was not affected differently by SBE-deficient or wild peas. The same lack of effect was shown in the case of pea flours.
- VAS was not affected differently by SBE-deficient or wild peas. The same lack of effect was shown in the case of pea flours.

Secondary findings looking at the effect of cellular structure

- GE half-time was not affected differently by wild pea seeds or flour consumption. The same finding was shown in the case of SBE-deficient pea seeds and flour.
- Plasma glucose was not affected differently by wild pea seeds or flour consumption. The same finding was shown in the case of SBE-deficient pea seeds and flour.
- Serum insulin was reduced following wild pea consumption when compared to wild flour consumption. The same reduction was observed following SBE-deficient pea consumption when compared to SBE-deficient flour consumption.

- Earlier secretion of plasma GLP-1 was increased following wild flour consumption when compared to wild pea consumption. The same effect was not shown in the case of SBE-deficient pea seeds and flour.
- Plasma PYY was not affected differently following wild pea and flour consumption. However, plasma PYY was increased following SBE-deficient flour consumption when compared to SBE-deficient pea seed consumption.
- VAS was not affected differently following wild pea and flour consumption. Moreover, VAS was not affected differently following SBE-deficient pea and SBE-deficient flour consumption.

3.5.2 Detailed discussion

Given the structural properties resulting from mutation in the starch branching enzyme, it is hypothesised that SBE-deficient peas can decrease/delay starch digestion, which would be reflected as a change in glycaemia. However, it is important to rule out the effect of GE, which is a major determinant of postprandial glycaemic response. This study tested the effect of a single starch assembly mutation of peas compared to the wild control and/or the effect of whole pea structure compared to processed pea flour on postprandial glycaemia and examined whether this response was a result of GE.

3.5.2.1 The effect of starch branching enzyme mutation

3.5.2.1.1 Gastric emptying

Whole pea seeds

GE half-times did not differ between SBE-deficient and wild pea seeds.

Pea flours

GE half-times did not differ between SBE-deficient and wild pea flours.

The ¹³C data tracked from breath samples showed that the GE rate was not affected by the starch branching enzyme mutation of peas when compared to controls with a similar structure,

suggesting it takes a similar amount of time for the different pea seed meals to be digested in the stomach. Thus, any differences in metabolic outcome resulting from these interventions would be attributed to structural differences and GI digestion beyond the stomach level.

3.5.2.1.2 Glucose, insulin, and GLP-1 responses

Whole pea seeds

Plasma glucose was higher following wild pea seed consumption, with a significant plasma glucose peak found at 30 minutes, followed by a decline at 60 minutes, compared to consumption of SBE-deficient pea seeds. Serum insulin and plasma GLP-1 were not affected differently when compared between SBE-deficient and wild pea seeds.

Pea flours

Plasma glucose was higher following wild pea flour consumption, with significant plasma glucose at 90 minutes, compared to SBE-deficient pea flour consumption. Serum insulin was not affected differently when compared between SBE-deficient and wild pea flours. Plasma GLP-1 was increased following wild pea flour consumption at 90 minutes, compared to SBE-deficient pea flour consumption.

Independent to GE, both physical forms (SBE-deficient pea seeds and flour) resulted in lower glucose response when compared to wild pea seeds and flour. Previous studies examining the effect of plant-based food structures on glucose and insulin responses have found that the amount of RS, specifically the amylose-to-amylopectin ratio, as well as food processing are significant factors determining glucose metabolism (Kristensen *et al.*, 2010; Priebe *et al.*, 2018). Atomic force microscopy on SBE-deficient peas has shown there is a different structure acquired due to the natural mutation in one of the starch branching enzymes (Bhattacharyya *et al.*, 1990; Bogracheva *et al.*, 1999). This has resulted in an amylose-to-amylopectin ratio of 70:30% within SBE-deficient pea granules, compared to 30:70% within wild pea granules (Bhattacharyya *et al.*, 1990). Due to its high surface area as a result of its high branching, amylopectin is more prone to amylase penetration and digestion (Zhang, Ao and Hamaker, 2006). Thus, it can be speculated that the differences shown between plasma glucose concentrations are driven by the higher

amylose-to-amylopectin ratio of SBE-deficient pea seeds and flour and the subsequent limited penetration of digestive amylase when compared with wild pea seeds and flour, respectively. These results are in line with previous studies showing that high-amylose starches can have a major impact on glucose and insulin responses by reducing the incremental area under the glucose and insulin concentration curves or lowering glucose and insulin responses at early time points (Panlasigui *et al.*, 1991; Behall and Scholfield, 2005; Brighenti *et al.*, 2006; Anderson *et al.*, 2010). Moreover, since the flour results are in line with the results from whole pea seeds, it can be further concluded that the differences in amylose-to-amylopectin ratios between the two lines of peas is the factor responsible for the differences in glucose response. This is because the structural integrity of whole pea seeds was lost in the process of milling to produce the flour, and this can make food particles smaller with a higher surface-to-volume ratio and also make the carbohydrate content readily available and thus penetrable by amylase. However, it is not possible from the current study to solely explain the differences in glucose levels by the relative digestion and breakdown of peas through the GI tract. To do that, sampling and tracking of ingested peas are needed, which are beyond the scope of this study but are addressed in the next experimental chapter of this thesis.

Despite the reduction seen in glycaemia, there was no change in serum insulin following SBE-deficient pea consumption. The lower plasma glucose seen following SBE-deficient pea seed and flour consumption suggests that the amount of serum insulin needed to clear that amount of glucose is also low, as was shown previously following higher amylose starch consumption (Behall, Scholfield and Canary, 1988). It is noteworthy to highlight the variability in insulin levels when compared to the tightly regulated glucose in this healthy data set of participants, which could explain the insulin results. Moreover, although serum insulin was lower following SBE-deficient flour consumption when compared to wild flour consumption, this difference was not significant. However, a sustained release of GLP-1 started at 30 minutes, with a significant peak at 90 minutes following wild flour consumption when compared to SBE-deficient flour consumption. In the context of the current study, this could be explained by the wild flour having more available starch to stimulate GLP-1 secretion than SBE-deficient flour does, as a positive

association was previously highlighted between glucose load and the increase in plasma GLP-1 (Pilichiewicz *et al.*, 2007).

3.5.2.1.3 Breath hydrogen, appetite measures, and plasma gut hormones

Whole pea seeds

Plasma GLP-1 and PYY were not affected differently by SBE-deficient peas and wild peas; the same was true for breath hydrogen and VAS.

Pea flours

Plasma PYY, subsequent secretion of plasma GLP-1, breath hydrogen, and VAS questions were not affected differently by SBE-deficient flour and wild flour.

It is proposed that as the digesta moves along the GI tract, the undigested components can serve as a substrate for microbiota fermentation, leading to SCFA production and gut hormone secretion. However, in the current study, there were no differences in breath hydrogen, which is a marker of gut fermentation. There were also no differences in PYY and subsequent secretions of GLP-1, and there was no effect on VAS. This might suggest that more time was required to address the effect of fermentation, if any, in the lower gut. However, it must be noted that, although it was not designed to, the current study has helped to estimate the time needed for fermentation to take place (> 6 hours). A future study discussed in this thesis has been planned to examine the effect of fermentation on the undigested pea remnants together with its impact on metabolism.

3.5.2.2 Secondary findings looking at the effect of cellular structure

3.5.2.2.1 Gastric emptying

Wild pea seeds and flour

GE half-times were not affected differently following wild flour and wild pea consumption.

SBE-deficient pea seeds and flour

GE half-times were not affected differently following SBE-deficient peas and SBE-deficient flour consumption.

The ^{13}C data tracked from breath samples showed that the GE rates were not affected differently by the structure of pea flour when compared to whole pea seeds with a similar genotype, suggesting it takes a similar amount of time for the different pea seed meals to be digested in the stomach. Thus, any differences in metabolic outcome resulting from these interventions would be attributed to the chemical composition differences and GI digestion beyond the stomach level.

3.5.2.2.2 Glucose, insulin, and GLP-1 responses

Wild pea seeds and flour

There was no difference in plasma glucose following wild flour consumption when compared to wild peas. This result was accompanied with an increase in serum insulin levels at time-point 60 minutes and an early secretion of plasma GLP-1 incretin (at 30 minutes), suggesting early stimulation by glucose during the digestive process and explaining the expected regulatory mechanism of glucose homeostasis in the currently healthy participants.

SBE-deficient pea seeds and flour

There was no difference in plasma glucose following SBE-deficient pea and flour consumption. Serum insulin was higher following the consumption of SBE-deficient flour when compared to SBE-deficient pea consumption. However, plasma GLP-1 was not affected differently by SBE-deficient pea seeds and flour.

Processing whole pea seeds into flour in both lines resulted in postprandial increase in insulin responses. These findings support the role of food matrices as a preventive barrier against digestive enzymes, thereby affecting the extent of postprandial metabolic response (Parada and Aguilera, 2011). Interestingly, plasma GLP-1 levels showed a significant peak at 30 minutes, with a sustained increase and another significant peak at 90 minutes following wild flour consumption

when compared to wild pea consumption. This means that wild flour had more available starch to stimulate GLP-1 secretion when compared to wild peas. Together, as the same effect was not apparent between SBE-deficient pea seeds and flour, these findings highlight the major impact of the food structure and intrinsic starch properties on the bioaccessibility of the substrate to pancreatic α -amylase, which is subsequently reflected in postprandial metabolic responses.

3.5.2.2.3 Breath hydrogen, appetite measures, and plasma gut hormones

Wild pea seeds and flour

Plasma PYY, subsequent secretion of plasma GLP-1, breath hydrogen, and VAS were not affected differently by wild pea seeds and flour.

SBE-deficient pea seeds and flour

Plasma GLP-1 and breath hydrogen were not affected differently by wild pea seeds and flour. However, plasma PYY was increased following SBE-deficient flour consumption when compared to SBE-deficient pea seed consumption. In contrast, the subjective desire to eat, appetite for a meal, and overall composite appetite scores were reduced following SBE-deficient pea consumption when compared to SBE-deficient flour consumption.

The differences in PYY secretion between SBE-deficient pea seeds and flour can be attributed to the effect of milling, which increases the surface area of the substrate and exposes entrapped nutrients, thus stimulating gut hormone secretion (Lim and Brubaker, 2006; Wachters-Hagedoorn *et al.*, 2006). Moreover, the different methods of cooking used for the peas and flour could have affected the availability and characteristics of the intrinsic starch. However, these factors did not affect the results between wild pea seeds and flour, leaving the difference in postprandial plasma PYY levels between SBE-deficient pea seed and flour consumption unexplained. Moreover, these results are not in line with the VAS and thus require further investigation.

3.5.3 Strengths, weaknesses, and future recommendations

The non-invasive use of the ^{13}C octanoic acid method to measure GE by tracking the label excretion in breath samples (Ghoos *et al.*, 1993; Geypens *et al.*, 1999) was a major strength of the current study. In addition following a cross-over design allowed for the removal of intra-individual variability. However, including other sources of carbohydrates in the mixed meal was a limitation in terms of measuring the metabolic response to the different pea interventions, as it could have masked the actual effects. Thus, it is not clear whether the glycaemic responses seen were related to the starch digestibility in peas or due to the peas limiting the absorption of other carbohydrate sources. Moreover, having different cooking protocols for peas and pea flours did not allow for valid comparison between structures. This is because different processing methods can affect starch digestibility; for example, boiling legumes can cause a loss of integrity of plant CWs, thus increasing permeability and amylase ingress during digestion (McDougall *et al.*, 1996). Moreover, boiling legumes in high-temperature excess water can increase granule swelling and hence the degree of gelatinisation and solubility, which increases starch digestibility (Slaughter *et al.*, 2002; Rehman and Shah, 2005). These changes in the physicochemical and structural characteristics of polysaccharides have been shown to influence the biological and metabolic properties of legumes, such as the glycaemic response (Jenkins *et al.*, 1982). Meanwhile, microwaving utilises less water and involves a shorter duration and thus causes different changes to the molecular structures compared to conventional cooking (Geng, Zongdao and Toledo, 2003; Anderson and Guraya, 2006; Zhang, Wang and Shi, 2009; Singh, Dartois and Kaur, 2010). Future work is required to determine the exact metabolic responses to pea ingestion solely without the inclusion of any confounders, ensuring similar handling of cooking protocols.

3.6 Conclusion

This study aimed to test the effect of different pea interventions on postprandial glycaemia in relation to GE. There were differences in glycaemic response following the ingestion of SBE-deficient peas as part of mixed meal when compared to both wild peas and SBE-deficient flour. However, these effects were independent of GE delays. Nevertheless, these results suggest that

these differences in glucose response were related to the amylose-to-amylopectin ratios and the effects of milling. The following study should help to further explain the effect of these factors on starch digestion and the mechanism of a single mutation and/or the effect of processing on energy homeostasis.

Chapter 4

4. Evaluating the impact of food structure from peas on small intestinal digestion

4.1 Background

The results from the previous experimental chapter confirmed the positive effects of SBE-deficient pea seeds on acute postprandial glycaemic response, which was independent of GE delaying. The current study seeks to understand whether starch digestion in the duodenum and consequent glucose absorption is responsible for their positive effects.

Starches in plant foods, depending on their botanical origin, can have complex matrix structures. Intracellular saccharides need to be released from tissue in order to be susceptible to amylase action and thus digested in the human small intestine. Differences in glycaemic response depend significantly on the rate and extent of starch amylolysis in the small intestine (O'Dea, Snow and Nestel, 1981; Jenkins *et al.*, 1982; Holm and Bjorck, 1992). Many factors have been shown to influence the kinetics of starch digestion, including the nature of the intrinsic starch and food processing (Singh, Dartois and Kaur, 2010). Manipulating existing foods and/or ingredients to limit digestion and subsequent nutrient absorption in the small intestine is an attractive strategy for controlling metabolic responses and reducing the amount of small positive energy consumed daily. Thus, understanding the interplay between food structure and small intestinal digestion can provide valuable information for combatting obesity in the long run.

Many studies have sought to understand the digestion behaviours of different food structures within the human GI tract. However, limitations of these studies, such as the use of in vitro digestion models or sampling from ileostomy patients, prevent the wider translation to the rest of the population (Englyst, Kingman and Cummings, 1992; Silvester, Englyst and Cummings, 1995; Englyst *et al.*, 1996a; Edwards, Grundy, *et al.*, 2015). In the current study, an in vivo method of the CORTRACK Enteral System was used, in which intubation of the small intestine allows for direct sampling during GI tract digestion in healthy participants without the need for radiation.

4.1.1 The interplay between food structure and small intestine digestion

Starches in plant foods are stored in the cotyledons and endosperm as granules, which vary in shape, structural organisation, and size depending on their botanical origin. Moreover, factors discussed in Chapter 1, such as granule porosity, degree of gelatinisation, granule morphology

and crystallinity, and amylose-to-amylopectin ratio, can all contribute to the extent of starch digestion, postprandial glucose, and RS delivery to the colon. It was interesting to discover that intact plant cells can still contain their enclosed nutrients (e.g. starch, lipid) in ileal effluent after the consumption of plant foods (Livesey *et al.*, 1995a; Noah *et al.*, 1998; Ellis *et al.*, 2004). The nutrient restoration was attributed to the resistance of the plant CWs to digestion in the upper GI tract, which might, if structurally intact, protect the enclosed nutrients from digestive enzymes, therefore delaying and/or limiting accessibility (McDougall *et al.*, 1996; Ellis *et al.*, 2004). Postprandial glucose and insulin responses to foods with similar starch contents or even to the same food can differ greatly depending purely on processing. Indeed, it was reported that postprandial glycaemia and insulinemia were attenuated in healthy participants following the consumption of structurally intact starchy foods as opposed to foods processed with no structure equivalents (i.e. ruptured cells) (Noah *et al.*, 1998; Edwards, Grundy, *et al.*, 2015). Similarly, the inclusion of undamaged legume cells in mixed meals has been shown to significantly lower the iAUC of blood glucose and insulin responses in patients with type 2 diabetes when compared with damaged legume cells (Golay *et al.*, 1986). These results confirm that encapsulated starch within leguminous cells is protected from digestion in the small intestine and can subsequently result in lower glycaemic response compared with non-encapsulated starch. Ultimately, unabsorbed plant food macronutrients and micronutrients in the small intestine reach the lower GI tract, serving as a substrate for microbiota fermentation to produce SCFAs (Cummings, 1981); otherwise, it would not be available for fermentation and would be excreted in faeces instead (McDougall *et al.*, 1996; Elia and Cummings, 2007).

Thus, nutrient composition alone is not enough to fully characterise a food product, as other properties like food structure and intrinsic starch are essential when considering the behaviour of foods in the GI tract and their subsequent postprandial metabolic effects. For example, the digestibility of plant foods with high-amylose starches is decreased due to limited starch digestibility. Limited digestibility was reflected by hindered salivary and pancreatic α -amylase access, affecting the release of starch hydrolysis products (e.g. maltotriose and maltose), which would be further converted into glucose by the action of the brush border enzymes (e.g. maltase-glucoamylase (MGAM) and sucrose-isomaltase (SI)) during intestinal digestion, subsequently

affecting postprandial glycaemia (Golay *et al.*, 1986; Livesey *et al.*, 1995b; Noah *et al.*, 1998). Therefore, measuring intestinal glucose concentration, the main site of glucose absorption (O'Dea, Snow and Nestel, 1981; Jenkins *et al.*, 1982; O'Donovan *et al.*, 2004), can help to identify the glucose release behaviour during starch amylolysis (Edwards *et al.*, 2014, 2018) and reflect the digestibility of the referenced starchy food (Englyst, Kingman and Cummings, 1992; Englyst *et al.*, 1996b), which will ultimately be reflected as a glycaemic plasma response (Jenkins *et al.*, 2000, 2002). Here, an attempt was made to understand the role of small intestinal digestion with regards to different foods structures and different intrinsic starch characteristics. This information can be used later to further explain the lower postprandial glucose levels seen in the previous experimental chapter in response to SBE-deficient pea seed consumption. Volunteers were asked to consume 50 g dry weight of pea seeds and flours from both genotypes, and postprandial parameters were collected for a period of 180 minutes. In addition, a collaborative work with the Quadram Institute of Food Research, Norwich, has kindly analysed the different lines of pea seeds with scanning electron microscopy (figures are presented).

4.2 Purpose of the study

4.2.1 Hypothesis

It was hypothesised that SBE-deficient whole pea seeds and flour would reduce postprandial glycaemia due to their digestion profiles in the small intestine, which differ from those of wild pea seeds and flour.

4.2.2 Aim

Measuring glucose concentrations at the duodenum level will give a better understanding of the digestion profiles of different pea interventions at the upper GI level, which would explain the plasma glycaemic response.

4.2.3 Outcome measures

Co-primary outcomes were:

- Plasma glucose levels
- Small intestinal glucose levels

Secondary outcomes were:

- Serum insulin and plasma gut hormones; GLP-1 and PYY levels
- VAS to assess subjective appetite

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Peas

Please refer to Chapter 2.

4.3.1.2 Randomisation

Participants were randomised using an independent telephone and internet company (sealedenvelope.com). Both the researcher and volunteers were blinded to the type of dietary intervention consumed on each study visit and/or during the study duration.

4.3.1.3 Sample size and power calculation

This was a descriptive exploratory study in a new area, and thus a power calculation was not possible. Twelve volunteers participated in this study.

4.3.1.4 Ethical approval

This study was approved by the South East-Coast Surrey Research Ethics Committee (REC project reference number 15/LO/0184) and the Imperial College Healthcare NHS Trust Joint Research Office. This study was performed in accordance with the Declaration of Helsinki.

4.3.1.5 Recruitment

The same recruitment strategy was used for all investigations. Volunteers were recruited through the National Institute for Health Research (NIHR)/Wellcome Trust Imperial CRF at Hammersmith Hospital healthy volunteers' panel database and through poster advertisements. PIS was provided to participants who expressed an interest in taking part in the study (Appendix 3). During the first stage, volunteer eligibility was determined through a brief phone interview. Before volunteers were able to participate in the study, they had to attend a screening visit at the NIHR/Imperial CRF at Hammersmith Hospital to further assess their eligibility. During the

screening visit, the study protocol was explained in full, and the participant provided full written informed consent before commencing any assessment (Appendix 4). The following measurements were collected: height, weight, BP, ECG, a standard screening blood sample, and a pregnancy test for all women of childbearing age. A research doctor had to check all of the volunteers' results before they were enrolled. Participants were then contacted with the results of their screening and given a start date for the study. Participants were asked not to start any intensive exercise regimen or new diet during the study period, as they could affect the results.

4.3.1.6 Volunteer inclusion and exclusion criteria

Healthy male and female volunteers between the ages of 18 and 65 years with a BMI of 20–35 kg/m² were invited to take part in this study. The exclusion criteria included the following: any gastrointestinal disease or syndrome; fluctuation in weight of ≥ 3 kg in the preceding two months; diabetes; pregnancy; excess alcohol intake; current smoking; history of substance abuse; cancer; cardiovascular, kidney, or liver diseases; pancreatitis; and the use of medications likely to interfere with hormonal balance, appetite regulation, and energy metabolism, including antibiotics, thyroid hormones, anti-inflammatory drugs or steroids, erythromycin, phenytoin, or androgens.

4.3.2 Method

4.3.2.1 Study day preparation and protocol

All participants were required to refrain from alcohol and strenuous exercise and to fast for 12 hours over night. The participants were asked to consume the same standard meal of their choice the evening before each visit to ensure consistency between the study visits.

Following overnight fasting from 09:00 p.m., volunteers were asked to attend the CRF at 09:00 a.m. (Figure 4.1). Before inserting the feeding tubes and consuming the test breakfast, participants were asked to empty their bladder in order to measure their weight and body composition using BIA. Participants were asked to stand barefoot on a Tanita segmental body composition analyser (Tanita MC-780MA analyser; Tanita Corporation, Japan). The BIA scale is

used to evaluate the total body water content with an electrical current. Then, TBW is used to estimate FFM, and fat mass is calculated by subtracting FFM from total body weight. Height was measured using a stadiometer. BMI was used to identify eligibility in order to recruit a homogenous cohort. After preparing for the study day, a gastroenterologist helped to place the nasoduodenal feeding tubes, which stayed in place for the four study days. Using the CORTAK (MedSystems, Halyard UK) feeding tube system made it possible to track the position of the tubes without the need for x-rays. To monitor placement and position, electromagnetic signals were received by the CORTACK system from a stylet at the tip of the tube. Samples were aspirated from the small intestinal content simultaneously using 50 ml syringes. To further confirm tube position, the pH of the fasting aspirated samples was tested.

4.3.2.2 Study design

This was an acute, randomised, controlled, double-blind, cross-over trial involving four study visits. Volunteers were required to attend a four-day study visit held in the Wellcome Trust Imperial CRF. Participants were asked to stay in the CRF for three nights.

After tube insertion, participants were given 30 minutes to rest, and later, a standardised breakfast was served at 0 minutes, which included pea treatments.

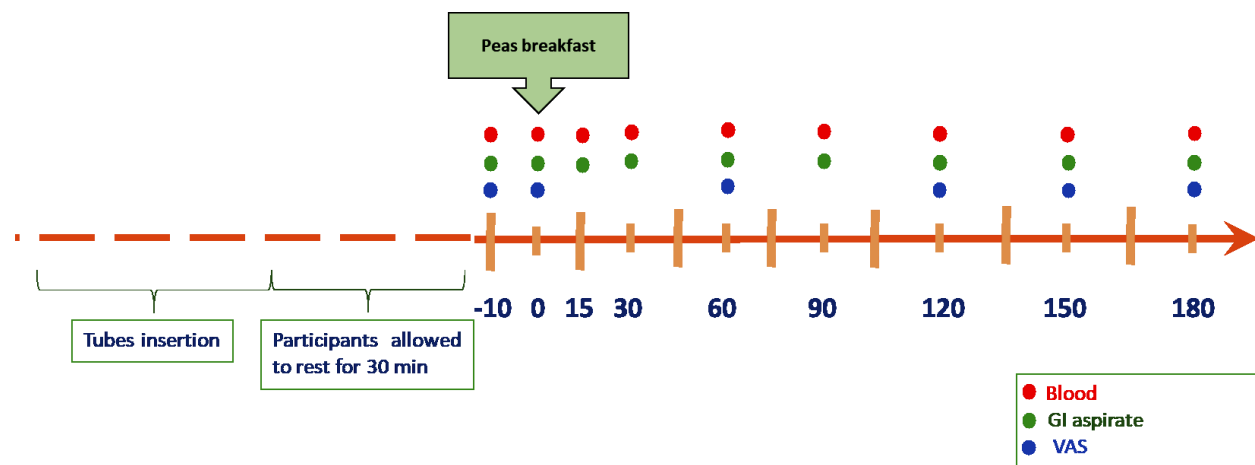


Figure 4.1: Study visit overview.

Overview of timings of blood sampling, VAS ratings and GI aspirates. Abbreviations: GI, gastrointestinal; VAS, visual analogue scale.

4.3.2.3 Study meal

A standardised breakfast was served at 0 minutes. In random order, the nutritionally matched test meals were freshly prepared so that they differed only in containing 50 g dry weight of each pea material (for more information about food material, please refer to chapter 2). The whole pea seeds and pea flours were soaked for 24 hours in 200 ml of water before cooking for 1 hour at medium temperature on the morning of the study visit. Test meals were served and consumed straight after cooking. Thus, any observed metabolic effects could be attributed to the starch properties or food structure of the test meals during GI digestive transit. Table 4.1 below lists the nutritional composition of study meal used in this study.

Table 4.1: Nutrition information of the current test meals provided from different pea lines

Food Products	Unit (g)	Kcal	CHO ^a (g)	Fat (g)	Protein (g)	Fibre (g)
Wild (peas & flour)	50	143	17.6	<0.5	10.6	18
SBE-deficient (peas and flour)	50	147	14	0.85	11.6	18.1

^a detailed carbohydrate analysis is not available

4.3.2.4 Duodenal samples

Samples of the duodenal content were collected. Fasting GI samples were collected in duplicate (at -10 and 0 minutes) before breakfast. Postprandial GI aspirates were collected at 15, 30, 60, 90, 120, 150, and 180 minutes after ingesting the test meal for glucose analysis (as previously detailed in chapter 2). Enteral tubes were removed after 180 minutes on the fourth day, and volunteers were discharged from the CRF. The % CV of this assay was 3.3%.

4.3.2.5 Subjective appetite scores

Please refer to Chapter 2.

4.3.2.6 Blood sampling, collection, and analysis

Please refer to Chapter 2 regarding sampling, collection, and analysis protocols.

4.3.2.6.1 Glucose analysis

The % CV of this assay was 3.1%. This CV is the average of twelve assays.

4.3.2.6.2 Insulin analysis

The % CV of this assay was 4.0%.

4.3.2.6.3 GLP-1 analysis

The % CV of this assay was 2.5%.

4.3.2.6.4 PYY analysis

The % CV of this assay was 4.1%.

4.3.2.7 Statistical analysis

Data were checked for normality using a Shapiro–Wilk test (defined as $P \geq 0.01$). In the case of non-normal distribution, data were normalised using log-transformation (the function LN or LN+1 where applicable). If the data were still not normally distributed, non-parametric tests were used. All figures are presented as raw data. The results are expressed as mean difference with $\pm$ standard error of the mean (SEM). The average pre and post data points were calculated and used to replace any missing data values. The two baseline samples for blood and VAS data were averaged and used as the 0 min measurement. Postprandial results are presented as tAUC for VAS and as iAUC for all blood parameters. tAUC and iAUC were calculated using the trapezoidal rule. iAUC data were presented as the mean values, and tAUC mean data were divided by the study visit time frame. AUC mean values were compared to look at the effect of starch branching enzyme mutation parameters (whole–whole and flour–flour) as well as the effect of cellular structure (wild whole pea seeds/flour and SBE-deficient whole pea seeds/flour), using a paired t-test or Wilcoxon signed-rank test where applicable. Repeated ANOVA measures were used with time and meal as ‘within’ factors to determine the effect of the test meals on postprandial

metabolic responses. Student's paired t-tests were used to identify time points with significantly different values between the two meals, and the Fisher LSD method was used to correct for multiple comparisons. The acute postprandial glucose response to foods was evaluated based on the incremental area of blood glucose concentration during a period of 2 hours post-ingestion (iAUC₀₋₁₂₀), which is the most dynamic period for measuring the metabolic response of a specific amount of food (usually 50 g of carbohydrates) compared to the response of a reference food (Jenkins *et al.*, 1982, 2002; American Diabetes Association *et al.*, 2008); thus, it was calculated for all blood parameters. A p-value ≤ 0.05 was considered statistically significant, with the significance level indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Statistical analysis was performed using IBM SPSS version 23. Graphs were prepared using GraphPad Prism version 7.04.

4.4 Results

4.4.1 Participant characteristics

Twelve adults were randomised to take part in the study. One of the participants only completed three of the four study visits. Consequently, the results of the biological samples involved only 11 volunteers for pea flours. All participants stayed in the CRF for four consecutive days. Thus, the demographic characteristics of the 12 participants were collected and averaged (Table 4.2).

Table 4.2: Demographic characteristics of participants recruited and completed the study (n=12)

<i>Characteristics</i>	Mean of 4 consecutive visits
<i>Male, female (n)</i>	7, 5
<i>Age (Y)</i>	45.8 ± 4.4
<i>Weight (Kg)</i>	69.5 ± 1.9
<i>BMI (Kg/m²)</i>	24.4 ± 0.6
<i>Body Fat (%)</i>	22.9 ± 2.4
<i>Data are means ± SEM or (n)</i>	

4.4.2 Plasma glucose

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 4.2 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on plasma glucose concentrations between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma glucose $iAUC_{0-180}$ between wild and SBE-deficient peas ($p=0.14$). However, the results indicated a trend towards a reduction when comparing plasma glucose $iAUC_{0-120}$ levels following the consumption of SBE deficient peas (39.1 ± 15.2 mmol/L) when compared to levels following the consumption of wild peas (72.7 ± 13.7 mmol/L) ($p=0.09$).

Main effects of meal ($p=0.02$) and meal X time interaction ($p=0.02$) were found. A significant plasma glucose peak was found at 60 minutes following wild pea consumption ($p<0.01$) when compared with SBE-deficient pea consumption.

Pea flours:

The results indicated no effect when comparing plasma glucose $iAUC_{0-180}$ between wild and SBE-deficient flour ($p=0.14$). The same lack of effect was observed in plasma glucose $iAUC_{0-120}$ concentrations between wild and SBE-deficient flour ($p=0.11$).

No main effect of peas was found ($p=0.41$), and a borderline effect of pea X time interaction was found ($p=0.06$).

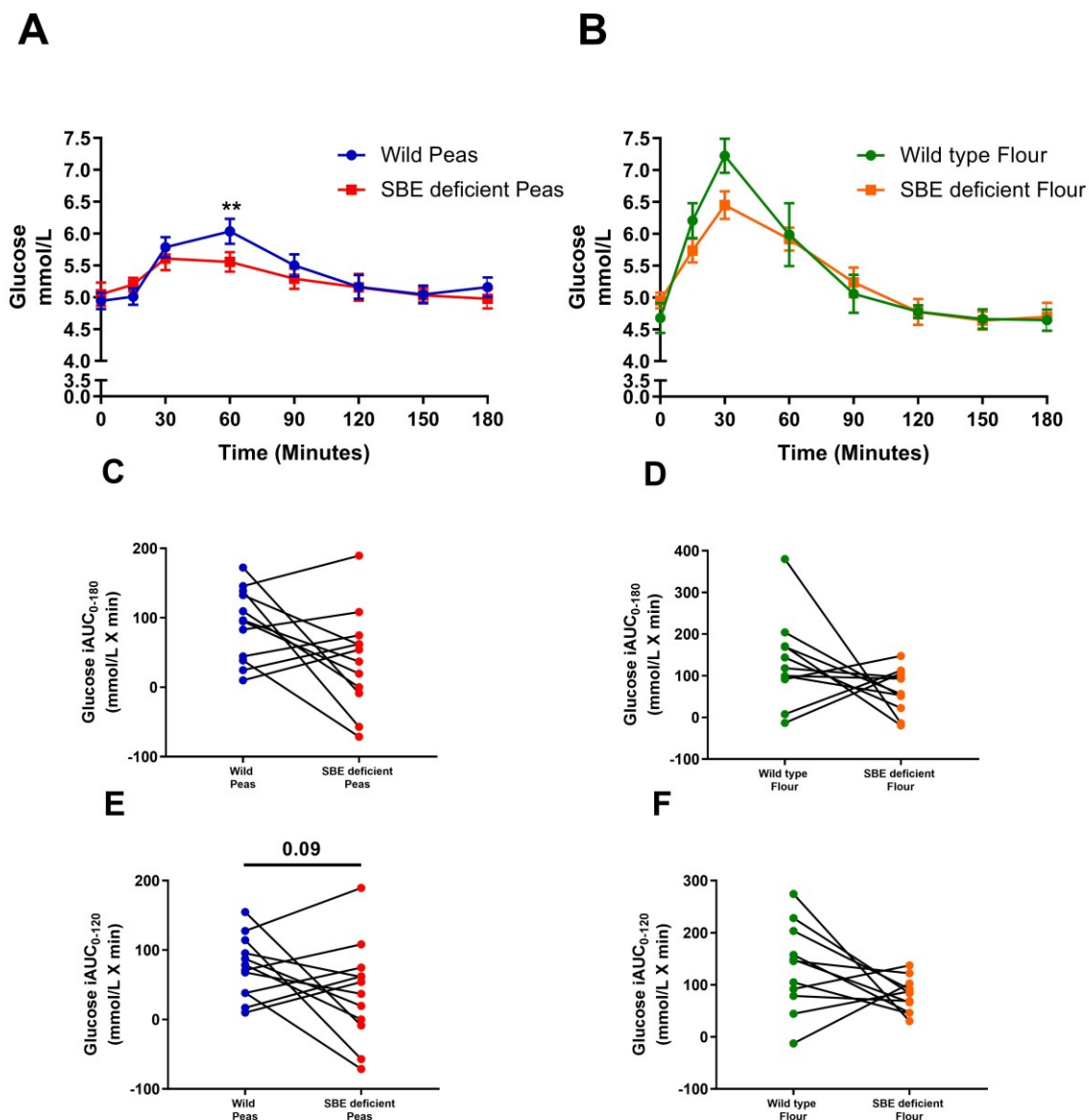


Figure 4.2 Postprandial plasma glucose in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=12) of (A) wild peas (---) and SBE-deficient peas (---). Data represent mean $\pm$ SEM (n=11) of (B) wild flour (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 4.3 shows time course, iAUC₀₋₁₈₀, and iAUC₀₋₁₂₀ data on plasma glucose concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma glucose iAUC₀₋₁₈₀ between wild peas and wild flour ($p=0.18$). The same effect was not observed in plasma glucose iAUC₀₋₁₂₀, as there was a significant reduction following the consumption of wild peas (72.7 ± 13.7 mmol/L x min) when compared to wild flour (133.2 ± 25.1 mmol/L x min) ($p=0.03$).

No main effect of peas was found ($p=0.73$). However, an effect of pea X time interaction was found ($p<0.01$). Following wild flour consumption, plasma glucose increased rapidly at 30 minutes ($P<0.01$) and reached a peak at 60 minutes ($p<0.01$), followed by reductions at 120, 150, and 180 minutes ($p=0.05$, $p<0.01$ and $p<0.01$, respectively), when compared with wild peas.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing plasma glucose iAUC₀₋₁₈₀ between SBE-deficient peas and SBE-deficient flour ($p=0.44$). The same effect was not observed in plasma glucose iAUC₀₋₁₂₀, as there was a significant reduction following the consumption of SBE-deficient peas (39.1 ± 15.2 mmol/L x min) when compared with SBE-deficient flour (80.2 ± 10.0 mmol/L x min) ($p=0.02$).

No main effect of peas was found ($p=0.59$). However, an effect of pea X time interaction was found ($p<0.01$). Following SBE-deficient flour consumption, plasma glucose increased rapidly at 15 minutes ($P=0.03$) and reached a peak at 30 minutes ($p<0.01$), followed by a rapid reduction at 60 minutes ($p=0.01$), with significant below-baseline reductions at 120 and 150 minutes ($p=0.04$ and $p=0.02$, respectively) when compared with SBE-deficient peas.

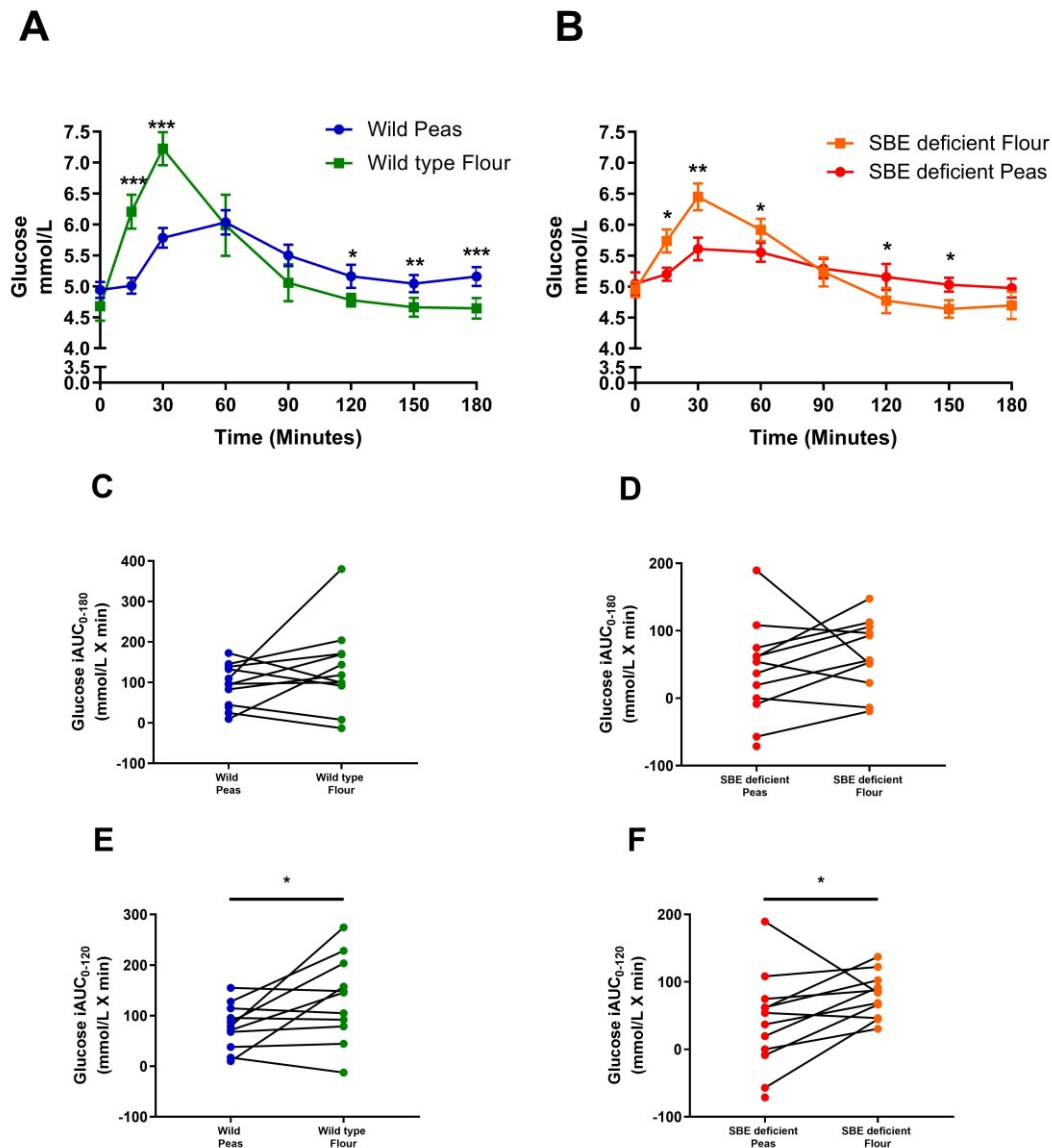


Figure 4.3: Postprandial plasma glucose in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM ($n=11$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

4.4.3 Serum insulin

Comparing the effects of mutation

Figure 4.4 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on serum insulin concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated a significant reduction in serum insulin $iAUC_{0-180}$ levels following SBE-deficient peas consumption ($911.3 \pm 179.6 \mu U/ml$) when compared with those following wild pea consumption ($1492.3 \pm 339.3 \mu U/ml$) ($p=0.02$). The same significant reduction was found in serum insulin $iAUC_{0-120}$ between SBE-deficient ($823.5 \pm 151.7 \mu U/ml$) and wild peas ($1439.6 \pm 317.6 \mu U/ml$) ($p=0.01$).

Significant effects of peas ($p=0.01$) and pea X time interaction ($p<0.01$) were found. Additionally, a significant increase in serum insulin was found at 90 and 120 minutes following wild pea consumption ($p<0.01$ and $p<0.01$, respectively) when compared with SBE-deficient pea consumption.

Pea flours:

The results indicated a significant reduction in serum insulin $iAUC_{0-180}$ following SBE-deficient flour consumption ($1773.4 \pm 191.0 \mu U/ml$) when compared with wild flour consumption ($2750.4 \pm 582.3 \mu U/ml$) ($p=0.05$). The same significant reduction was found in serum insulin $iAUC_{0-120}$ between SBE-deficient ($1710.6 \pm 179.2 \mu U/ml$) and wild flour ($2723.3 \pm 580.7 \mu U/ml$) ($p=0.04$).

Borderline main effects of flour ($p=0.07$) and flour X time interaction ($p=0.09$) were found.

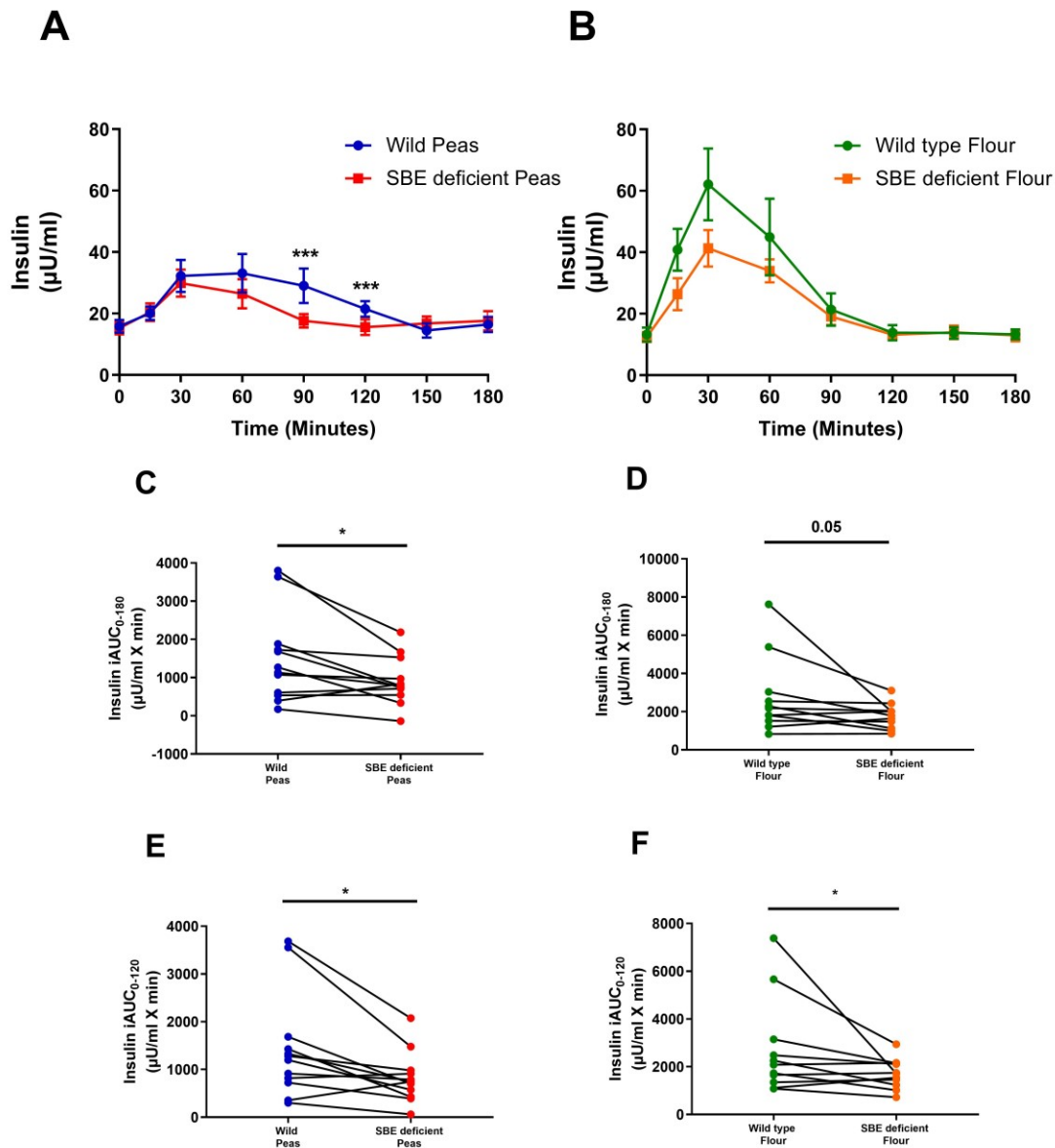


Figure 4.4: Postprandial serum insulin in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=12) of (A) wild peas (---) and SBE-deficient peas (---). Data represent mean $\pm$ SEM (n=11) of (B) wild flour (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 4.5 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on serum insulin concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated a significant reduction in serum insulin $iAUC_{0-180}$ levels following wild pea consumption ($1492.4 \pm 339.3 \mu U/ml$) when compared with those following wild flour consumption ($2750.4 \pm 608.2 \mu U/ml$) ($p < 0.01$). The same significant reduction was shown in serum insulin $iAUC_{0-120}$ following wild pea consumption ($1439.6 \pm 317.6 \mu U/ml$) when compared with wild flour consumption ($2723.3 \pm 580.7 \mu U/ml$) ($p < 0.01$).

Main meal effects of peas ($p = 0.01$) and pea X time interaction ($p < 0.01$) were found. Following wild flour consumption, plasma glucose increased rapidly at 15 minutes ($P = 0.01$) and reached a peak at 30 minutes ($p < 0.01$), followed by reductions at 90 and 120 minutes ($p = 0.03$ and $p < 0.01$, respectively) when compared with wild peas.

SBE-deficient pea seeds and flour:

The results indicated a significant reduction in serum insulin $iAUC_{0-180}$ following SBE-deficient pea consumption ($911.3 \pm 179.6 \mu U/ml$) when compared with SBE-deficient flour consumption ($1773.4 \pm 199.5 \mu U/ml$) ($p < 0.01$). The same significant reduction in serum insulin $iAUC_{0-120}$ concentrations was found following SBE-deficient pea consumption ($823.5 \pm 151.7 \mu U/ml$) when compared with SBE-deficient flour consumption ($1710.6 \pm 179.2 \mu U/ml$) ($p < 0.01$).

No main effect of peas was found ($p = 0.22$). However, an effect of pea X time interaction was found ($p < 0.01$). Serum insulin reached a peak at 30 minutes following both peas forms, with a significant difference at 60 minutes ($p = 0.01$) following SBE-deficient flour consumption when compared with SBE-deficient pea consumption.

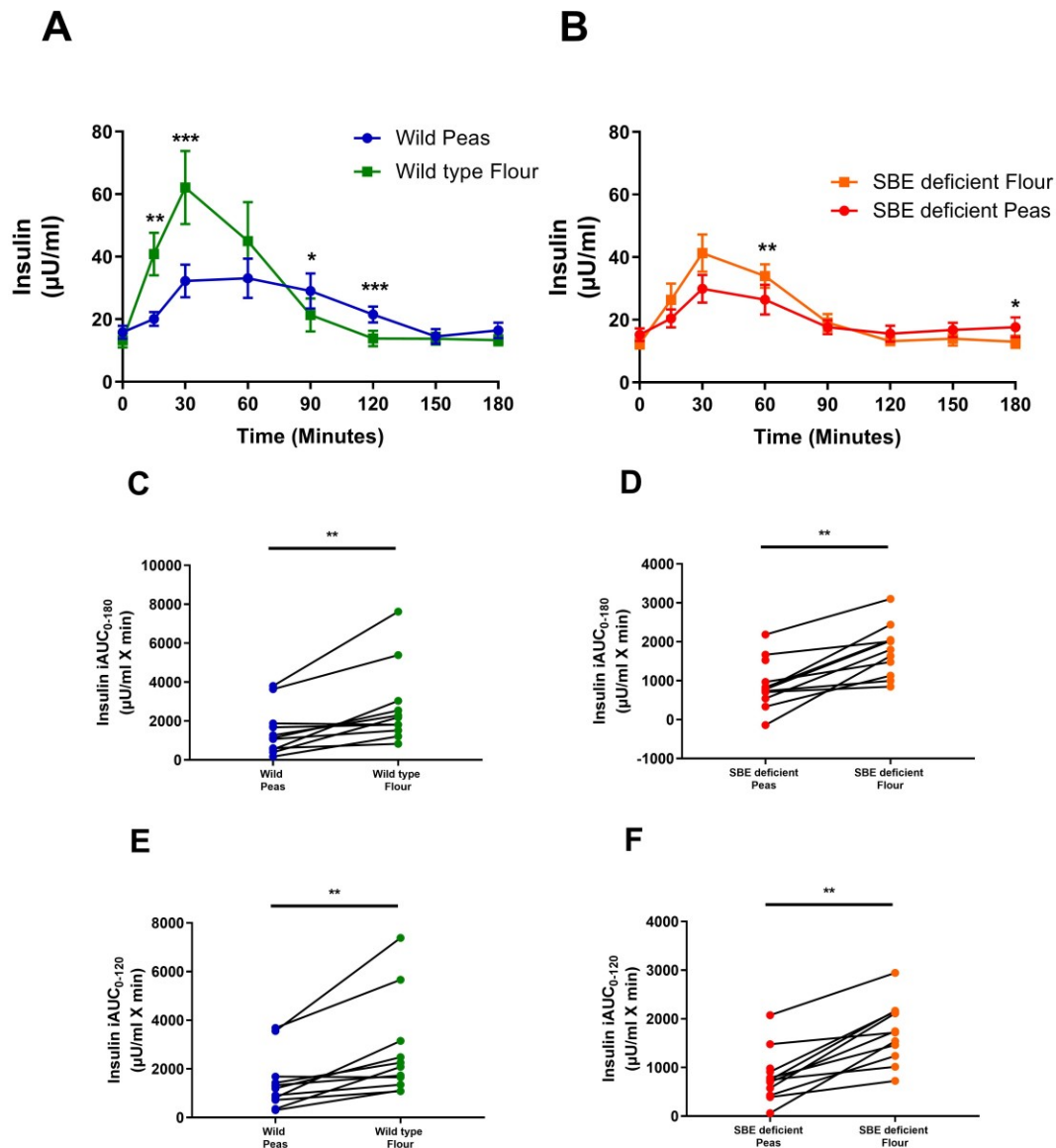


Figure 4.5: Postprandial serum insulin in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM ($n=11$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

4.4.4 Small intestinal digestion of starch structures as reflected by glucose content

Unlike the other parameters, complete data sets were only collected for seven participants.

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 4.6 shows time course, AUC₀₋₁₈₀, and AUC₀₋₁₂₀ data on small intestinal glucose, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated a significant reduction in duodenal glucose AUC₀₋₁₈₀ levels following SBE-deficient peas consumption (177.6 ± 35.5 mmol/L) when compared to those following wild pea consumption (371.2 ± 59.2 mmol/L) ($p=0.01$). The same effect was observed when comparing duodenal glucose AUC₀₋₁₂₀ following SBE-deficient pea consumption (129.5 ± 28.9 mmol/L) when compared to wild pea consumption (275.0 ± 43.6 mmol/L) ($p=0.04$).

It is also noted that small intestinal glucose reached peak levels at 30 minutes following both wild pea (3.77 ± 2.28 mmol/L) and SBE-deficient pea (1.92 ± 2.21 mmol/L) consumption.

Pea flours:

The results indicated no effect on duodenal glucose AUC₀₋₁₈₀ between wild and SBE-deficient flour ($p=0.75$). The same lack of effect was observed when comparing duodenal glucose AUC₀₋₁₂₀ levels between wild and SBE-deficient flour ($p=0.80$).

It is also noted that small intestinal glucose reached peak levels at 30 minutes following both wild flour (3.81 ± 2.2 mmol/L) and SBE-deficient flour consumption (3.43 ± 1.9 mmol/L).

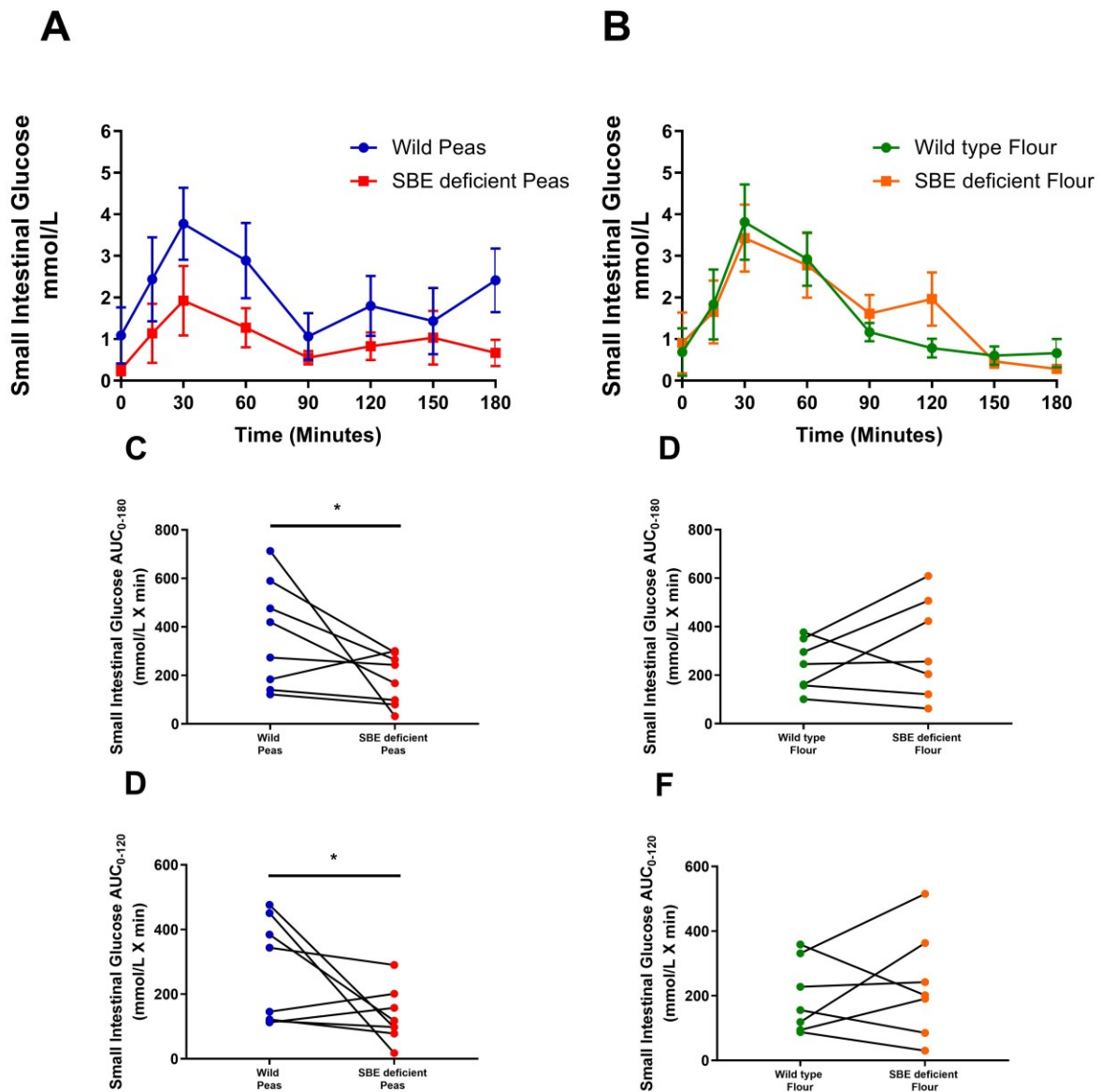


Figure 4.6: Postprandial small intestinal glucose in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM ($n=7$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent (C) iAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 4.7 shows time course, AUC_{0-180} , and AUC_{0-120} data on small intestinal glucose concentrations, comparing the effects of structure between wild pea seed and flour and between SBE-deficient pea seeds and flour.

Wild pea seed and flour:

The results indicated no effect on duodenal glucose AUC_{0-180} levels between wild peas and wild flour ($p=0.54$). The same lack of effect was observed when comparing duodenal glucose AUC_{0-120} between wild peas and wild flour ($p=0.72$)

SBE-deficient pea seeds and flour:

The results indicated no effect on duodenal glucose AUC_{0-180} levels between SBE-deficient peas and SBE-deficient flour ($p=0.13$). The same lack of effect was observed when comparing duodenal glucose AUC_{0-120} between SBE-deficient peas and SBE-deficient flour ($p=0.10$).

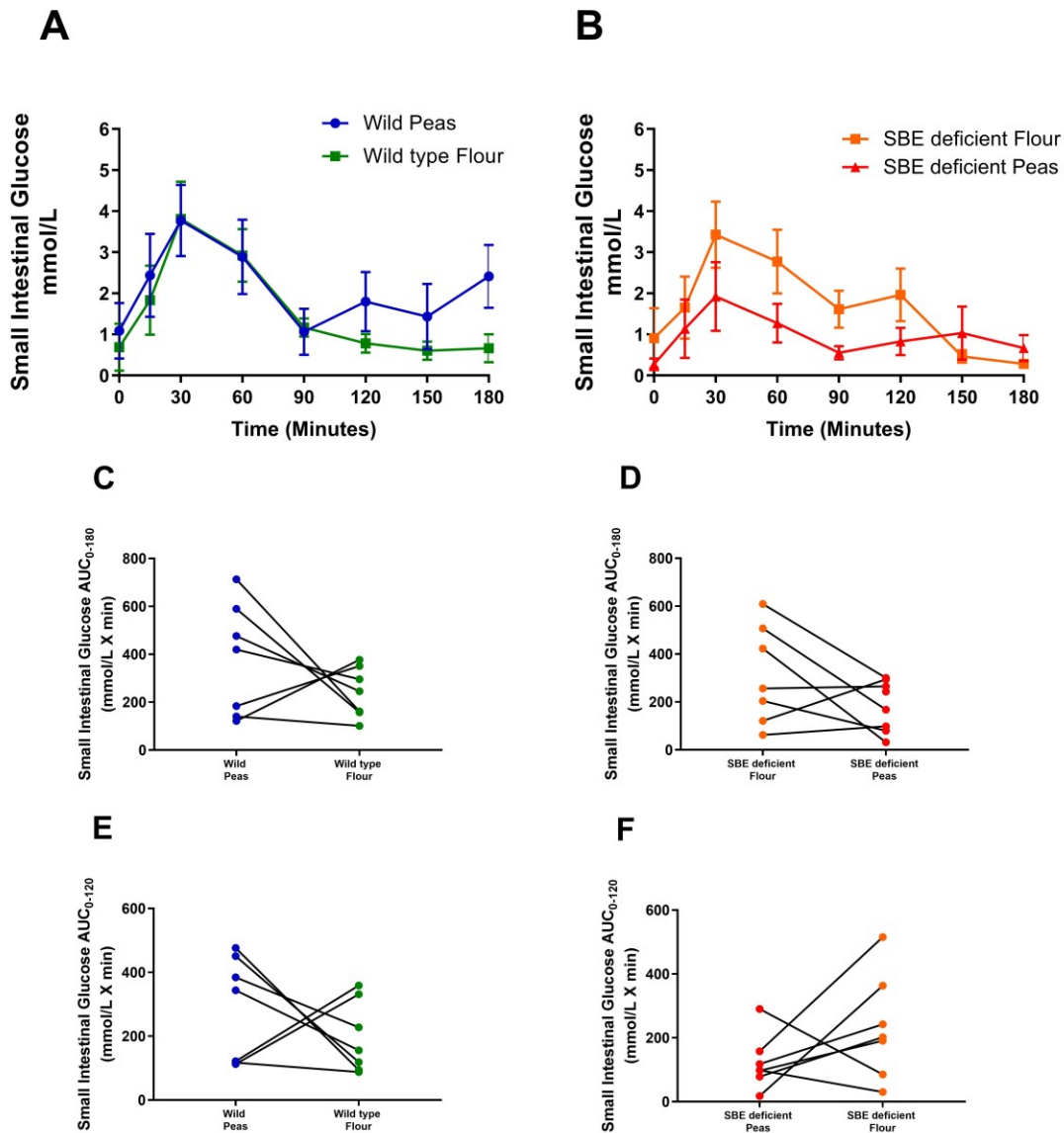


Figure 4.7: Postprandial small intestinal glucose in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=7) (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: AUC, area under the curve.

4.4.5 Plasma GLP-1

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 4.8 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on plasma GLP-1 concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-180}$ between wild and SBE-deficient peas ($p=0.49$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.59$).

No main effects of peas ($p=0.85$) or pea X time interaction ($p=0.50$) were found.

Pea flours:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-180}$ levels between wild and SBE-deficient flour consumption ($p=0.88$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ levels between wild and SBE-deficient flour consumption ($p=0.82$).

No main effect of peas was found ($p=0.33$). However, a significant pea X time interaction was found ($p<0.01$). Following wild flour consumption, plasma GLP-1 reached a significant peak at 15 minutes ($p<0.01$) when compared with SBE-deficient flour consumption.

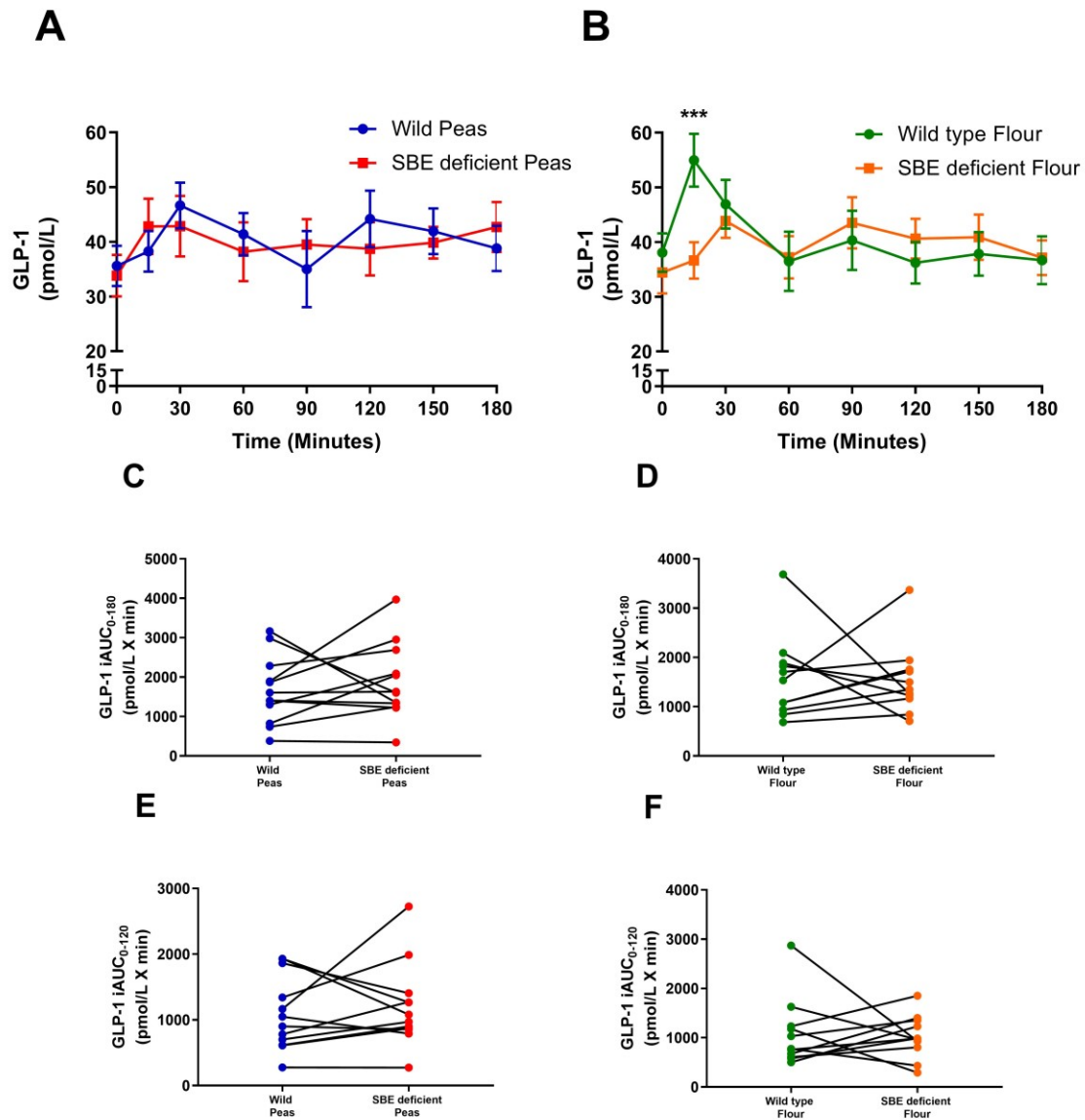


Figure 4.8: Postprandial plasma GLP-1 in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=12) of (A) wild peas (---) and SBE-deficient peas (---). Data represent mean $\pm$ SEM (n=11) of (B) wild flour (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 4.9 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on plasma GLP-1 concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-180}$ levels between wild peas and wild flour ($p=0.83$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between wild peas and wild flour ($p=0.69$).

No main effect of peas was found ($p=0.75$). However, a significant pea X time interaction was found ($p=0.02$). Following wild flour consumption, plasma GLP-1 levels reached a significant peak at 15 minutes ($p<0.01$) when compared with wild pea consumption.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing plasma GLP-1 $iAUC_{0-180}$ levels between SBE-deficient pea and SBE-deficient flour consumption ($p=0.20$). The same lack of effect was observed when comparing plasma GLP-1 $iAUC_{0-120}$ between SBE-deficient peas and SBE-deficient flour ($p=0.48$).

No main effects of peas ($p=0.73$) or pea X time interaction ($p=0.42$) were found.

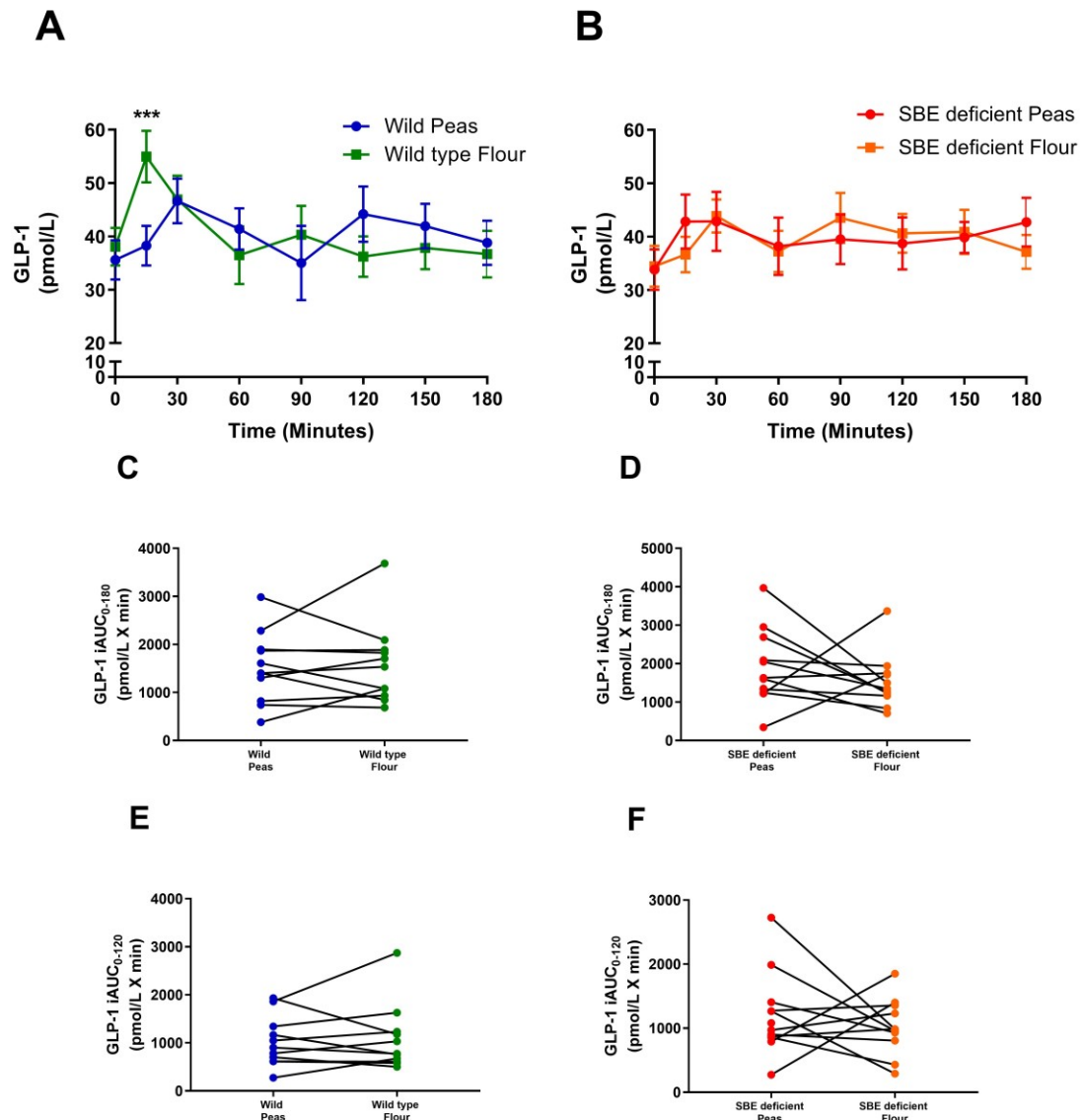


Figure 4.9: Postprandial plasma GLP-1 in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM ($n=11$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

4.4.6 Plasma PYY

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 4.10 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on plasma PYY concentrations, comparing the effects of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma PYY $iAUC_{0-180}$ between wild and SBE-deficient peas ($p=0.55$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.53$).

No main effects of peas ($p=0.13$) or pea X time interaction ($p=0.89$) were found.

Pea flours:

The results indicated no effect when comparing plasma PYY $iAUC_{0-180}$ levels between wild and SBE-deficient flour ($p=0.67$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.57$).

No main effects of flour ($p=0.22$) or flour X time interaction ($p=0.87$) were found.

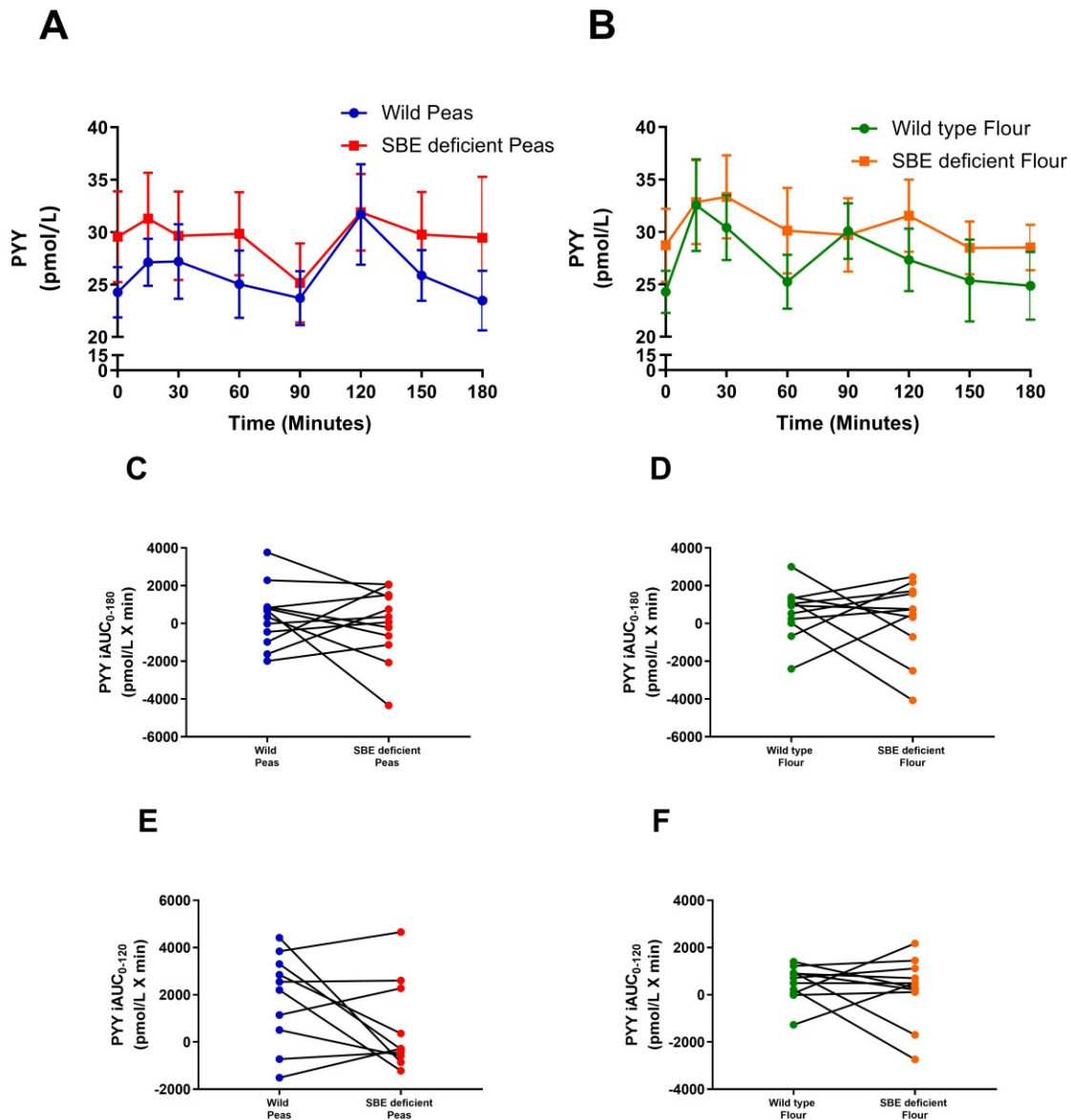


Figure 4.10: Postprandial plasma PYY in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=12) of (A) wild peas (---) and SBE-deficient peas (---). Data represent mean $\pm$ SEM (n=11) of (B) wild flour (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 4.11 shows time course, $iAUC_{0-180}$, and $iAUC_{0-120}$ data on plasma PYY concentrations, comparing the effects of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing plasma PYY $iAUC_{0-180}$ between wild peas and flour ($p=0.69$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild peas and flour ($p=0.48$).

No main effects of meal ($p=0.67$) or meal X time interaction ($p=0.32$) were found.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing plasma PYY $iAUC_{0-180}$ between SBE-deficient peas and SBE-deficient flour ($p=0.87$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between SBE-deficient peas and flour ($p=0.95$).

No main effects of meal ($p=0.79$) or meal X time interaction ($p=0.74$) were found.

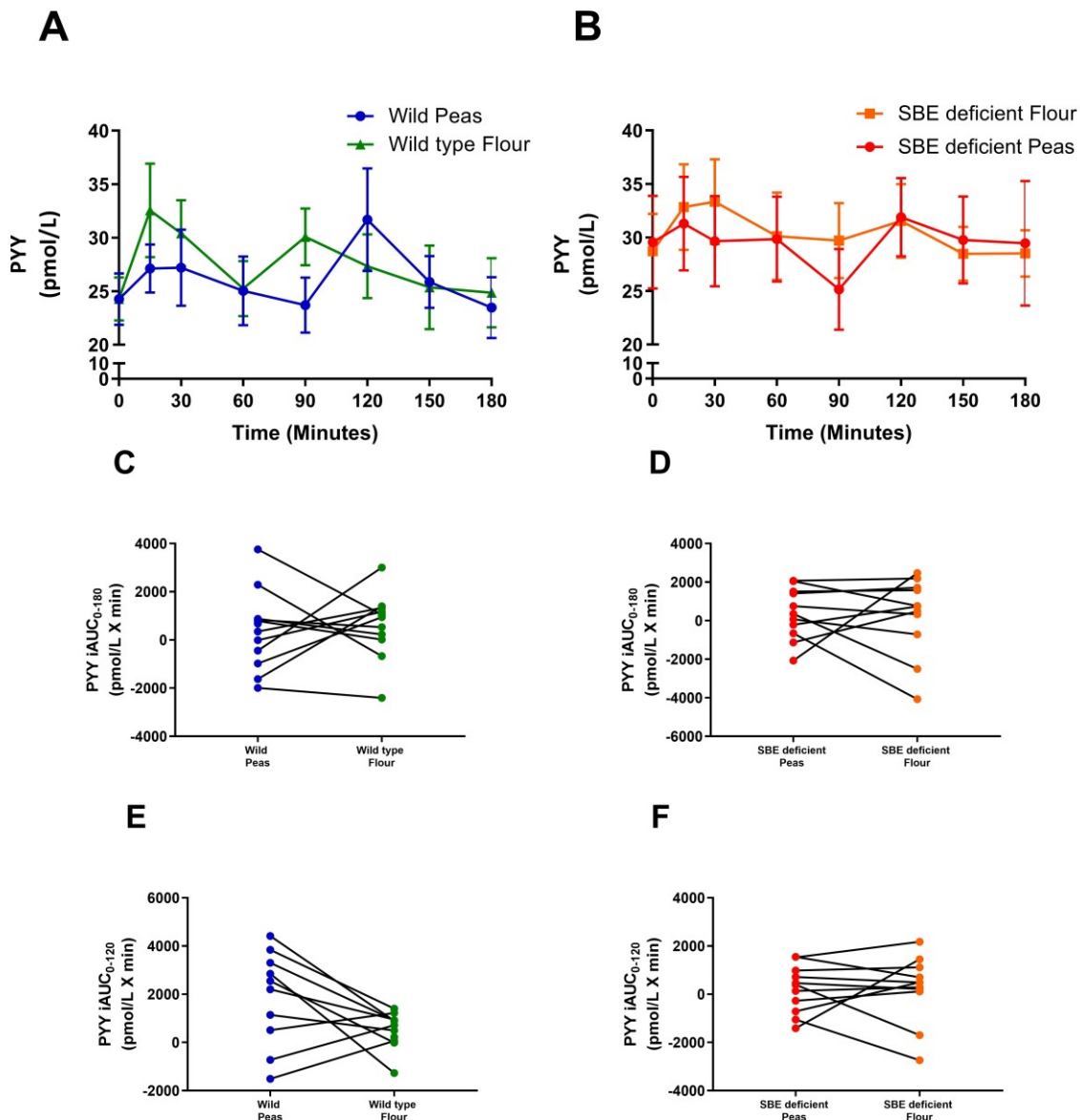


Figure 4.11: Postprandial plasma PYY in response to ingesting 50g dry weight of pure peas test meal.

Data represent mean $\pm$ SEM (n=11) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---) as analysis was performed on available paired data. Insets represent mean $\pm$ SEM of (C) iAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

4.4.7 Visual analogue scale questions

4.4.7.1 Hunger

Figure 4.12 presents time course and tAUC data on subjective hunger, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective hunger scores between wild and SBE-deficient peas consumption ($p=0.99$).

Pea flours:

There were no differences in subjective hunger scores between SBE deficient flour and wild flour consumption ($p=0.70$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There was a significant reduction in subjective hunger scores following the consumption of wild peas (42.5 ± 7.9 mm) when compared to wild flour consumption (48.7 ± 9.5 mm) ($p=0.03$).

Mutant pea seeds:

There was no significant difference in subjective hunger scores between SBE-deficient peas and flour consumption ($p=0.75$).

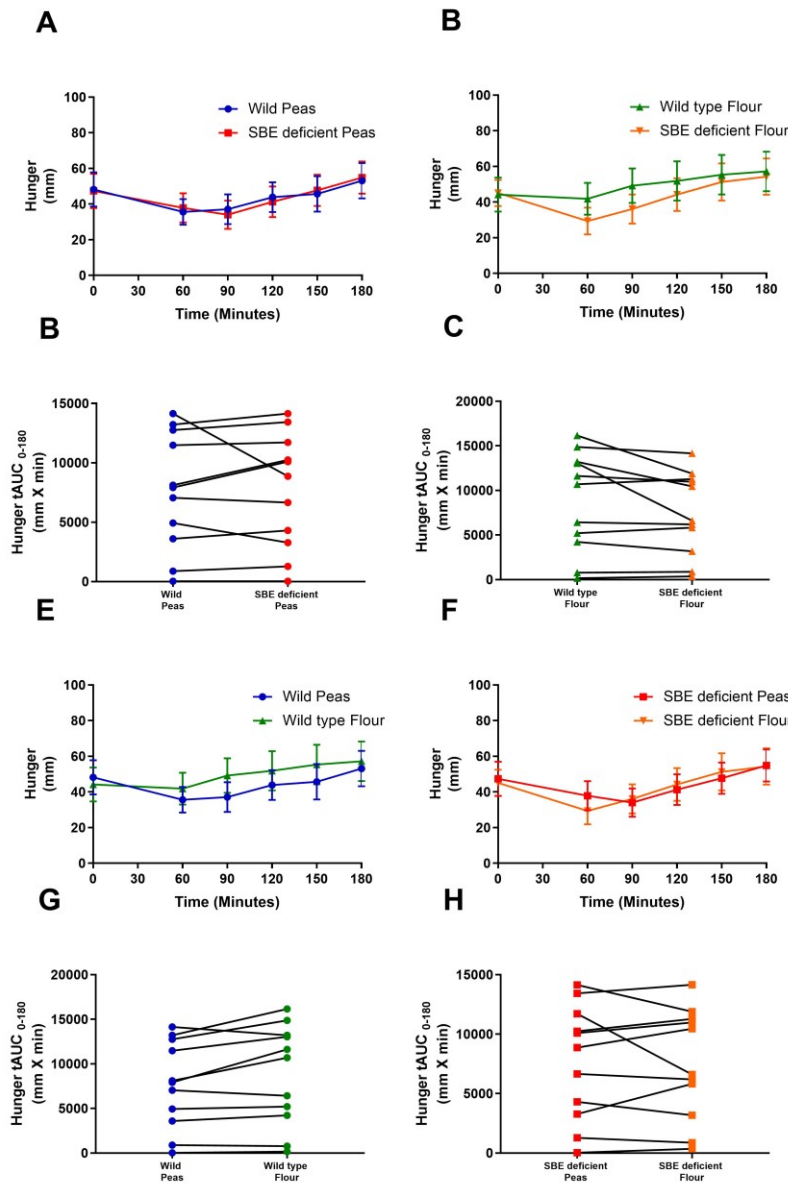


Figure 4.12 Subjective hunger score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.4.7.2 Fullness

Figure 4.13 presents time course and tAUC data on subjective fullness, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective fullness scores between wild pea and SBE-deficient pea consumption ($p=0.61$).

Pea flours:

There were no differences in subjective fullness scores between wild and SBE-deficient flour consumption ($p=0.38$).

Secondary comparisons looking at the effects of cellular structure

Wild pea seeds:

There were no differences in subjective fullness scores between wild peas and wild flour consumption ($p=0.83$).

Mutant pea seeds:

There were no differences in subjective fullness scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.15$).

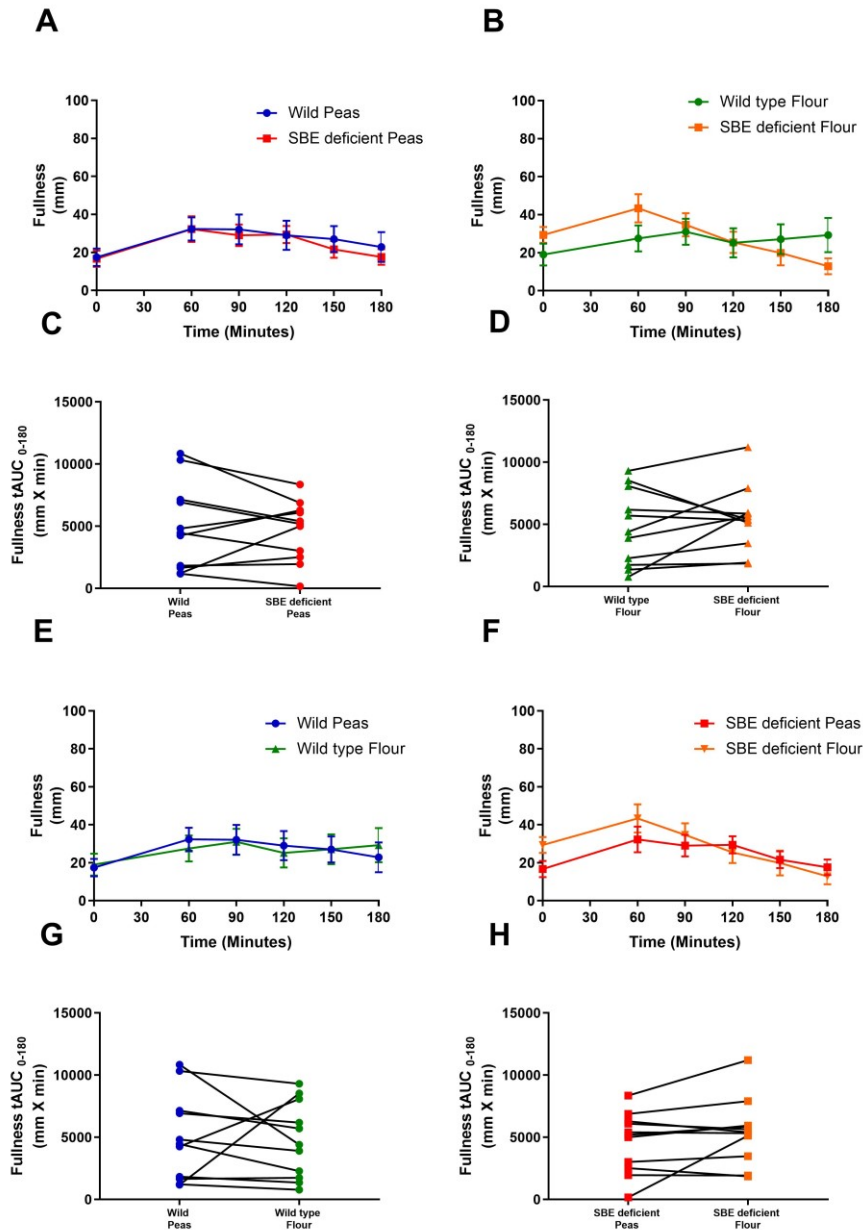


Figure 4.13 Subjective fullness score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.4.7.3 Desire to eat

Figure 4.14 shows time course and tAUC data for subjective desire to eat scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient peas consumption ($p=0.77$).

Flour pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient flour consumption ($p=0.16$).

Secondary comparisons looking at the effects of cellular structure

Wild pea seeds:

There was a significant reduction in subjective desire to eat scores following the consumption of wild peas (42.2 ± 8.2 mm) when compared to the consumption of wild flour (48.8 ± 10.2 mm) ($p=0.02$).

Mutant pea seeds:

There were no significant differences in subjective desire to eat scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.83$).

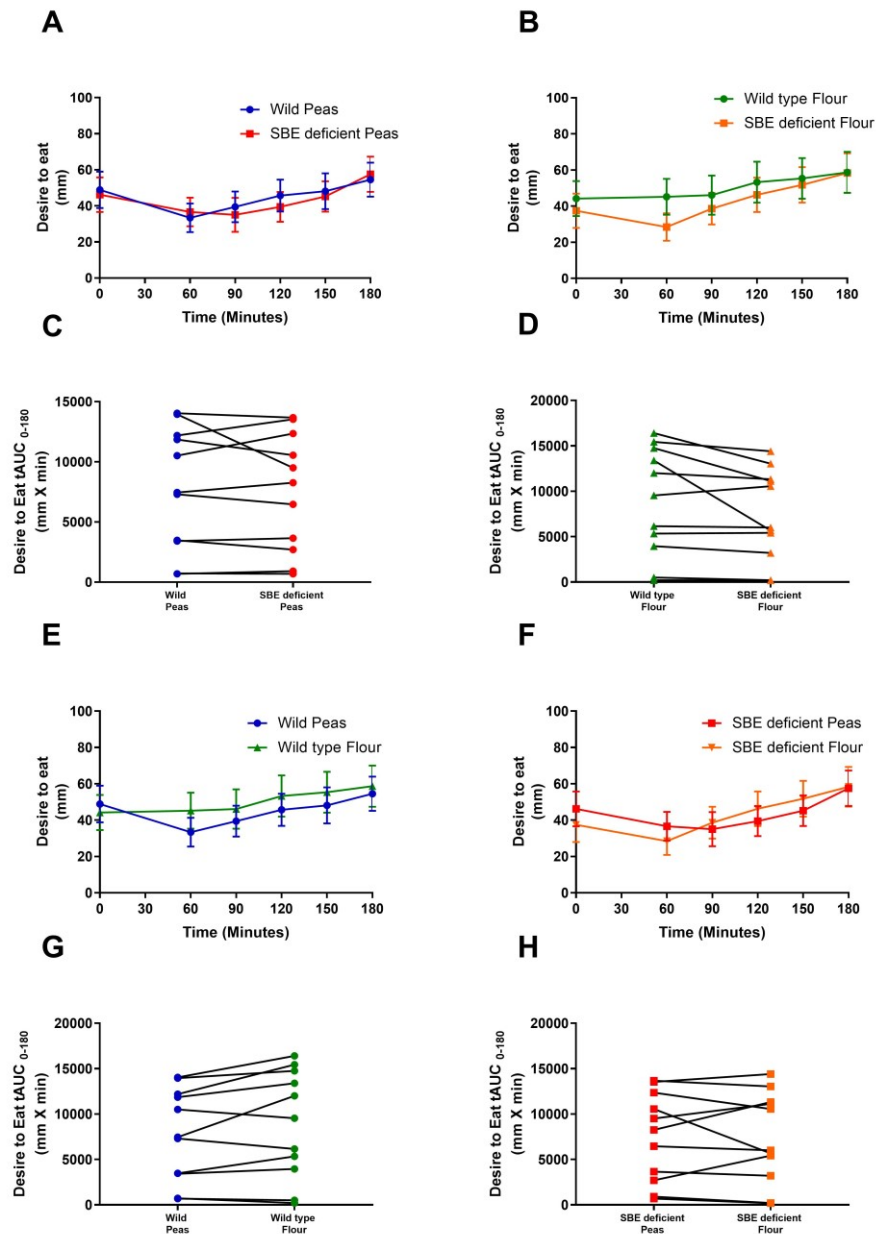


Figure 4.14 Subjective desire to eat score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.4.7.4 Appetite for a meal

Figure 4.15 presents time course and tAUC data for subjective appetite for a meal scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective appetite for a meal scores between wild and SBE-deficient peas consumption ($p=0.56$).

Flour pea seeds:

There were no differences in subjective appetite for a meal scores between SBE deficient flour and wild flour consumption ($p=0.70$).

Secondary comparisons looking at the effects of cellular structure

Wild pea seeds:

There were no differences in subjective appetite for a meal scores between wild peas and wild flour consumption ($p=0.70$).

Mutant pea seeds:

There were no differences in subjective appetite for a meal scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.87$).

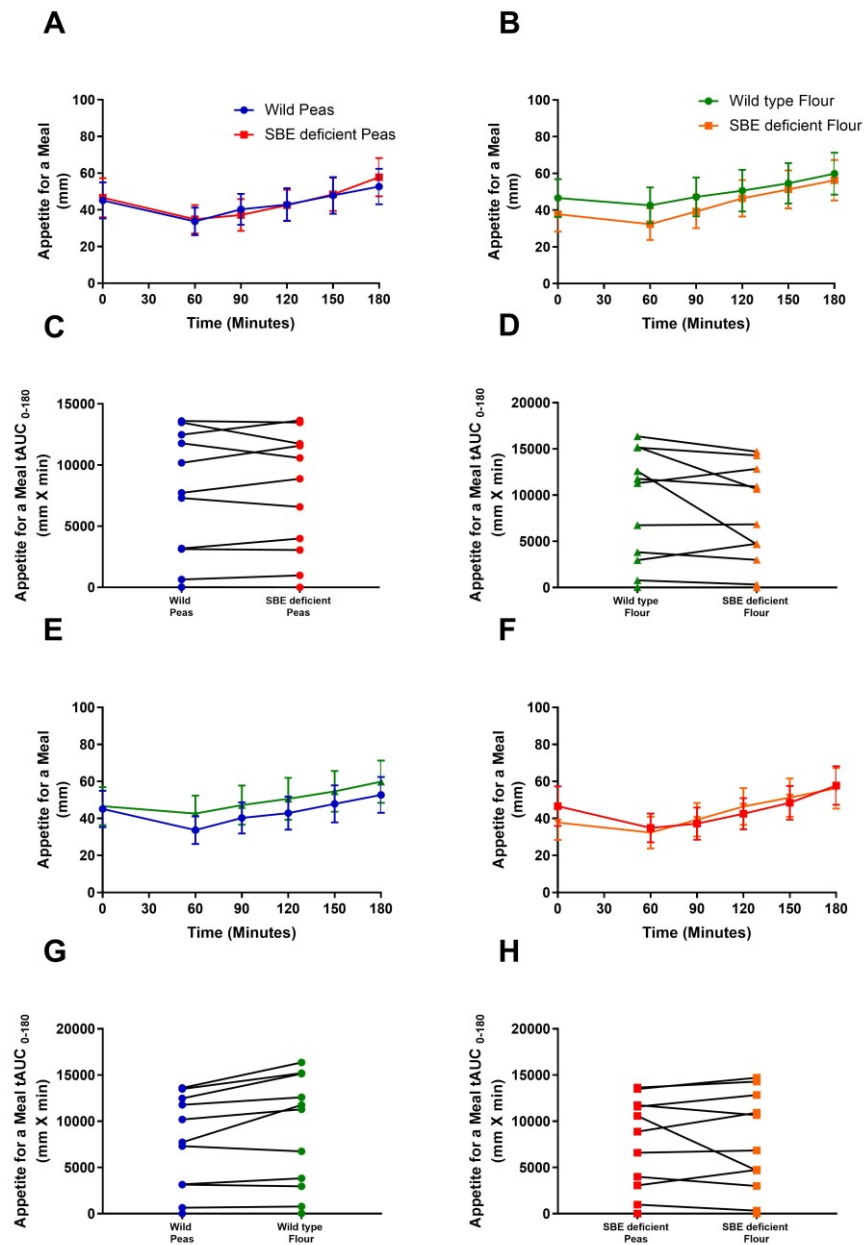


Figure 4.15 Subjective appetite for a meal score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.4.7.5 Composite appetite score

Figure 4.16 shows time course and tAUC data on composite appetite scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective composite appetite scores between wild and SBE-deficient peas consumption ($p=0.92$).

Flour pea seeds:

There was a borderline reduction in composite appetite scores following the consumption of SBE deficient flour (48.5 ± 6.4 mm) when compared with the consumption of wild flour (55.1 ± 7.8 mm) ($p=0.06$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There was a trend towards a reduction in composite appetite scores following the consumption of wild peas (50.1 ± 6.2 mm) when compared to the consumption of wild flour (55.1 ± 7.8 mm) ($p=0.05$).

Mutant pea seeds:

There were no significant differences in subjective hunger scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.58$).

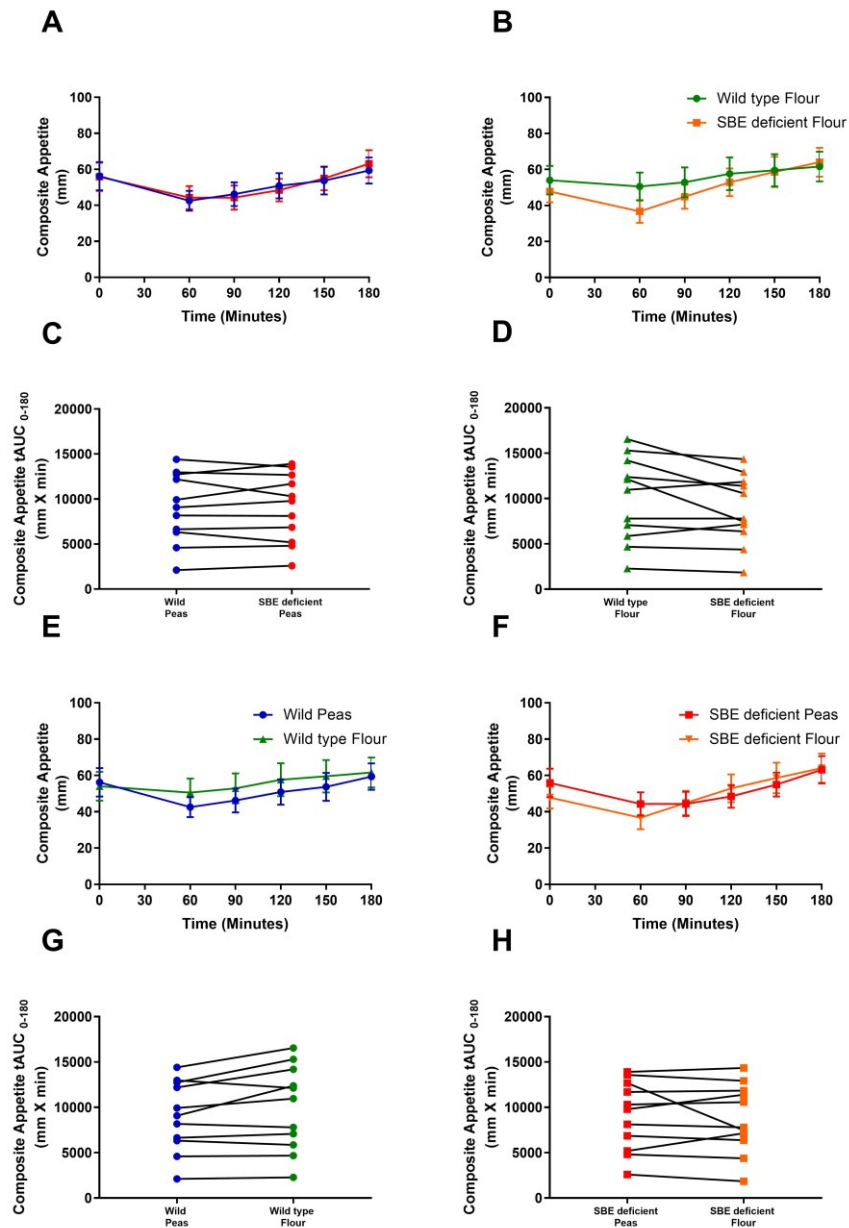


Figure 4.16 Subjective composite appetite score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.4.7.6 Nausea

Figure 4.17 presents time course and tAUC data on nausea scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient peas consumption ($p=0.78$).

Flour pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient flour consumption ($p=0.91$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective nausea scores between wild peas and wild flour consumption ($p=0.39$).

Mutant pea seeds:

There were no differences in subjective nausea scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.31$).

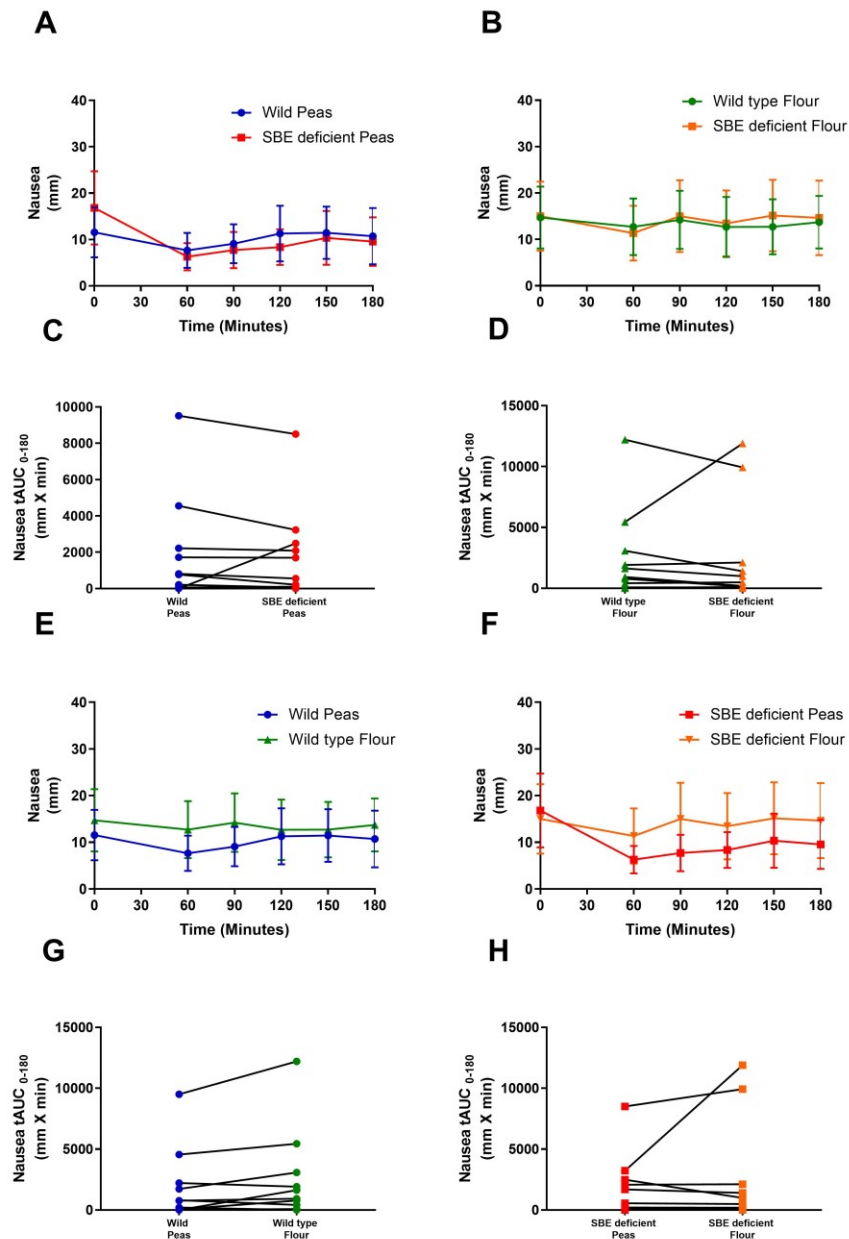


Figure 4.17 Subjective nausea score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₁₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₁₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₁₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₁₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Analysis was performed on available paired data. Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve.

4.5 Discussion

4.5.1 Main findings

Primary findings looking at the effect of starch branching enzyme mutation

- Plasma glucose was reduced following SBE-deficient consumption compared to wild pea consumption. The same effect was not shown in the case of pea flours.
- Insulin response was lower following SBE-deficient pea consumption when compared with wild pea consumption. The same effect was shown in the case of pea flours.
- Small intestinal glucose content was reduced following SBE-deficient pea consumption when compared with wild pea consumption. The same effect was not shown in the case of pea flours.
- Plasma GLP-1 levels were not affected differently by SBE-deficient or wild peas. The same effect was not shown in the case of pea flours, where there was a significant peak at 15 minutes following wild flour consumption when compared with SBE-deficient flour consumption.
- Plasma PYY was not affected differently by SBE-deficient or wild peas. The same lack of effect was shown in the case of pea flours.
- VAS did not differ between SBE-deficient and wild peas. The same effect was not shown in the case of pea flours, where there were reductions in subjective hunger, appetite for a meal, and composite appetite scores following the consumption of SBE-deficient flour when compared with the consumption of wild flour.

Secondary findings looking at the effect of cellular structure

- Plasma glucose was higher following wild flour consumption compared to wild pea consumption. The same effect was shown in the case of SBE-deficient pea seeds and flour.
- Serum insulin was higher following wild flour consumption compared to wild pea consumption. The same effect was shown in the case of SBE-deficient pea seeds and flour.

- Small intestinal glucose content did not differ following wild pea seeds or wild flour consumption. The same lack of effect was shown in the case of SBE-deficient pea seeds and flour.
- Plasma GLP-1 reached a significant peak at 15 minutes following wild flour consumption when compared with wild pea consumption. The same effect was not shown in the case of SBE-deficient pea seeds and flour.
- Plasma PYY did not differ following wild pea or wild flour consumption. The same lack of effect was shown in the case of SBE-deficient pea seeds and flour.
- Subjective hunger, desire to eat, appetite for a meal, and composite appetite scores were reduced following wild pea consumption when compared with wild flour consumption. The same effects were not shown in the case of SBE-deficient pea seeds and flour.

4.5.2 Detailed discussion

The results from experimental Chapter 3 indicated improvement in plasma glucose following the consumption of SBE-deficient peas independent of the rate of GE. However, those results were limited by the fact that the mixed meal included other carbohydrate sources, so it was not clear whether the peas had limited digestibility. This study was conducted to understand digestion behaviour with pea interventions served alone as a test meal. By measuring small intestinal glucose content, the digestibility of different pea treatments with different structural forms and its impact on postprandial glucose response following ingestion can be identified.

4.5.2.1 The effect of starch branching enzyme mutation

4.5.2.1.1 Glucose, insulin, and GLP-1 responses

Whole pea seeds

Plasma glucose and serum insulin levels were significantly reduced following SBE-deficient pea consumption compared to wild pea consumption. Plasma GLP-1 was not affected by the genetic mutation when comparing both of the whole pea seeds.

Pea flours

Plasma glucose was borderline reduced following SBE-deficient flour consumption compared to wild flour consumption. Serum insulin followed the same pattern, as it was significantly decreased following SBE-deficient flour consumption. Plasma GLP-1 levels increased rapidly to reach a peak during the first 15 minutes following wild flour consumption when compared with SBE deficient flour consumption.

These observations support the results from Chapter 3 and demonstrate that plant genotype has an impact on postprandial glycaemia. Specifically, high-amylose starches can have a major impact on glucose and insulin responses (Behall and Scholfield, 2005; Brighenti *et al.*, 2006; Anderson *et al.*, 2010). In the current study, having a high amylose content was a result of a mutation in the gene coding for the starch branching enzyme, which caused pleiotropic effects to the resultant SBE-deficient peas, a factor known to significantly delay/limit starch digestion and thereby affect postprandial metabolic responses. Small intestinal glucose availability data should explain pea starch digestibility further.

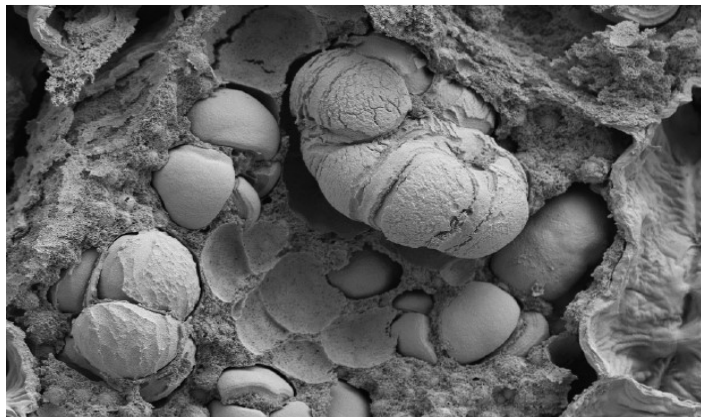
These results also show that wild flour stimulated the release of plasma GLP-1, reaching a peak at 15 minutes. This effect that was not apparent between whole pea seeds. This early increase in incretin response might suggest stimulation due to the rapid increase in glucose early in the digestive process. It was shown previously that high concentrations of duodenal glucose infusion were associated with increases in plasma GLP-1 (Pilichiewicz *et al.*, 2007). Although no differences were shown in small intestinal glucose availability, differences in plasma glucose were apparent. Future metabolomics results should reveal the composition breakdown of those peas, including glucose and other starch digestion products (e.g. maltose and maltotriose) in the duodenum to confirm these observations. These results suggest that the higher postprandial insulin response following wild flour consumption is driven by higher glucose availability and subsequent incretin stimulation.

These positive glucose and insulin responses are relevant for controlling postprandial glycaemia by increasing the intake of low glycaemic index foods and thus can serve as protective tools against the development of diabetes. It must be noted, however, that the current sample consists

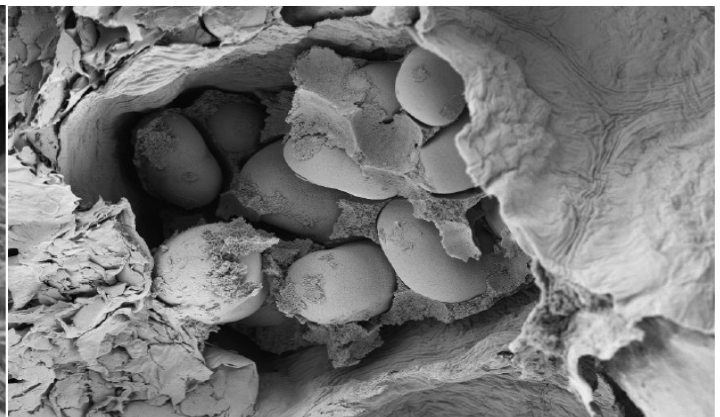
of healthy volunteers, so having a tightly regulated glucose response as a result of the functional insulin action is expected.

4.5.2.1.2 Collaborative in vitro work

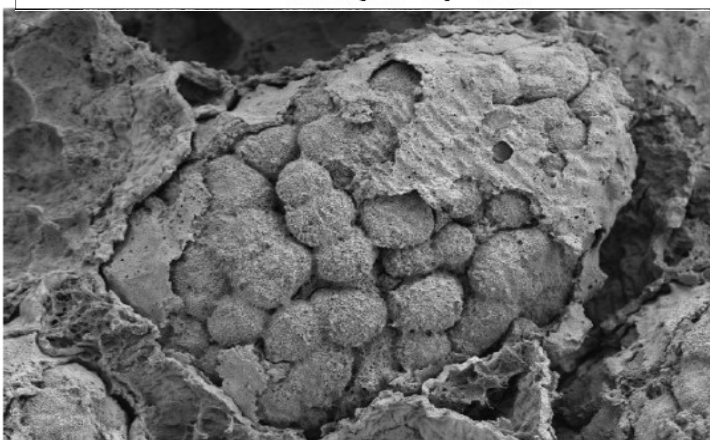
In support of these data, our collaborative in-vitro work on peas concentrated on the effect of cooking on the tissue structure of peas and starch granules within the pea seeds. With the aid of a scanning electron microscope, it was found that cooking changed the structure of the starch in wild pea seeds extensively (figure 4.18, below). However, the SBE-deficient pea seeds retained the same granular structure (as before) inside the cells. Moreover, the SBE-deficient pea CWs were found to be 40% thicker than the wild pea seed CWs. This finding confirms the proposed hypothesis that a thick CW can limit the gelatinisation process, and these factors should lead to different digestibility effects in the gut and subsequent fermentation. These in vitro results strengthen the assumption that SBE-deficient peas might be a suitable product for fermentation, as they retain their structure during upper GI digestion and thus maintain glucose homeostasis.



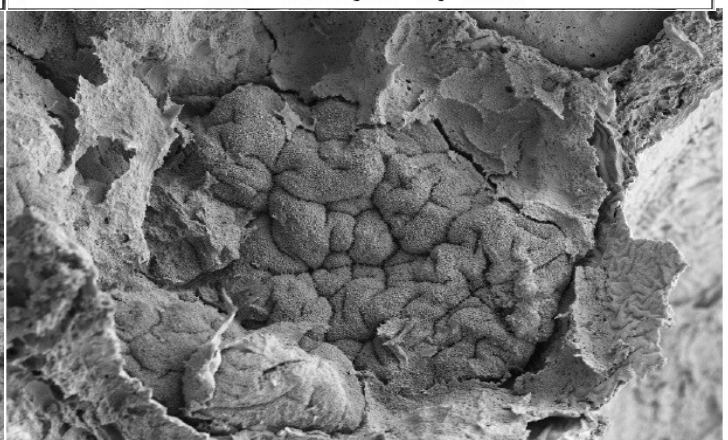
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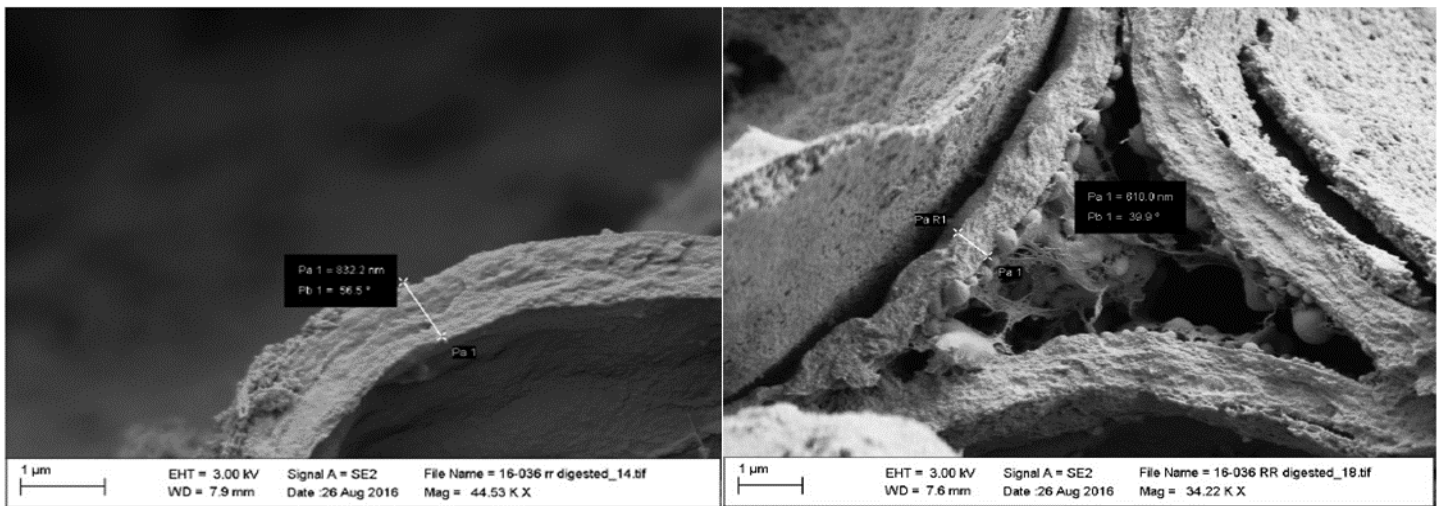


Figure 4.18 wild (right) and mutant (left) pea seeds under the scanning electron microscope (SEM).

These were taken by project collaborators in Quadrum Institute of food research, Norwich.

4.5.2.1.3 Availability of glucose in the small intestine

Whole pea seeds

Luminal glucose levels were significantly lower following the consumption of SBE-deficient peas when compared with the consumption of wild peas. Moreover, peak levels of luminal glucose were observed 30 minutes after the ingestion of both lines of whole pea seeds, with glucose release being more attenuated after consuming SBE-deficient peas. In contrast, plasma glucose data showed a peak at 60 minutes following the consumption of both lines of whole pea seeds.

Pea flours

Luminal glucose levels did not vary between pea flours of both genotypes. Moreover, peak levels of luminal glucose were observed 30 minutes after the ingestion of both lines of pea flours, which is in line with the same peak duration observed in plasma glucose data after consuming both pea flours (i.e. at 30 minutes).

These data suggest that plasma glucose concentration is driven by the availability of carbohydrates in the small intestine. More RS structures, such as in SBE-deficient whole pea seeds, demonstrated lower duodenal glucose and thus lower postprandial glucose response. These observations can be expected when looking at the collaborative *in vitro* microscopy (figure 4.18 in the current chapter), where SBE-deficient peas had a thicker CW, which acted as a physical barrier protecting the entrapped starch from digestion and limiting the space available for water absorption in response to hydrothermal processing (i.e. cooking), consequently restricting gelatinisation (Edwards, Warren, *et al.*, 2015). In addition, the high amylose content can lead to higher level of retrogradation and subsequently hinder amylase digestion when compared with high amylopectin starches, such as those found in wild peas (Englyst and Cummings, 1985; Haralampu, 2000). Together, these characteristics are in line with the *in vitro* results published earlier, where SBE deficient peas were confirmed to be 31% less digestible than wild peas (Edwards *et al.*, 2018). In order to explain the digestibility and the amylolysis behaviour, the same researchers demonstrated that around 82% of the starch fraction of the wild peas was digested in the first rapid phase, whereas only 57% of the SBE-deficient pea starch was digested in the same phase (Edwards *et al.*, 2018). Differences in starch structure, CWs, and hydrolysis rates can all have significant effects on starch breakdown in the small intestine. Further, they can explain the differences observed in plasma glucose and affect RS delivery to the colon. Together, it can be concluded that, as both of the pea lines are equally susceptible to digestion by amylase in humans, wild peas have more readily available starches and are more vulnerable to digestion when compared to SBE-deficient peas.

However, the same effects were not observed between pea flours. To explain these findings, previous *in vitro* studies have shown that, depending on the botanical origin of plants, the outer layer of starches in legumes can be composed of an amorphous starch while the inner core is crystalline (Edwards *et al.*, 2014, 2018). Moreover, the initial stages of amylase digestion target the amorphous regions of starches (Gallant *et al.*, 1992). The rate of amylase digestion has been shown to be initially the same between the currently investigated pea genotypes but decrease in the SBE-deficient line over time (Skrabanja *et al.*, 1999; Tahir *et al.*, 2011; Edwards *et al.*, 2018). These findings imply that the amorphous starches in both lines of peas following the destruction

of the CW are digested at a comparable rate while resident in the duodenum, which is a relatively short time to complete the digestion of both outer and inner starches. These data highlight the role of the CW matrix as a barrier to enzymatic digestion.

Due to practicality and the invasive process, a complete data set was only collected from seven participants for this parameter. However, this was an exploratory study in a new area and power calculation was not possible. Another point to be questioned is the validity of choosing glucose as the only product to reflect starch digestibility at the duodenal level because other starch digestion products (maltose and maltotriose) might have been missed, and thus the overall extent of starch digestion might have been underestimated. Future work should include analysis of total amylose by-products as maltose and maltotriose, as measuring total reducing sugars may be more informative than glucose only which may under represent the total amylose products digestion.

4.5.2.1.4 Appetite measures and plasma gut hormones

Whole pea seeds

Visual analogue and nausea scores were not affected when compared between SBE-deficient and wild peas. Moreover, plasma GLP-1 and PYY were not affected when compared between SBE-deficient and wild peas.

Pea flours

Subjective composite appetite score was reduced following SBE-deficient flour consumption when compared to wild flour consumption. However, plasma PYY was not affected differently when compared between SBE-deficient and wild flour. The same lack of effect was observed in plasma GLP-1, as no second/sustained peak reflecting the nutrient-sensing of colonic cells (Lim and Brubaker, 2006; Wachters-Hagedoorn *et al.*, 2006) was observed.

It is crucial to consider the invasive aspect of this trial, as participants were intubated for four consecutive days. Therefore, since VAS is sensitive to the trial conditions (Stubbs *et al.*, 2000), these subjective scores might be less valid when compared to everyday appetite response.

4.5.2.2 Secondary findings looking at the effect of cellular structure

4.5.2.2.1 Glucose, insulin, and GLP-1 responses

In both genotypes, processing whole pea seeds into flour significantly increased plasma glucose and serum insulin responses following the study visit time course. Again, plasma GLP-1 levels increased rapidly, reaching a peak during the first 15 minutes following wild flour consumption when compared to wild pea consumption. The same response was not observed in the case of SBE-deficient pea seeds and flour

These observations support the major impact of plant cell structure in both of pea lines on the extent of glucose absorption into the portal blood and the subsequent insulin release, suggesting high permeability of the processed peas to α -amylase due to the absence of CWs (Ellis *et al.*, 2004; Grassby *et al.*, 2012; Edwards, Grundy, *et al.*, 2015). Thus, having a whole, intact structure can limit the rate and/or extent of energy uptake post-ingestion, with SBE-deficient whole pea seeds significantly lowering glucose and insulin postprandial responses. This also implies that the structural integrity of pea seeds is a major factor influencing starch bioaccessibility and affecting postprandial metabolism.

These results also show that wild pea flour stimulated the release of plasma GLP-1, an effect that was not apparent in the case of SBE-deficient pea seeds and flour. Previous findings showed a positive association between high concentrations of duodenal glucose infusion and increases in plasma GLP-1 (Pilichiewicz *et al.*, 2007). Although no differences were shown in small intestinal glucose availability, differences in plasma glucose were apparent. Future metabolomics results should reveal the composition breakdown of these peas, including glucose and other starch digestion products (e.g. maltose and maltotriose) in the duodenum to confirm these

observations. These results suggest that the higher postprandial insulin response following wild flour is driven by higher glucose availability and subsequent incretin stimulation.

4.5.2.2.2 Availability of glucose in the small intestine

There were no differences in small intestinal glucose concentrations between peas and pea flours of both lines.

This could suggest that, at the small intestinal digestion level, the chemical composition of nutrients ingested is more important to exerting metabolic effects than the physical form and subsequent processing effects of foods.

4.5.2.2.3 Appetite measures and plasma gut hormones

Subjective hunger, desire to eat, and composite appetite scores were reduced following the consumption of wild peas when compared with the consumption of wild flour. The same effects were not shown in the case of SBE-deficient pea seeds and flour. In addition, there were no differences in plasma GLP-1 and PYY levels between peas and pea flours of both lines.

As mentioned above, it is crucial to remember the invasive conditions of this trial, in which the participants were intubated for four consecutive days. Again, as VAS is sensitive to the trial conditions (Stubbs *et al.*, 2000), these subjective scores might be less valid when compared to everyday appetite response. Moreover, plasma GLP-1 and PYY were not affected when compared between whole pea seeds and pea flour from both botanical lines. However, the short duration of the current study may not have provided sufficient time for nutrients to fully stimulate the release of GLP-1 and PYY from L-cells located in the distal small intestine and colon. A future study looking primarily at appetite response should be carefully designed.

4.5.3 Strengths, weaknesses, and future recommendations

Designing this study in a cross-over manner was a strength. Furthermore, unlike the first study, in which peas were served within mixed meals, serving the pea treatments without other ingredients in this study allowed for the accurate interpretation of the postprandial metabolic

profile in response to pea seed ingestion. This approach was practical for representing the effect of meal choice in everyday life.

Moreover, to date, this is the first study known to measure in vivo intraduodenal glucose content in healthy participants, who are not on ileostomy bag. However, having a small number of participants could have been masked the real significance of the results. Future properly powered studies based on the current findings are needed.

Notably, this study was designed to assess the metabolomics profiles governing these different pea seeds. This assessment should have helped in broadly identifying the different impacts of these pea interventions on metabolites generated from the lumen, which ultimately influence other gut physiological responses and can further confirm the pathway of a metabolic process governed by those peas. However, by the time this thesis was submitted, it was still not feasible to analyse and interpret the results from the metabolomics assessment. Future work based on this study should reflect the by-products resulting from different pea interventions to identify the differences responsible for the metabolic responses accordingly.

4.6 Conclusion

The analysis of the current study supported the lower vulnerability of SBE-deficient pea seeds to digestion, as reflected by the lower glucose transport to the duodenum and subsequent glycaemic and insulin responses. As RS is beneficial for health due to the lower glucose and insulin response as well as the increased rate of fermentation, the next experimental chapter will be aimed to determine whether SBE-deficient pea seeds and flour leave the small intestine relatively unaffected to deliver more RS to the lower gut for microbiota fermentation to produce more SCFAs.

Chapter 5

5. The impact of food structure on lower gastrointestinal digestion: a focus on the fermentation.

5.1 Introduction

The results so far have shown that both lines of peas leave the stomach at the same time, indicating no effects on GE. However, there was a clear effect on plasma glucose profiles, with the SBE-deficient peas resulting in lower concentrations of glucose compared to wild pea seeds. Duodenal glucose concentrations following the consumption of SBE-deficient peas, when compared with those following wild pea seed consumption, suggest lower breakdown and thus a higher amount of RS delivery to the lower gut for fermentation. The current study aimed to understand whether SBE-deficient peas increase fermentation to a greater extent than wild pea seeds and SBE-deficient flour in the lower gut.

Consuming the same foods, but with different structures, has been shown to result in different digestion profiles and consequent metabolic responses (Jenkins *et al.*, 1982). For instance, while rapidly digestible starches result in higher postprandial responses of glucose and insulin, thereby increasing risk factors of developing metabolism defects such as insulin resistance and obesity, NDC can modulate glucose response and improve insulin sensitivity, thus reducing the risk of developing metabolic conditions and diseases (Jenkins *et al.*, 2002). Upon ingestion, NDC can escape amylase digestion, reaching the lower GI fairly intact, where gut microbiota anaerobically hydrolyse the complex structure in the ileum, caecum, and colon (Topping and Clifton, 2001). Hence, given their lower digestibility, NDC can be classified as low glycaemic index foods (Lappi *et al.*, 2013). Moreover, other beneficial effects are thought to be driven by SCFA production, resulting from microbiota fermentation. These volatile fatty acids can act both as substrate and signalling molecules to regulate appetite and energy homeostasis and may thus drive the beneficial effects of NDCs. The amount of SCFAs produced in response to this process is highly related to the amount of substrate available for microbial fermentation. Due to the inaccessibility of the colon in healthy, free living humans, it is difficult to estimate SCFA colonic production and SCFA bioavailability to the host. However, a general assumption is that 10% of ingested starch is subjected to fermentation (McNeil, 1984; Baghurst, Baghurst and Record, 1996).

Consuming NDCs, as RS, is considered a natural way to increase SCFA production in the lower GI (Englyst and Macfarlane, 1986; Asp, van Amelsvoort and Hautvast, 1996). As such, Young and colleagues have shown that rats fed high-amylose foods had increased propionate concentrations when compared to controls (Young *et al.*, 2012). Similarly, Brown *et al.* found that total faecal SCFA excretion was significantly higher in pigs fed high-amylose starch when compared to controls (Brown *et al.*, 1997). Moreover, altering the physical structure of food plays an important role in fermentation and the amount of SCFA production. Milling, for example, can expose the intracellular content to mechanical and enzymatic actions during upper GI digestion due to an increase in substrate surface area, consequently increasing digestibility and decreasing susceptibility to fermentation (Noah *et al.*, 1998; Bhattarai *et al.*, 2017). The same factors affecting starch digestibility in the upper GI tract can significantly affect the fermentation process in the GI later on and the subsequent level of SCFA production. However, it should be noted that a considerable proportion of less-soluble NDCs are non-fermentable and remain intact until excreted to induce faecal bulk (McDougall *et al.*, 1996).

Importantly, there are inconsistencies in the literature regarding the individual effects of SCFAs, and some studies show no effects after RS supplementation in the long run (Hylla *et al.*, 1998). These discrepancies might be attributed to the amount and type of RS used, differences in the experimental design, and variations within volunteers' responses. Moreover, different RS types have been shown to produce different SCFA profiles. The consumption of RS type II has been shown in both rats and humans to increase butyrate levels (Cummings *et al.*, 1996; Ferguson *et al.*, 2000; Henningsson *et al.*, 2003), while RS type III resulted in higher acetate levels in pigs but not in humans (Cummings *et al.*, 1996; Martin *et al.*, 2000). It is also important to mention that there are difficulties in quantifying starch digestion in the gut, including the type of RS reaching the lower GI to be available for fermentation and the type of SCFA produced consequently. This is because of the challenges of aspirating samples from the upper and lower GI. In vitro assays can provide a considerable amount of information and mechanistic insight about starch digestibility (Englyst *et al.*, 1999), but they do not always reflect the in vivo starch digestion (Priebe *et al.*, 2008).

Here, in an attempt to understand the effect of the food structure of plant-based starchy foods on lower GI fermentation and SCFA production, stable isotope-labelled whole pea seeds and pea flour with natural ^{13}C -enrichment were used. This is a non-ionising radiation method for labelling pea starches located in endosperm, cotyledons and CW that uses elements that are less abundant in the biological environment (ranging from 0.02 to 1%) (Nargund and Sriram, 2013; Umpleby, 2015). Both lines of peas used in the current study were grown in a $^{13}\text{CO}_2$ -enriched environment in a greenhouse to produce pea starch with an enrichment of ~1–2 atom per cent above natural abundance. This is a sufficient enrichment level for identifying metabolites using isotope ratio mass spectrometry (IRMS) technology. This approach has been used successfully by Professor Tom Preston at the University of Glasgow. In a previous *in vitro* study, Professor Preston and his group produced ^{13}C -enriched wheat to make whole-meal wheat bread, aiming to investigate whether starch hydrolysis rates reflect postprandial glucose kinetics in a group of healthy volunteers (Priebe *et al.*, 2008). Thus, this chapter will provide insights about the gut fermentation process and SCFA production in healthy human volunteers after the consumption of ^{13}C wild and SBE-deficient peas and flour.

For the purpose of the current study, participants were requested to consume 50 g dry weight of ^{13}C peas and ^{13}C flour from both genotypes, which were included in a mixed meal test (grown by Prof Tom Preston, University of Glasgow). With advanced mass spectrometry technology, this study aimed to investigate the metabolism of pea starch-derived glucose in the blood by measuring exogenous glucose production (^{13}C plasma glucose) as well as the fermentation activity by measuring fractional ^{13}C SCFAs enrichment in urine between the pea and flour consumption groups.

5.2 Purpose of the study

5.2.1 Hypothesis

It was hypothesised that ^{13}C SBE-deficient peas would result in lower plasma ^{13}C glucose due to lower digestion in the upper GI tract and would deliver more RS to the large intestine and thus produce higher levels of fractional ^{13}C SCFA enrichment in urine compared to wild pea seeds and pea flour.

5.2.2 Aim

This study aimed to investigate the influence of starch delivery to the lower gut and understand whether differences in ^{13}C plasma glucose result in differences in ^{13}C SCFA profiles.

5.2.3 Outcome measures

Co-primary outcomes:

- ^{13}C enrichment profiles in plasma glucose
- Fractional ^{13}C SCFA enrichment in urine

Secondary outcomes

- Breath hydrogen levels as a broader marker of fermentation
- Plasma glucose and serum insulin concentrations
- GLP-1 and PYY concentrations
- VAS to assess subjective appetite

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Peas

Please refer to Chapter 2.

5.3.1.2 Randomisation

Participants were randomised using an independent telephone and internet company (sealedenvelope.com). Both the researcher and volunteers were blinded as to the type of dietary intervention consumed on each study visit and/or during the study duration.

5.3.1.3 Sample size and power calculation

This was a descriptive exploratory study in a new area, and therefore a power calculation was not possible. Ten volunteers participated in this study.

5.3.1.4 Ethical approval

This study was approved by the South East-Coast Surrey Research Ethics Committee (REC project reference number 15/LO/0184) and the Imperial College Healthcare NHS Trust Joint Research Office. This study was performed in accordance with the Declaration of Helsinki.

5.3.1.5 Recruitment

The same recruitment strategy was used for all investigations. Volunteers were recruited through the National Institute for Health Research (NIHR)/Wellcome Trust Imperial CRF at Hammersmith Hospital healthy volunteers' panel database and through poster advertisements. PIS was provided to participants who expressed an interest in taking part in the study (Appendix 5). During the first stage, volunteer eligibility was determined via a brief phone interview. Before volunteers were able to participate in the study, they had to attend a screening visit at the NIHR/Imperial CRF at Hammersmith Hospital to further assess their eligibility. During the

screening visit, the study protocol was explained in full, and the participant provided full written informed consent before commencing any assessment (Appendix 6). The following measurements were collected: height, weight, BP, ECG, a standard screening blood sample, and a pregnancy test for all women of childbearing age. A research doctor had to check all of the volunteers' results before they were enrolled. Participants were then contacted with the results of their screening and given a start date for the study. They were asked not to start any intensive exercise regimen or new diet during the study period, as they could affect with the results.

5.3.1.6 Volunteer inclusion and exclusion criteria

Healthy male and female volunteers between the ages of 18 and 65 years with a BMI of 20–35 kg/m² were invited to take part in this study. The exclusion criteria included the following: any gastrointestinal disease or syndrome; fluctuation in weight of ≥ 3 kg in the preceding two months; diabetes; pregnancy; excess alcohol intake; current smoking; history of substance abuse; cancer; cardiovascular, kidney, or liver diseases; pancreatitis; and the use of medications likely to interfere with hormonal balance, appetite regulation, or energy metabolism including antibiotics, thyroid hormones, anti-inflammatory drugs or steroids, erythromycin, phenytoin, or androgens.

5.3.2 Method

5.3.2.1 Study day preparation and protocol

All participants were required to refrain from alcohol and strenuous exercise and to fast for 12 hours over night. The participants were asked to consume the same standard meal of their choice the evening before each visit to insure consistency between study visits.

Following overnight fasting from 09:00 p.m., volunteers were asked to attend the CRF at 09:00 a.m. (Figure 5.1). Before consuming the test breakfast, participants were asked to empty their bladder in order to measure their weight and body composition using BIA. Participants were asked to stand barefoot on a Tanita segmental body composition analyser (Tanita MC-780MA analyser; Tanita Corporation, Japan). The BIA scale is used to evaluate total body water content with an electrical current. Then, TBW is used to estimate FFM, and the fat mass is calculated by

subtracting FFM from total body weight. Height was measured using a stadiometer. BMI was used to identify eligibility in order to recruit a homogenous cohort.

5.3.2.2 Study design

This was an acute, randomised, controlled, double-blind, cross-over trial involving four study visits. Volunteers were required to attend four study visits held in the CRF. A seven-day interval was proposed between each study visit to washout any ^{13}C residues from the previous visit.

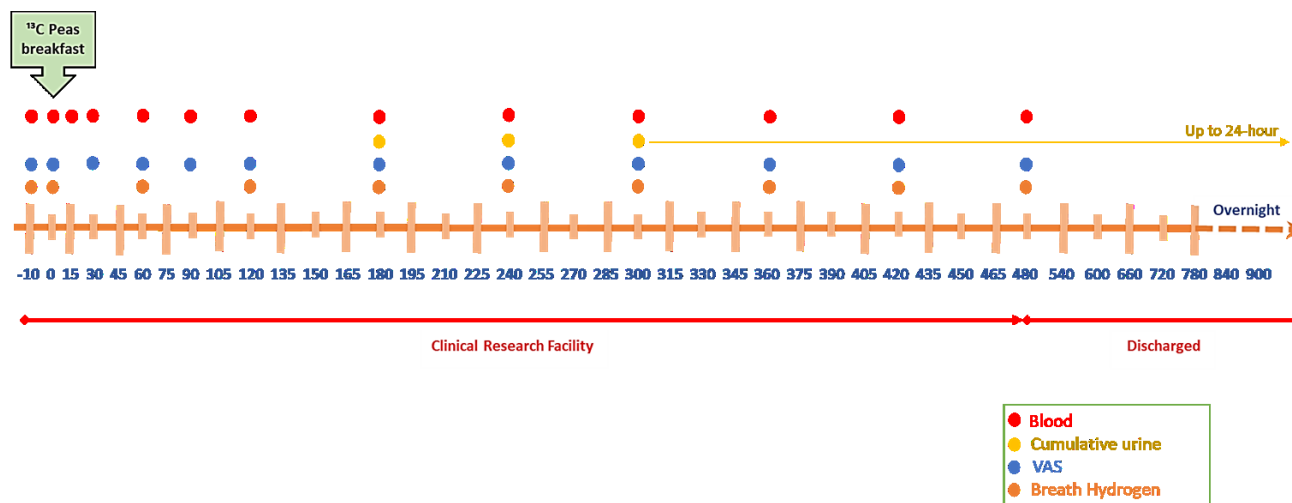


Figure 5.1: Study visit overview.

Overview of timings of blood sampling, cumulative urine samples and VAS ratings. Abbreviations: ^{13}C , stable isotope ^{13}C carbon; VAS, visual analogue scale.

5.3.2.3 Study meal

A standardised breakfast was served at 0 minutes. In random order, the nutritionally matched test meals were freshly prepared to differ only in containing 50 g dry weight of each ^{13}C pea material (for more information about food material, please refer to Chapter 2). The whole pea seeds were soaked for 24 hours in 200 ml of water before cooking for 1 hour at medium temperature on the morning of the study visit. The pea flour was mixed with the tomato soup and microwaved for 2 minutes, and the test meal contained the same food products listed in Table 5.1. Test meals were served and consumed immediately after cooking. Thus, any observed metabolic effects could be attributed to the starch properties or food structure of the test meals

during GI digestive transit. Table 5.1 below lists the nutrition composition of the study meal used in this experiment.

Table 5.1: Nutrition information of the current test meals provided from different pea lines						
Food Products	Unit (g)	Kcal	CHO ^a (g)	Fat (g)	Protein (g)	Fibre (g)
Heinz tomato soup	280	167	20	8.4	1.96	1.68
Hovis soft white bread	80	93.0	17.9	0.7	3.5	1.0
Flora butter	10	40	0.5	4.5	0.5	0
Wild (peas & flour)	50	143	17.6	<0.5	10.6	18
Total (with wild pea seeds & flour)	-	443	56	14.1	16.5	20.68
SBE-deficient (peas and flour)	50	147	14	0.85	11.6	18.1
Total (with SBE-deficient peas & flour)	-	445	53.4	14.9	17.5	20.69

^a detailed carbohydrate analysis is not available

5.3.2.4 Post study visit protocol

In order to keep tracking fractional ¹³C SCFA enrichment in 24-hour urine, participants were asked to empty their bladder before the consumption of the test meal and to collect urine after they had left the CRF to complete a duration of 24 hours. After 480 minutes, participants were discharged from the CRF and provided with a convenient bottle to collect urine after their study visit.

5.3.2.5 Tracking starch metabolism

5.3.2.5.1 Exogenous ¹³C plasma glucose

Liquid chromatography with isotope ratio mass spectrometry (LC/IRMS) was used for the quantification of ¹³C plasma glucose, and analysis was conducted by Dr. Douglas Morrison at the University of Glasgow, as previously described (Morrison *et al.*, 2011). Briefly, plasma samples were diluted 1:5 with L-fucose internal standard. The ¹³C natural abundance of L-fucose was

separately calibrated against the Vienna Pee Dee Belemnite (VPDB) scale and used as a chemical and isotopic internal standard (IS). A total of 0.5 ml of plasma was diluted with 2 ml internal standard. Then, to remove proteins and other high molecular weight compounds, samples underwent ultrafiltration using 30000 molecular weight cut-off ultrafiltration devices (Amicon Ultra 4; Millipore, Watford, UK) at 3600 g for 45 minutes. After this step, samples were stored in two separate aliquots at -20°C for further analysis. Analysis by LC/IRMS was performed as previously described (Morrison *et al.*, 2011). Fucose and glucose peak areas and background-corrected isotope ratios were exported to a spreadsheet for analysis. Glucose enrichment ($\delta^{13}\text{C}$ (‰)) was calculated following an in-house routine using a relative ratio analysis approach against the IS for each sample to report the enrichment of glucose relative to VPDB, and glucose ^{13}C concentration was calculated as the product of enrichment X concentration at each time point. Glucose concentration was calculated from the area ratio of the glucose peak area relative to fucose.

5.3.2.5.2 Fractional ^{13}C SCFAs enrichment in 24-hour urine

Samples were analysed using a previously described procedure (Morrison *et al.*, 2004), which was modified to increase the sensitivity of the analysis. In brief, urine samples (7 ml) were spiked with 200 nmoles 3-methyl valerate (3mV; internal standard) and 200 L NaOH (300 mmoles/L). A 'process blank' was prepared containing freshly deionised water and identical spikes of 3mV and NaOH. Samples and blanks for each run were dried with a vacuum concentrator (Jouan RC10 Vacuum Centrifuge, ThermoFisher, Paisley, UK) at ambient temperature. Dried samples were acidified with 100 μl HCl and SCFA extracted with 400 μl methyl-tert butyl ether (MTBE). A total of 300 μl of the MTBE phase was removed to clean the vials for analysis by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), as previously described (Morrison *et al.*, 2004). The isotopic enrichment of each SCFA was calculated relative to 3mV, which itself had been calibrated against laboratory standards and VPDB. Enrichment of each SCFA with time was expressed relative to the enrichment of the starting pea material ingested to derive a fractional ^{13}C enrichment curve for each SCFA.

5.3.2.5.3 Breath hydrogen concentration

Please refer to Chapter 2.

5.3.2.6 Subjective appetite scales

Please refer to Chapter 2.

5.3.2.7 Blood sampling, collection and analysis

Please refer to Chapter 2 regarding sampling and collecting methods.

5.3.2.7.1 Glucose analysis

For the analysis protocols, please refer to Chapter 2.

5.3.2.7.2 Insulin analysis

For the analysis protocols, please refer to Chapter 2. The % CV of this assay was 3.3%

5.3.2.7.3 GLP-1 analysis

For the analysis protocols, please refer to Chapter 2. The % CV of this assay was 3.1%

5.3.2.7.4 PYY analysis

For the analysis protocols, please refer to Chapter 2. The % CV for this assay was 3.9%

5.3.3 Statistical analysis

Data were checked for normality using a Shapiro–Wilk test (defined as $P \geq 0.01$). In the case of non-normal distribution, data were normalised using log-transformation (the function LN or LN+1 where applicable). If the data were still not normally distributed, non-parametric tests were used. All figures are presented as raw data. Results are expressed as mean difference $\pm$ standard error of the mean (SEM). The average pre and post data points were calculated and used to replace any missing data values. The two baseline samples for blood, VAS, and breath hydrogen data were averaged and used as the 0 min measurement. Postprandial results are presented as tAUC

for breath hydrogen and VAS and as iAUC for all blood parameters. tAUC and iAUC were calculated using the trapezoidal rule. iAUC data presented were the mean values, whereas tAUC mean data were divided based on the study visit time frame. AUC mean values were compared to first look at the effect of starch branching enzyme mutation parameters (whole-whole and flour-flour) and then the effect of cellular structure (wild whole pea seeds/flour and SBE-deficient whole pea seed/-flour), using a paired t-test or Wilcoxon signed-rank test where applicable. Repeated ANOVA measures were used with time and meal as 'within' factors to determine the effect of the test meals on postprandial metabolic responses. Student's paired t-tests were used to identify time points with significantly different values between the two meals, and the Fisher LSD method was used to correct for multiple comparisons. The acute postprandial glucose response to foods is evaluated by the incremental area of blood glucose concentration during a period of 2 hours post-ingestion (iAUC₀₋₁₂₀), which is the most dynamic period to measure metabolic response of a specific amount of food (usually 50 g of carbohydrates) compared to the response of a reference food (Jenkins *et al.*, 1982, 2002; American Diabetes Association *et al.*, 2008); thus, it was calculated for all blood parameters. A p-value ≤ 0.05 was considered to be significant, with the significance level indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Statistical analysis was performed using IBM SPSS version 23. Graphs were prepared using GraphPad Prism version 7.04.

5.4 Results

5.4.1 Baseline characteristics

Ten volunteers were recruited and randomised to take part in this study. Baseline characteristics of these participants are described in Table 5.2

Table 5.2: Demographic characteristics of participants recruited and completed the study (n=10)

Variable	Study visit 1	Study visit 2	Study visit 3	Study visit 4	p-value
Male, female (n)		4, 6			-
Age (Y)		45.5 ± 4.9			-
Weight (Kg)	70.1 ± 2.5	70.2 ± 2.6	69.4 ± 3.4	69.8 ± 2.9	0.40
BMI (Kg/m ²)	24.0 ± 0.9	24.1 ± 0.9	24.2 ± 1.0	24.1 ± 1.0	0.42
Body Fat (%)	28.5 ± 1.8	27.7 ± 2.0	27.2 ± 1.9	27.8 ± 2.0	0.81
<i>Data are means ± SEM or (n)</i>					

5.4.2 Exogenous ^{13}C plasma glucose

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.2 shows time course, iAUC_{0-480} , and iAUC_{0-120} data on ^{13}C plasma glucose concentrations, comparing the effect of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated a significant effect when comparing postprandial plasma ^{13}C glucose AUC_{0-480} following the consumption of SBE-deficient peas ($1.3 \pm 0.2 \mu\text{M}$) with that following the consumption of wild peas ($1.7 \pm 0.1 \mu\text{M}$) ($p=0.04$). The same effect was not observed when comparing postprandial plasma ^{13}C glucose AUC_{0-120} between wild and SBE-deficient peas ($p=0.85$).

A borderline main effect of peas was found ($p=0.09$). However, no pea X time interaction was found ($p=0.31$). Significant late peaks in ^{13}C plasma glucose were found at 120 minutes ($p=0.03$), 180 minutes ($p=0.03$), and 240 minutes ($p<0.01$) following wild pea consumption when compared to SBE-deficient pea consumption.

Pea flours:

The results indicated no effect when comparing postprandial ^{13}C plasma glucose AUC_{0-480} between wild and SBE-deficient flour ($p=0.94$). The same lack of effect was observed in ^{13}C glucose AUC_{0-120} between wild and SBE-deficient flour ($p=0.61$).

No main effects of flour ($p=0.81$) or flour X time interaction ($p=0.98$) were found. There was an early peak of ^{13}C plasma glucose concentrations in the flour interventions (30–90 minutes).

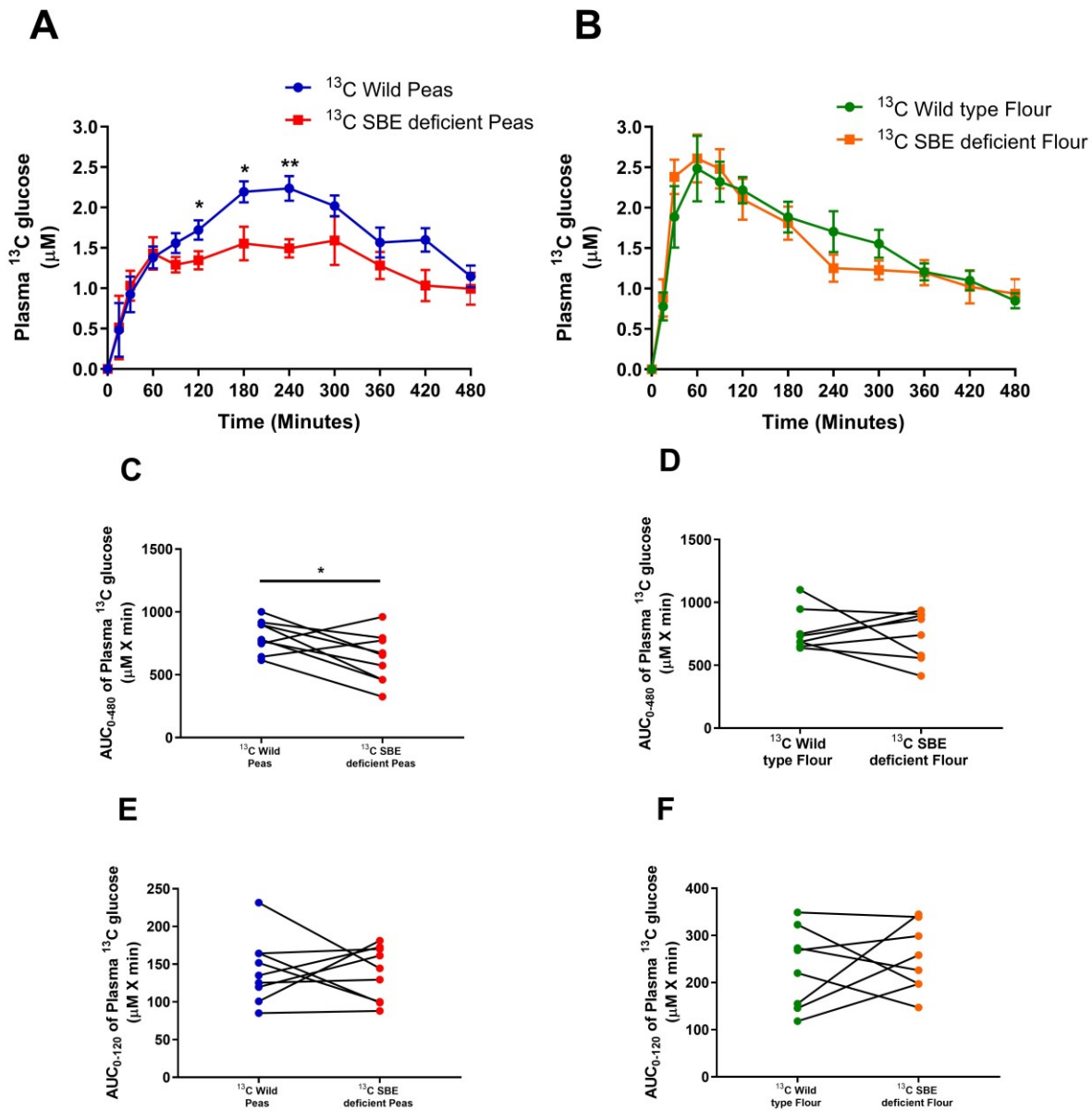


Figure 5.2: ^{13}C plasma glucose in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) AUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) AUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) AUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for AUCs calculations. Time-points and data at which values differed significantly, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: AUC, area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.3 shows time course, iAUC₀₋₄₈₀, and iAUC₀₋₁₂₀ data on plasma ¹³C glucose concentrations, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seed and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing postprandial plasma ¹³C glucose AUC₀₋₄₈₀ between wild peas and wild flour (p=0.94). There was a borderline reduction in postprandial plasma ¹³C glucose AUC₀₋₁₂₀ following the consumption of wild peas ($0.3 \pm 0.1 \mu\text{M}$) when compared to flour consumption ($0.5 \pm 0.1 \mu\text{M}$) (p=0.06).

No main effect of meal found (p=0.53). However, a significant effect of meal X time interaction was found (p<0.01). Significant early ¹³C plasma glucose peaks were found at 60 minutes (p=0.04) and 90 minutes (p=0.04), followed by significant reductions at 300 minutes (p=0.03) and 420 minutes (p=0.03) after wild flour consumption when compared to wild pea consumption.

SBE-deficient pea seeds and flour:

The results indicated a significant effect when comparing ¹³C plasma glucose AUC₀₋₄₈₀ following SBE-deficient peas consumption ($1.3 \pm 0.1 \mu\text{M}$) with that following SBE-deficient flour consumption ($1.6 \pm 0.1 \mu\text{M}$) (p=0.01). The same effect was observed in postprandial plasma ¹³C glucose AUC₀₋₁₂₀ following SBE-deficient peas consumption ($0.3 \pm 0.1 \mu\text{M}$) when compared to SBE-deficient flour consumption ($0.5 \pm 0.1 \mu\text{M}$) (p<0.01).

Significant main effects of meal (p<0.01) and meal X time interaction (p<0.01) were found. Significant early peaks of ¹³C plasma glucose were found at 30 minutes (p<0.01), 60 minutes (p<0.01), 90 minutes (p<0.01), and 120 minutes (p<0.01) following SBE-deficient flour consumption when compared to SBE-deficient pea consumption.

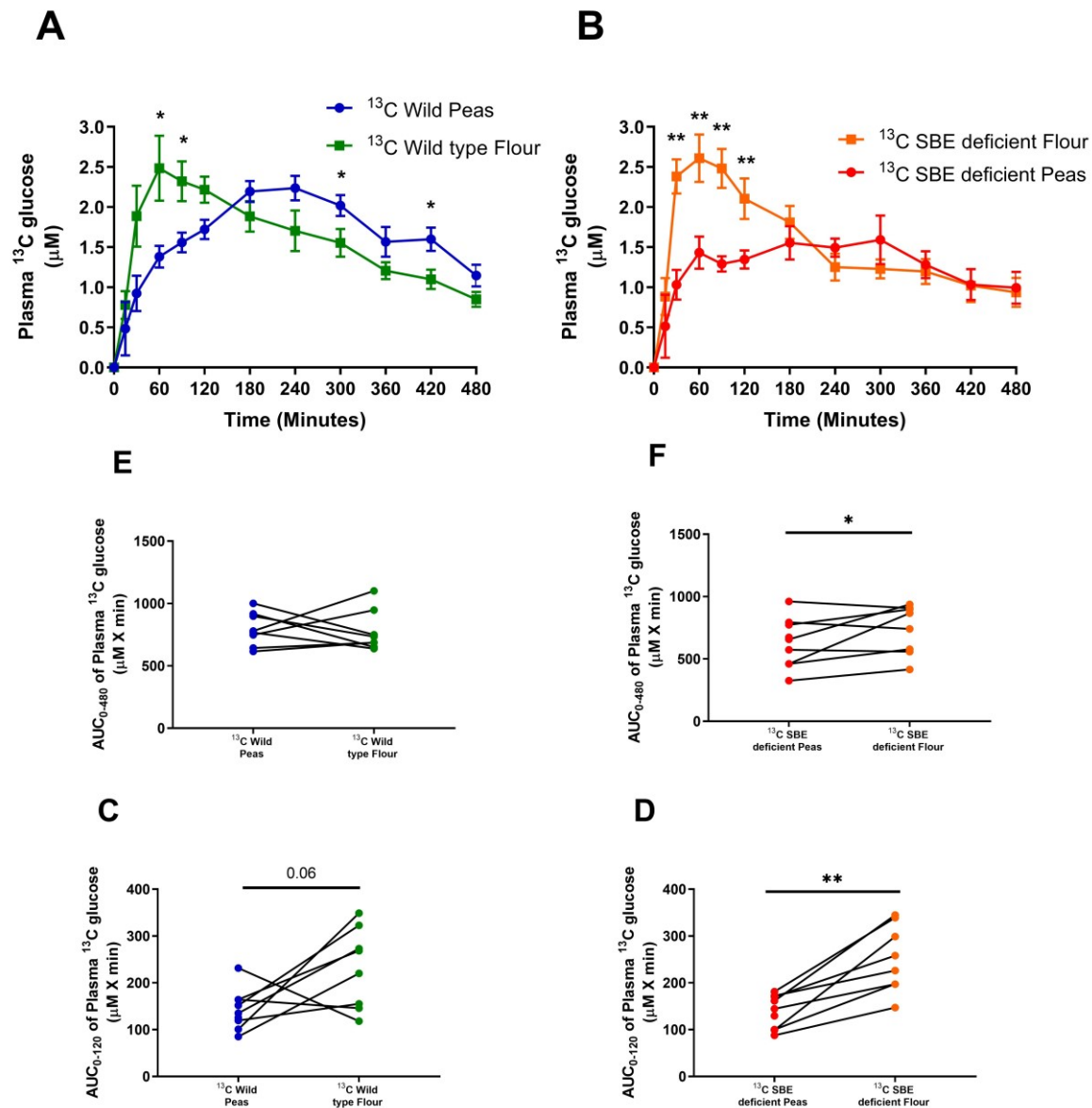


Figure 5.3: ^{13}C plasma glucose in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and wild flour (---), (B) SBE-deficient peas (---) and SBE-deficient flour (---). Insets represent (C) AUC_{0-480} for wild peas (●) and wild flour (●), (D) AUC_{0-480} for SBE-deficient peas (●) and SBE-deficient flour (●), (E) AUC_{0-120} for wild peas (●) and wild flour (●), (F) AUC_{0-120} for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for AUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: AUC, area under the curve.

5.4.3 Fractional ^{13}C SCFA enrichment in 24-hour urine

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.4 shows time course and cumulative data on fractional ^{13}C SCFA enrichment in 24-hour urine, comparing the effect of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing fractional ^{13}C acetate enrichment in urine between wild and SBE-deficient peas ($p=0.98$). However, the results indicated a significant effect when comparing fractional ^{13}C propionate enrichment in urine following SBE-deficient pea consumption (13.9 ± 0.6 ppm) with the enrichment following wild pea consumption (8.5 ± 1.5 ppm) ($p=0.01$). The same effect was observed in fractional ^{13}C butyrate enrichment in urine following SBE-deficient pea consumption (10.4 ± 1.0 ppm) compared to wild pea consumption (6.2 ± 1.1 ppm) ($p=0.01$).

Pea flours:

The results indicated no effect when comparing fractional ^{13}C acetate enrichment in urine between wild and SBE-deficient flour ($p=0.94$). However, the results indicated a significant effect when comparing fractional ^{13}C propionate enrichment in urine following SBE-deficient flour consumption (14.8 ± 1.0 ppm) compared to wild flour consumption (10.2 ± 0.9 ppm) ($p\leq0.01$) and a trend towards an increase in fractional ^{13}C butyrate enrichment in urine following SBE-deficient flour consumption (13.1 ± 1.7 ppm) compared to wild flour consumption (8.0 ± 0.5 ppm) ($p=0.05$).

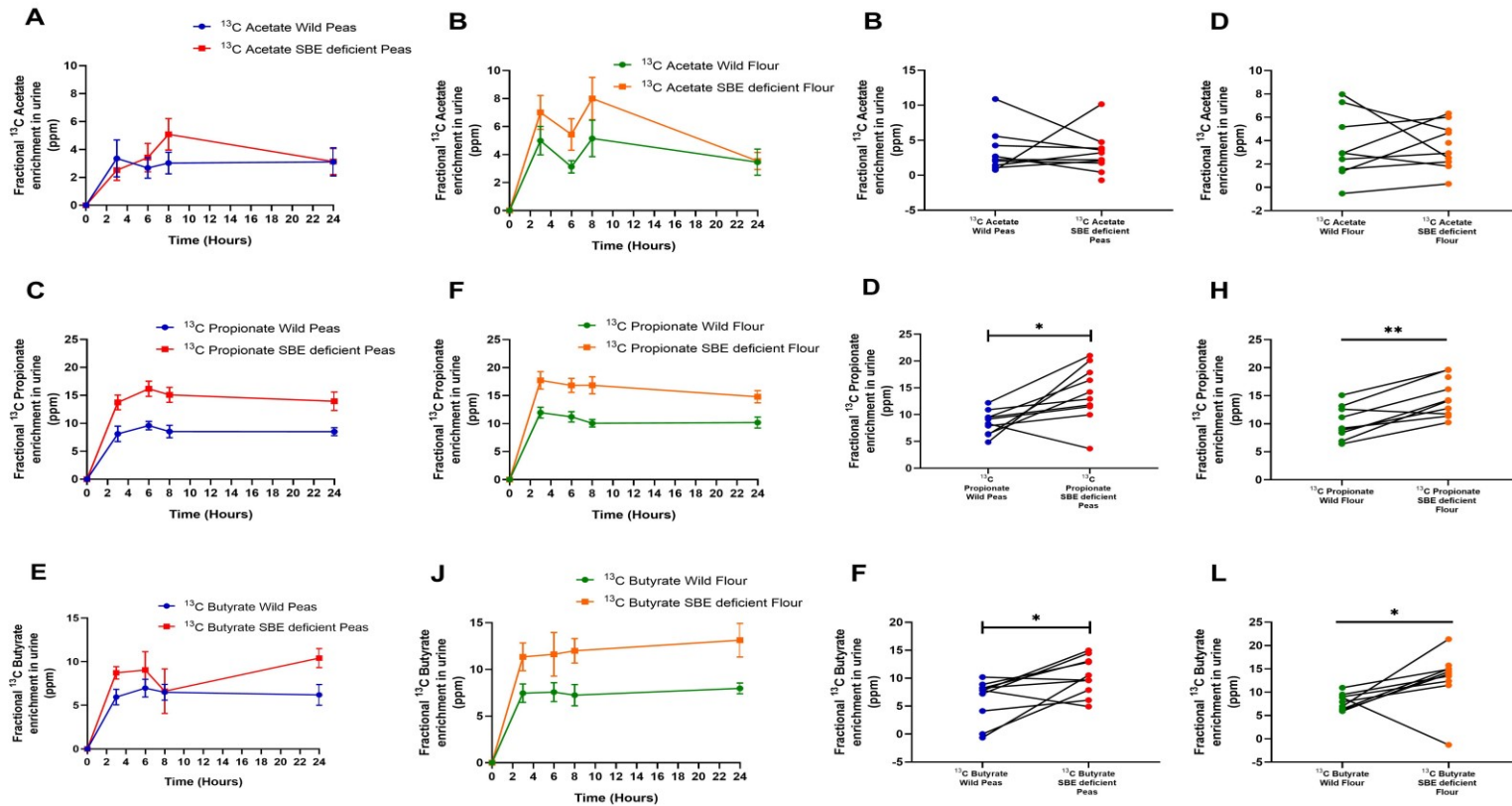


Figure 5.4: Fractional ^{13}C SCFAs enrichment in 24 hour urine in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) Acetate, (E) Propionate, (I) Butyrate following consumption of wild peas (---) and SBE-deficient peas (---), mean $\pm$ SEM of (B) Acetate, (F) Propionate, (J) Butyrate following consumption of wild flour (---) and SBE-deficient flour (---), Urinary cumulative fraction of (C) Acetate, (G) Propionate, (K) Butyrate 24 hours postprandial following consumption of wild peas (●) and SBE-deficient peas (●), Urinary cumulative fraction of (D) Acetate, (H) Propionate, (L) Butyrate 24 hours postprandial following consumption of wild flour (●) and SBE-deficient flour (●). Paired t-tests were used for cumulative fraction calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: SCFAs, Short Chain Fatty Acids.

Secondary comparisons looking at the effect of cellular structure

Figure 5.5 shows time course and cumulative data for fractional ^{13}C SCFA enrichment in 24-hour urine, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seed and flour.

Wild pea seeds and flour:

The results indicated no effects when comparing fractional ^{13}C acetate ($p=0.92$) and ^{13}C butyrate ($p=0.34$) enrichment in urine between wild peas and flour. There was a significant effect when comparing fractional ^{13}C propionate enrichment in urine following wild flour consumption (10.2 ± 0.9 ppm) to that following wild pea consumption (8.5 ± 1.5 ppm) ($p<0.01$).

SBE-deficient pea seeds and flour:

The results indicated no effects when comparing fractional ^{13}C acetate ($p=0.62$), ^{13}C propionate ($p=0.61$), and ^{13}C butyrate ($p=0.14$) enrichment in urine between SBE-deficient peas and flour.

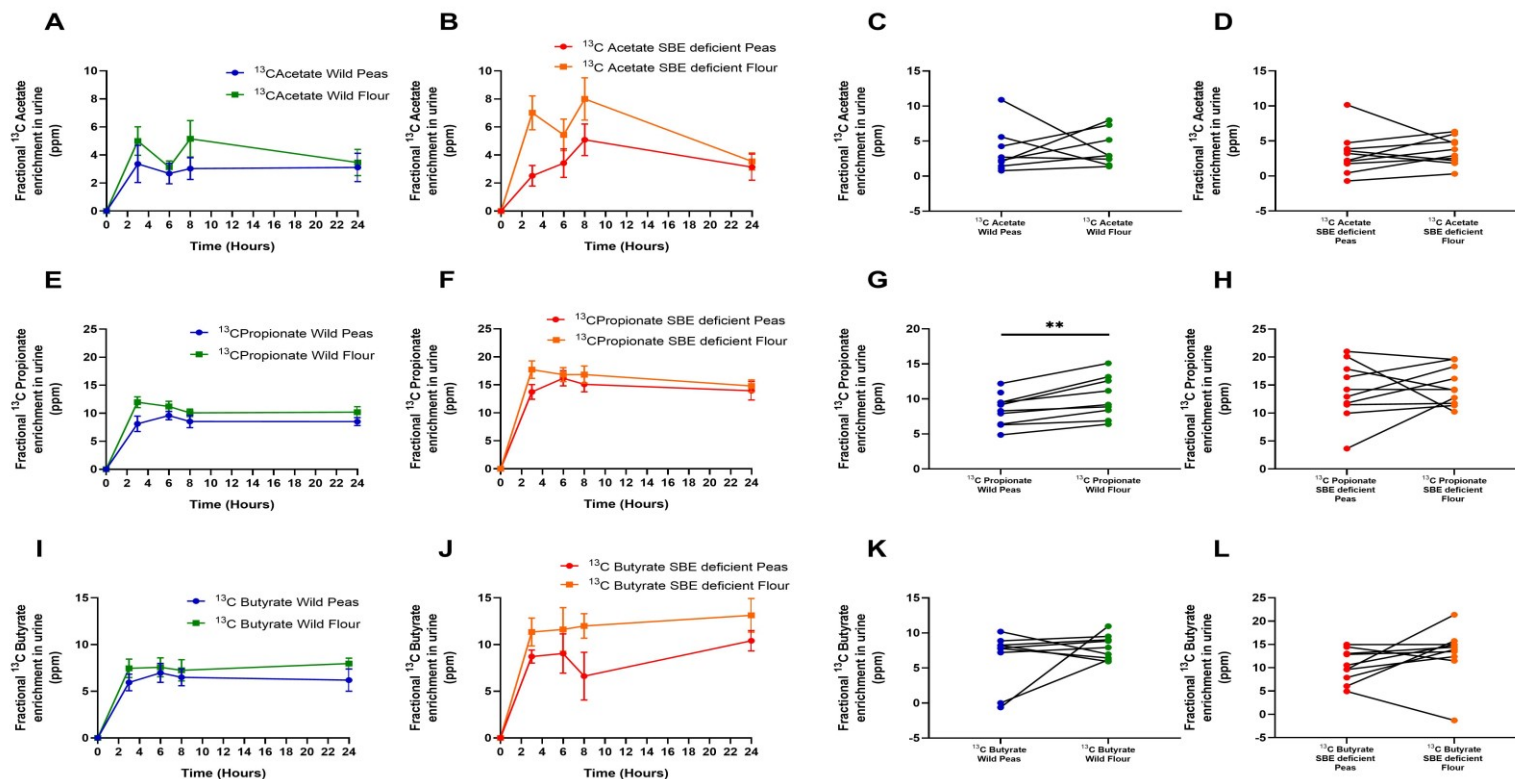


Figure 5.5: Fractional ^{13}C SCFAs enrichment in 24 hour urine in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) Acetate, (E) Propionate, (I) Butyrate following consumption of wild peas (---) and wild flour (---), mean $\pm$ SEM of (B) Acetate, (F) Propionate, (J) following consumption of SBE-deficient peas (---) and SBE-deficient flour (---), Urinary cumulative fraction of (C) Acetate, (G) Propionate, (K) Butyrate 24 hours postprandial following consumption of wild peas (●) and wild flour (●), Urinary cumulative fraction of (D) Acetate, (H) Propionate, (L) Butyrate 24 hours postprandial following consumption of SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for cumulative fraction calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: SCFAs, Short Chain Fatty Acids

5.4.4 Breath H₂

The time-course graphs of breath H₂ suggest that fermentation starts to occur after 180–240 minutes postprandial.

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.6 shows time course and tAUC data on breath hydrogen excretion meter readings, comparing the effect of mutation between whole pea seeds and between pea flours.

There were no differences in baseline values.

Whole pea seeds:

The results indicated no effect when comparing tAUC₀₋₄₈₀ breath hydrogen excretion between wild and SBE-deficient peas ($p=0.22$).

No main effects of peas ($p=0.51$) and pea X time interaction ($p=0.17$) were found.

Pea flours:

The results indicated a significant effect when comparing tAUC₀₋₄₈₀ breath hydrogen excretion following the consumption of SBE-deficient flour (19.3 ± 1.8 ppm) with that following wild flour consumption (12.7 ± 2.2 ppm) ($p \leq 0.01$).

A main effect of flour was found ($p=0.03$). However, no flour X time interaction was found ($p=0.19$). Significant increases in breath hydrogen excretion were found at 240 minutes ($p < 0.01$), 300 minutes ($p < 0.01$), 360 minutes ($p < 0.01$), 420 minutes ($p = 0.01$), and 480 minutes ($p < 0.01$) following SBE-deficient flour consumption when compared with wild flour consumption.

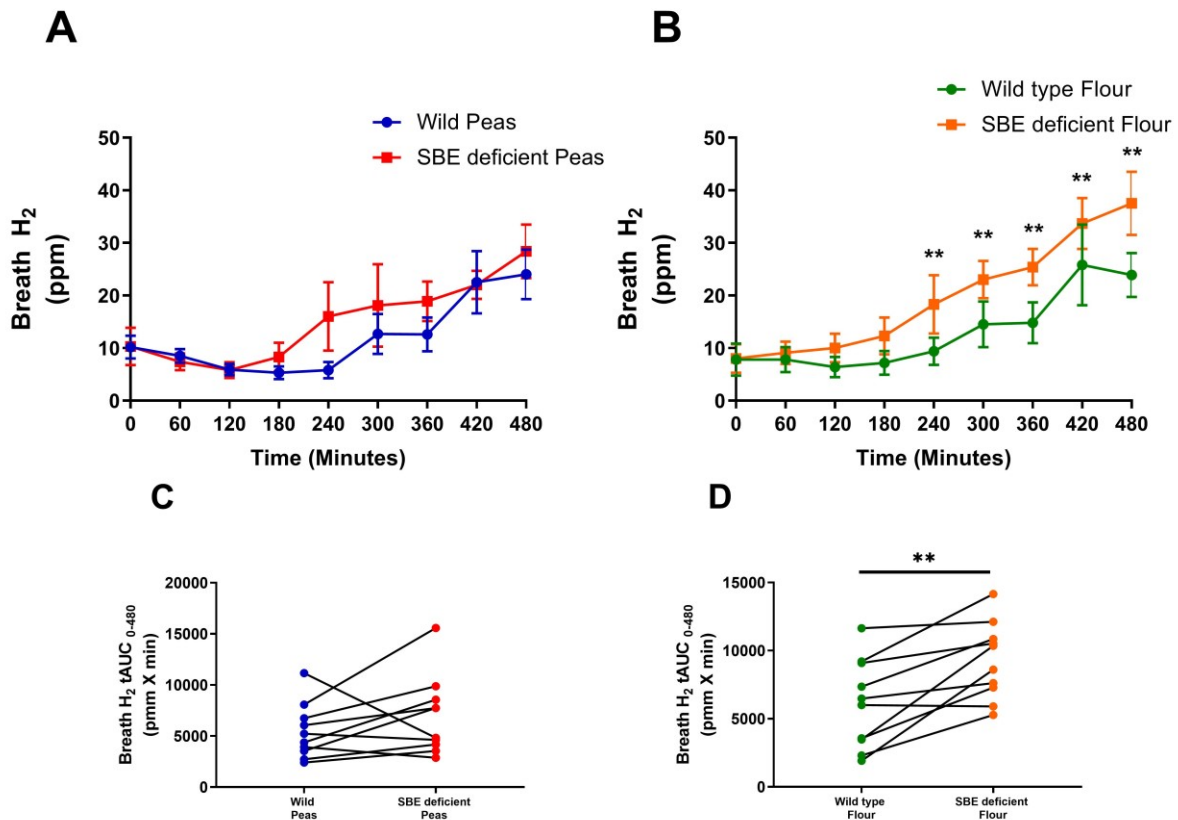


Figure 5.6: Breath hydrogen excretion in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for tAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.7 shows time course and tAUC data on breath hydrogen excretion meter readings, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

There were no differences in baseline values.

Wild pea seeds and flour:

The results indicated no effect when comparing tAUC₀₋₄₈₀ breath hydrogen excretion between wild peas and wild flour ($p=0.57$).

No main effects of meal ($p=0.98$) and meal X time interaction ($p=0.19$) were found.

SBE-deficient pea seeds and flour:

The results indicated a borderline increase when comparing tAUC₀₋₄₈₀ breath hydrogen excretion following SBE-deficient flour consumption (19.3 ± 1.8 ppm) with that following SBE-deficient pea consumption (14.5 ± 2.3 ppm) ($p=0.05$).

No main effect of meal was found ($p=0.10$). However, significant meal X time interaction was found ($p=0.01$). A significant increase in breath hydrogen excretion was found at 420 minutes ($p<0.01$) following SBE-deficient flour consumption when compared to SBE-deficient pea consumption.

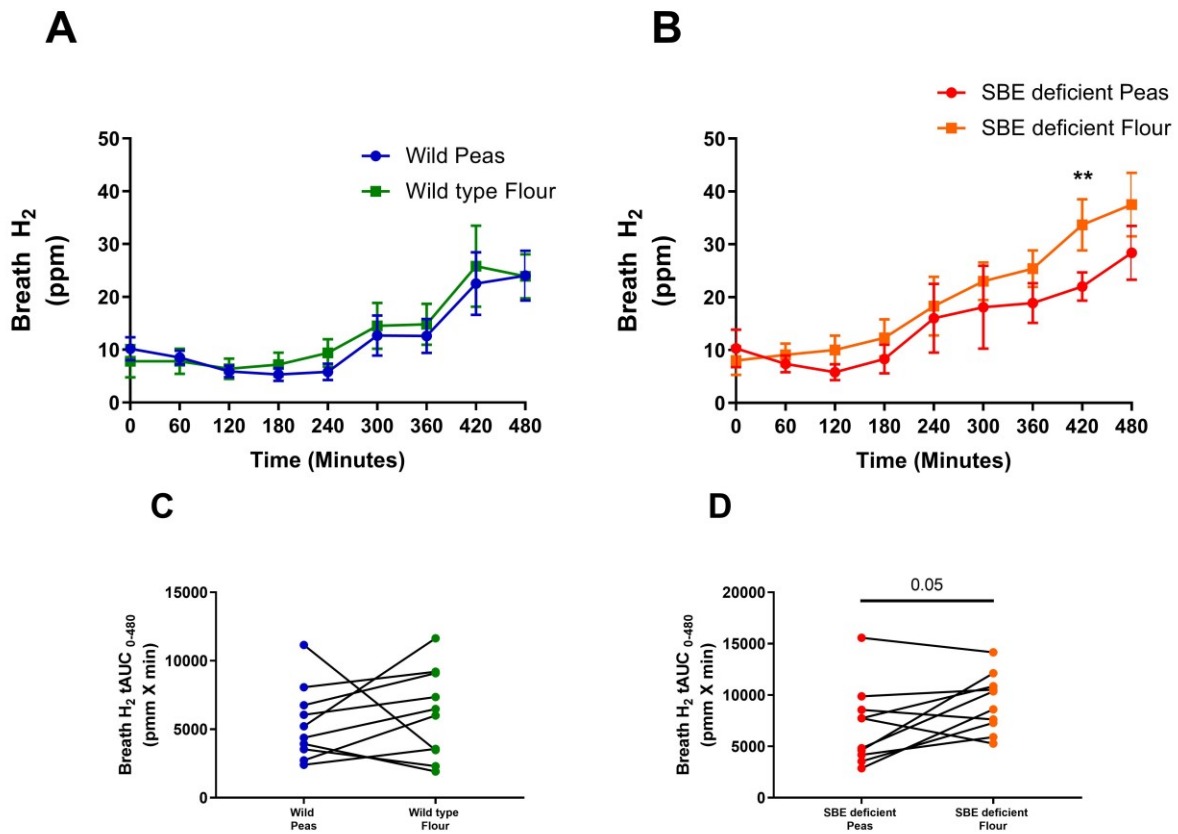


Figure 5.7: Breath hydrogen excretion in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (D) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for tAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve.

5.4.5 Plasma glucose

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.8 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on plasma glucose concentrations, comparing the effect of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma glucose $iAUC_{0-480}$ between wild and SBE-deficient peas ($p=0.57$). The same lack of effect was observed when comparing plasma glucose $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.95$).

No main effects of peas ($p=0.31$) and pea X time interaction ($p=0.70$) were found.

Pea flours:

The results indicated no effect when comparing plasma glucose $iAUC_{0-480}$ wild and SBE-deficient flour ($p=0.68$). The same lack of effect was observed when comparing plasma glucose $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.20$).

No main effects of flour ($p=0.86$) and flour X time interaction ($p=0.96$) were found.

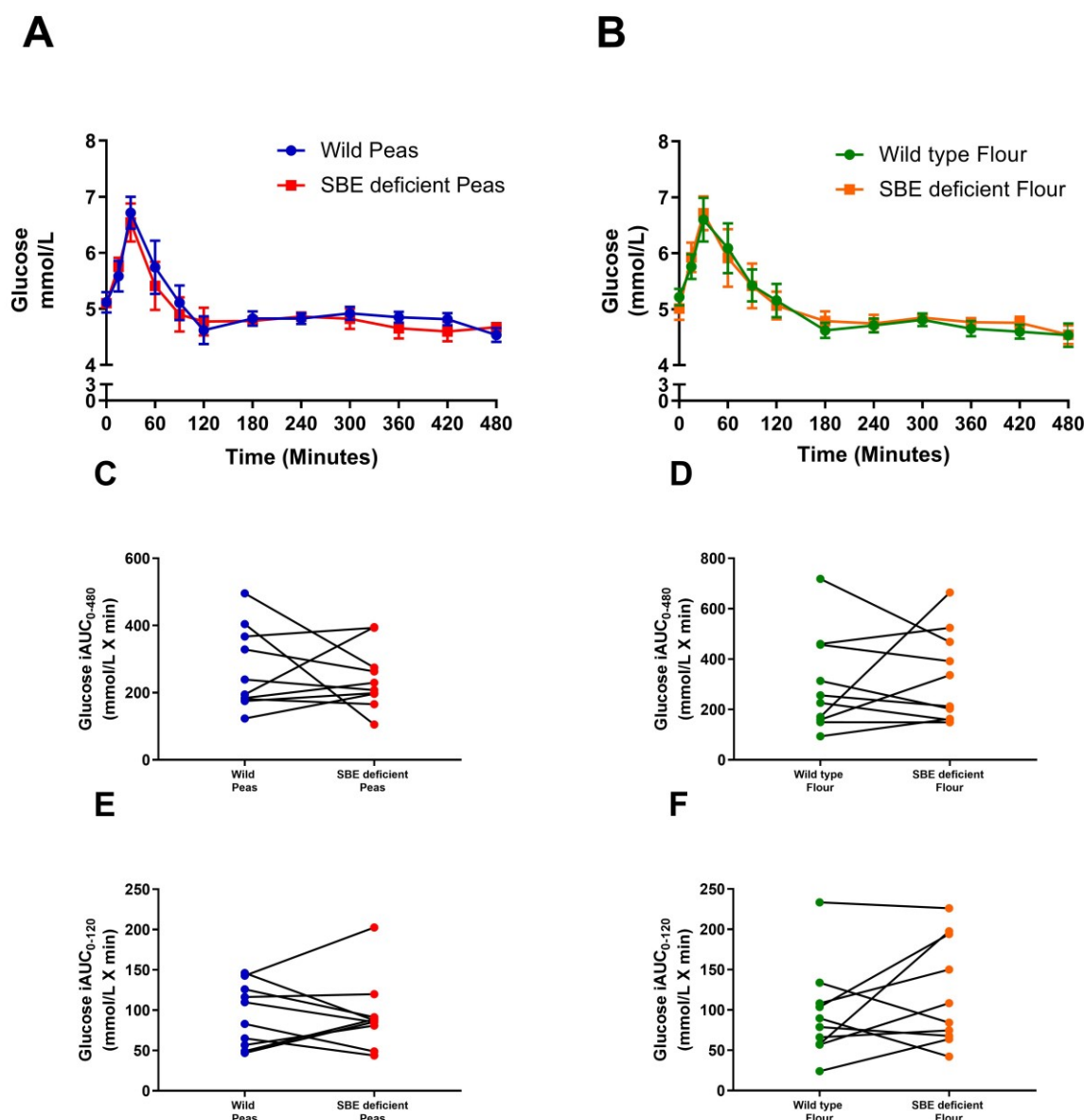


Figure 5.8: Postprandial plasma glucose concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.9 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on plasma glucose concentrations, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing $iAUC_{0-480}$ plasma glucose between wild peas and wild flour ($p=0.55$). The same lack of effect was observed when comparing $iAUC_{0-120}$ plasma glucose between wild peas and wild flour ($p=0.95$).

No main effects of meal ($p=0.54$) and meal X time interaction ($p=0.16$) were found.

SBE-deficient pea seeds and flour:

The results indicated a borderline reduction in $iAUC_{0-480}$ plasma glucose following SBE-deficient pea consumption (243.0 ± 26.8 mmol/L x min) compared to that following SBE-deficient flour consumption (327.0 ± 57.1 mmol/L x min) ($p=0.07$). The results indicated no effect when comparing $iAUC_{0-120}$ plasma glucose levels between SBE-deficient peas and flour ($p=0.76$).

No main effects of meal ($p=0.42$) and meal X time interaction ($p=0.53$) were found.

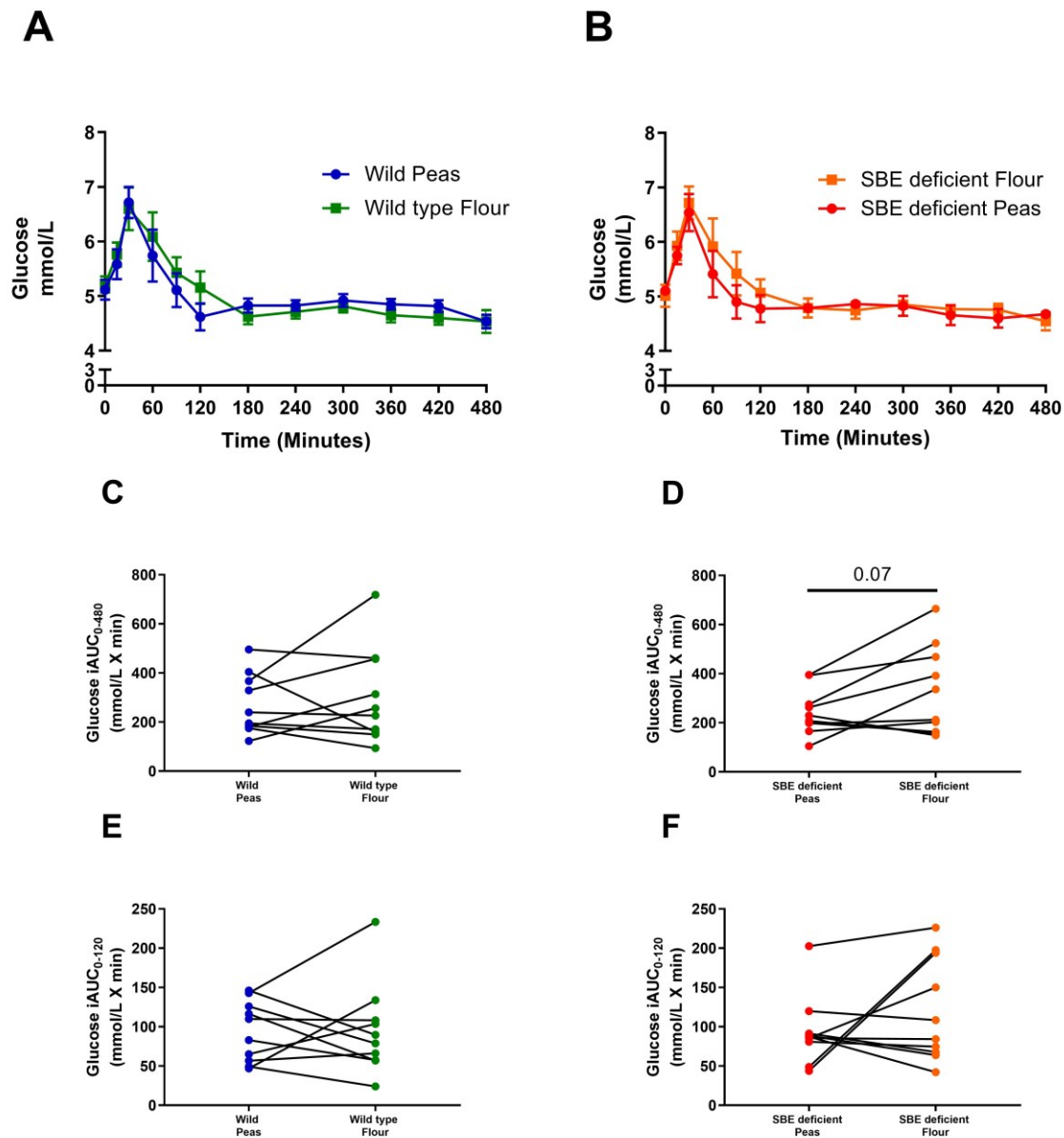


Figure 5.9: Postprandial plasma glucose concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ for wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

5.4.6 Serum insulin

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.10 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on serum insulin concentrations, comparing the effect of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing serum insulin $iAUC_{0-480}$ between wild and SBE-deficient peas ($p=0.73$). However, the results indicated a significant decrease when comparing serum insulin $iAUC_{0-120}$ following SBE-deficient pea consumption ($3056.1 \pm 657.6 \mu U/ml$) with that following wild pea consumption ($3677.6 \pm 588.4 \mu U/ml$) ($p=0.03$).

No main effects of peas ($p=0.16$) and pea X time interaction ($p=0.16$) were found.

Pea flours:

The results indicated no effect when comparing serum insulin $iAUC_{0-480}$ between wild and SBE-deficient flour ($p=0.65$). The same effect was not observed when comparing serum insulin $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.81$).

No main effects of meal ($p=0.10$) and meal X time interaction ($p=0.52$) were found.

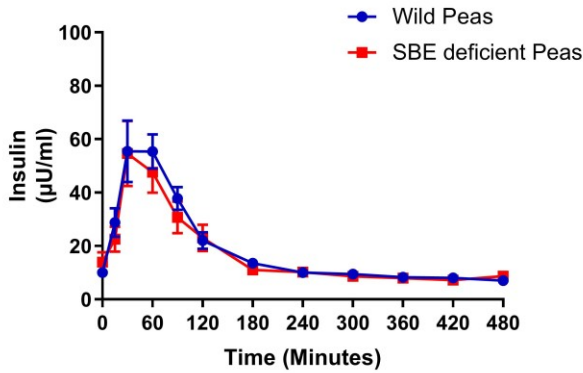
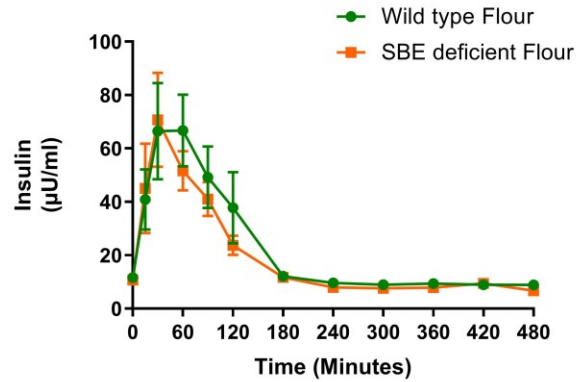
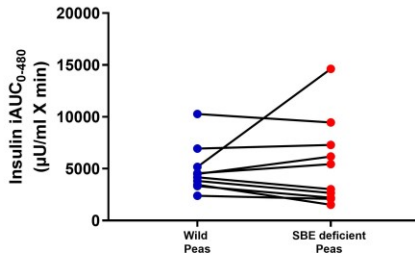
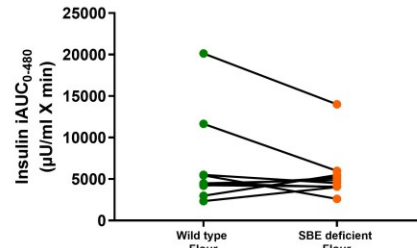
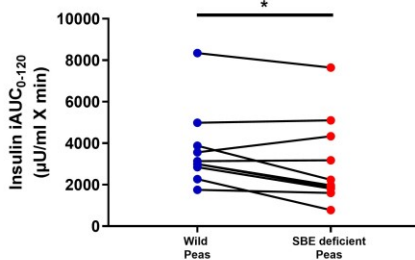
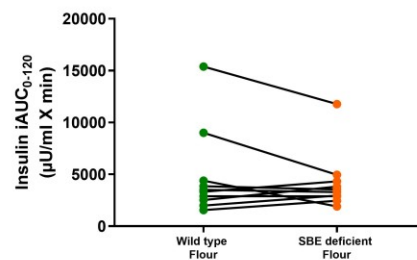
A**B****C****D****E****F**

Figure 5.10: Postprandial serum insulin concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.11 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on serum insulin concentrations, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing serum insulin $iAUC_{0-480}$ between wild peas and flour ($p=0.36$). The same lack of effect was observed when comparing $iAUC_{0-120}$ serum insulin following wild pea and wild flour consumption ($p=0.49$).

No main effects of meal ($p=0.17$) and meal X time interaction ($p=0.26$) were found.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing serum insulin $iAUC_{0-480}$ between SBE-deficient peas and SBE-deficient flour ($p=0.43$). The same effect was observed when comparing $iAUC_{0-120}$ serum insulin between SBE-deficient peas (3056.1 ± 657.6) and SBE-deficient flour (4193.8 ± 888.6) ($p=0.04$).

No main effect of meal ($p=0.14$) was found. However, a significant meal X time interaction ($p<0.01$) was found. Significant increases of serum insulin were found at 15 minutes ($p=0.03$) and 90 minutes ($p=0.02$), followed by reductions to below baseline at 240 minutes ($p=0.01$) and 480 minutes ($p=0.02$) following SBE-deficient flour consumption when compared to SBE-deficient pea consumption.

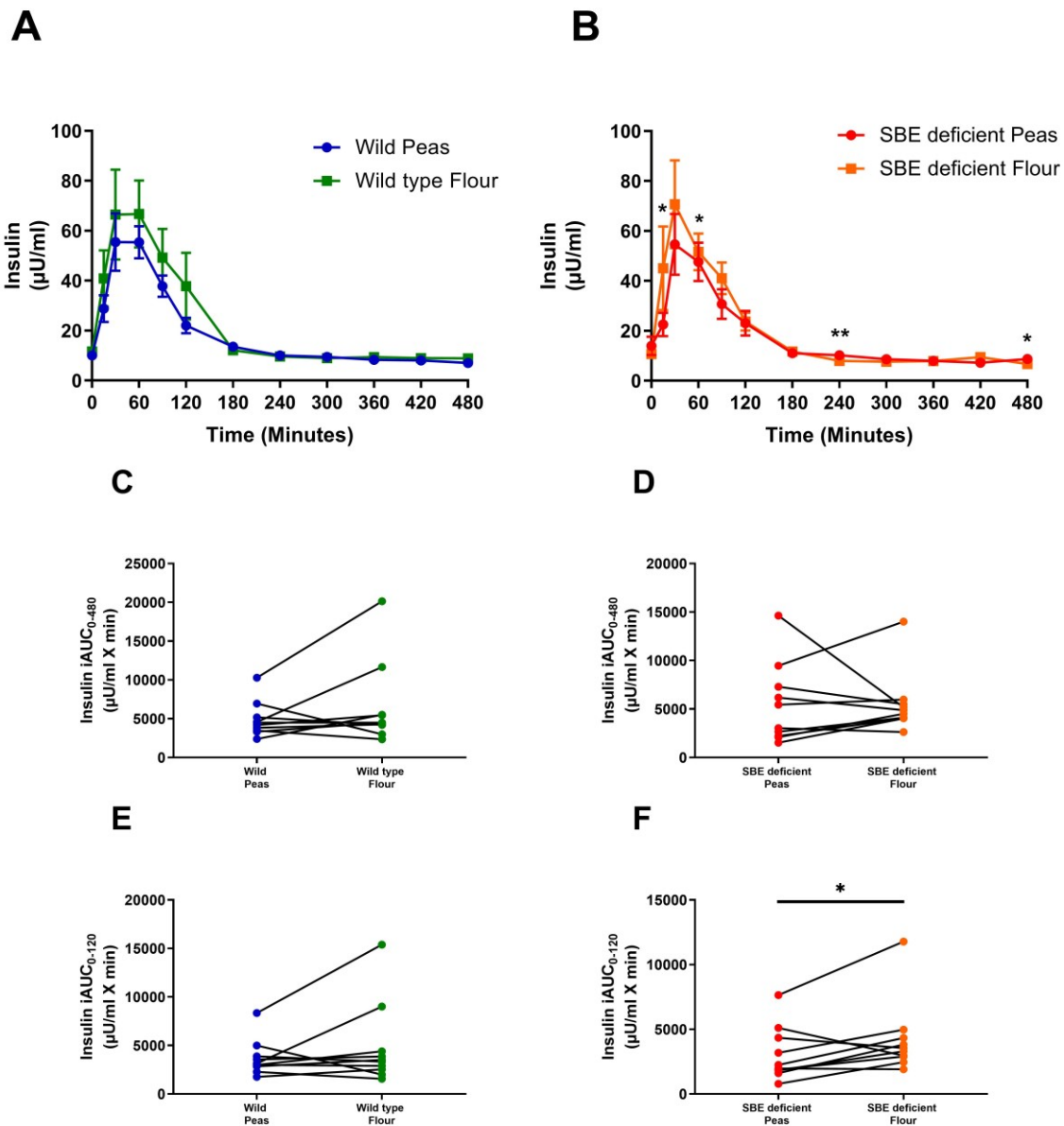


Figure 5.11: Postprandial serum insulin concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ for wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

5.4.7 Plasma GLP-1

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.12 shows time course, iAUC₀₋₄₈₀, and iAUC₀₋₁₂₀ data on plasma GLP-1 concentrations, comparing the effect of mutation between whole pea seeds and between Pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma GLP-1 iAUC₀₋₄₈₀ between wild and SBE-deficient peas ($p=0.89$). The same lack of effect was observed when comparing plasma GLP-1 iAUC₀₋₁₂₀ between wild and SBE-deficient peas ($p=0.42$).

No main effects of peas ($p=0.63$) and peas X time interaction ($p=0.19$) were found.

Pea flours:

The results indicated no effect when comparing plasma GLP-1 iAUC₀₋₄₈₀ between wild and SBE-deficient flour ($p=0.64$). However, the results indicated a significant increase when comparing plasma GLP-1 iAUC₀₋₁₂₀ following SBE-deficient flour consumption (4020.6 ± 686.0 pmol/L) to that following wild flour consumption (2631.9 ± 505.7 pmol/L) ($p=0.02$).

No main effects of flour ($p=0.53$) and flour X time interaction ($p=0.64$) were found.

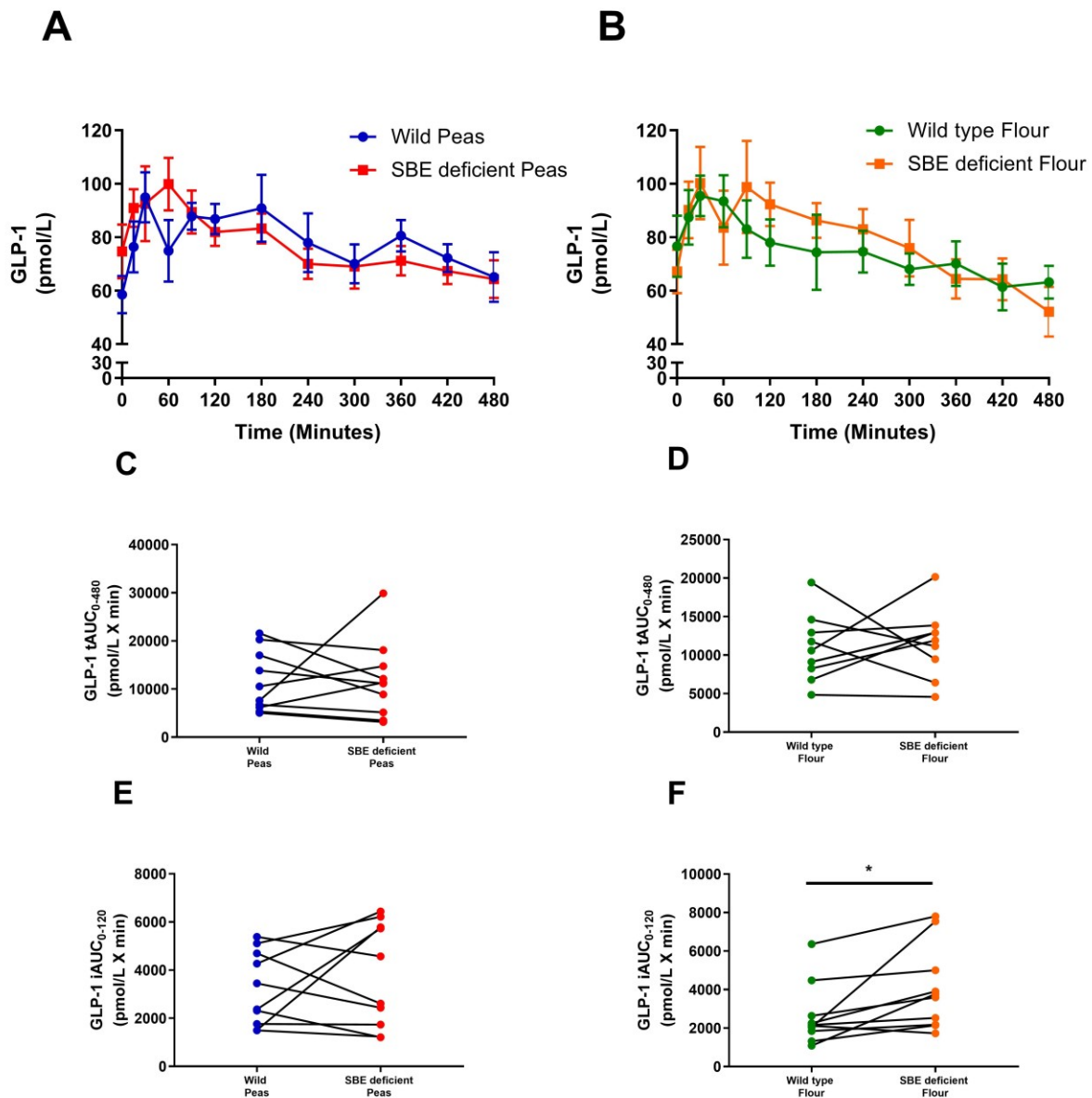


Figure 5.12: Postprandial plasma GLP-1 concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.13 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on plasma GLP-1 concentrations, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated no effect when comparing $iAUC_{0-480}$ plasma GLP-1 between wild peas and flour ($p=0.77$). The same lack of effect was observed when comparing $iAUC_{0-120}$ plasma GLP-1 between wild peas and flour ($p=0.29$)

No main effects of meal ($p=0.91$) and meal X time interaction ($p=0.10$) were found.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing $iAUC_{0-480}$ plasma GLP-1 levels between SBE-deficient peas and SBE-deficient flour ($p=0.94$). The same lack of effect was observed when comparing $iAUC_{0-120}$ plasma GLP-1 levels between SBE-deficient peas and SBE-deficient flour ($p=0.76$).

No main effects of meal ($p=0.95$) and meal X time interaction ($p=0.64$) were found.

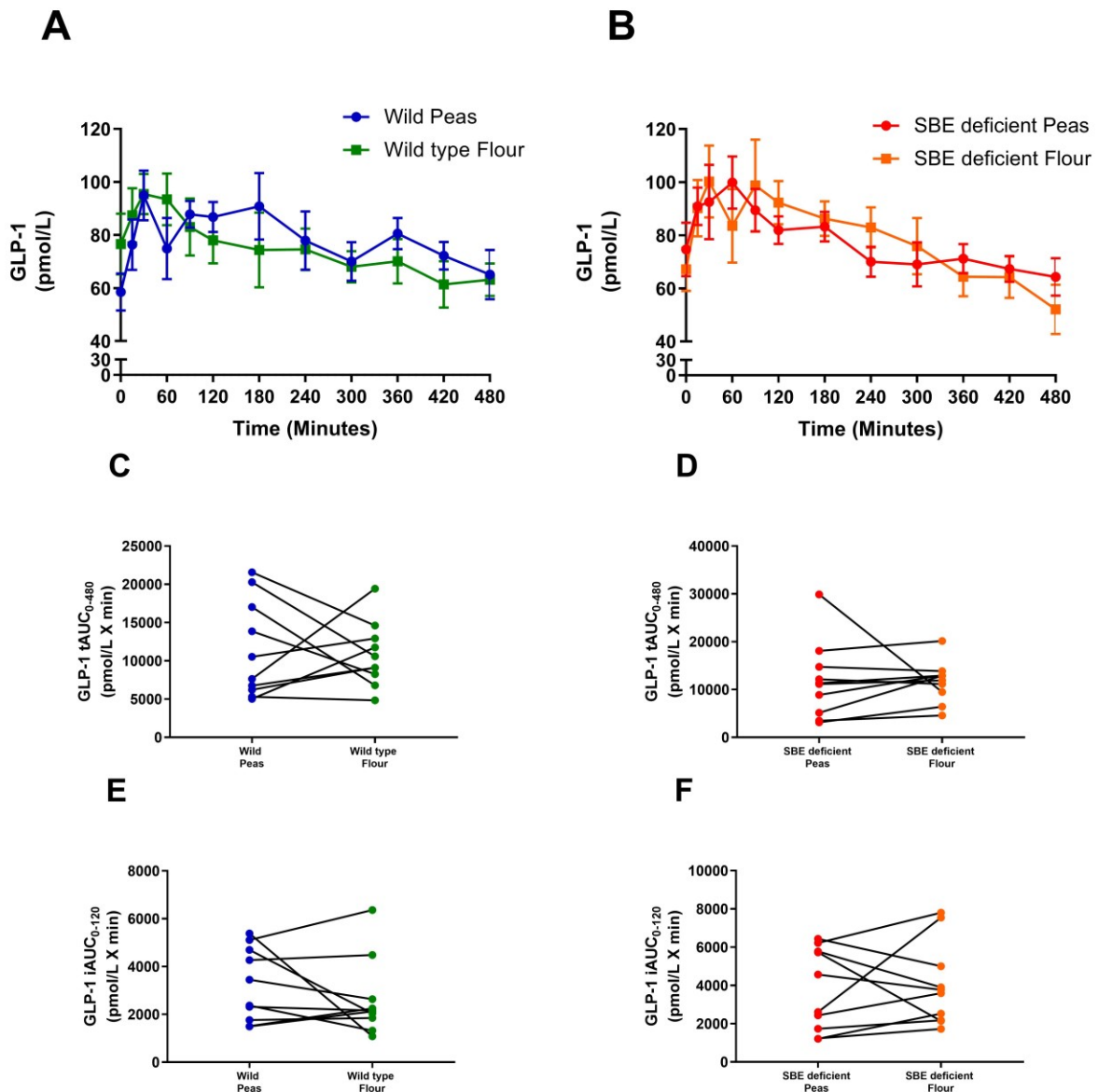


Figure 5.13: Postprandial plasma GLP-1 concentration in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ for wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

5.4.8 Plasma PYY

Primary comparisons looking at the effect of starch branching enzyme mutation

Figure 5.14 shows time course, $iAUC_{0-480}$, and $iAUC_{0-120}$ data on plasma PYY concentrations, comparing the effect of mutation between whole pea seeds and between pea flours.

Whole pea seeds:

The results indicated no effect when comparing plasma PYY $iAUC_{0-480}$ between wild and SBE-deficient peas ($p=0.82$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient peas ($p=0.88$).

No main effects of peas ($p=0.24$) and pea X time interaction ($p=0.92$) were found.

Pea flours:

The results indicated no effect when comparing plasma PYY $iAUC_{0-480}$ between wild and SBE-deficient flour ($p=0.88$). The same lack of effect was observed when comparing plasma PYY $iAUC_{0-120}$ between wild and SBE-deficient flour ($p=0.65$).

No main effects of flour ($p=0.71$) and flour X time interaction ($p=0.91$) were found.

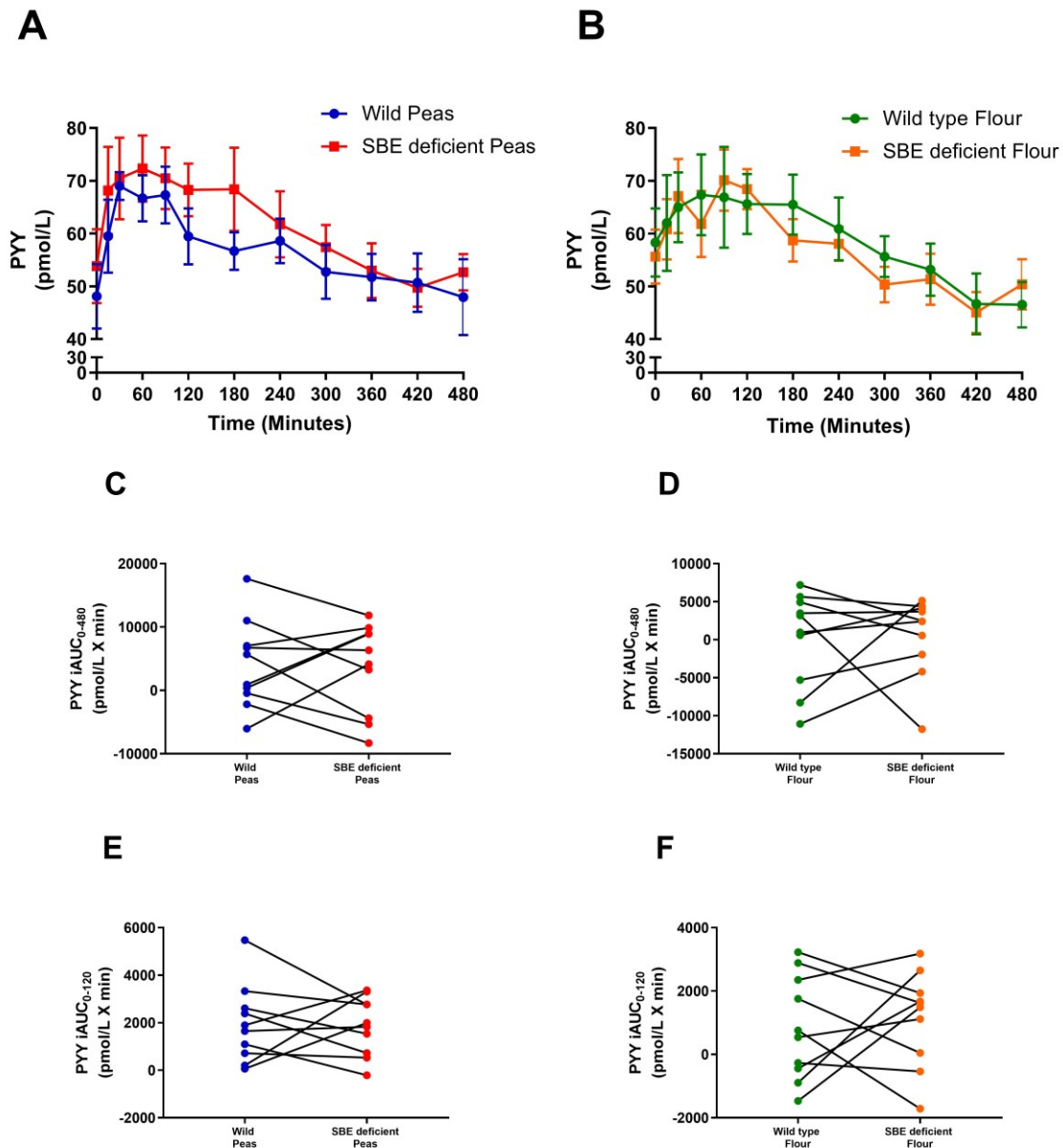


Figure 5.14: Postprandial plasma PYY in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) iAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (●) and SBE-deficient peas (●), (F) iAUC₀₋₁₂₀ for wild flour (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

Secondary comparisons looking at the effect of cellular structure

Figure 5.15 shows time course, iAUC₀₋₄₈₀, and iAUC₀₋₁₂₀ data on plasma PYY concentrations, comparing the effect of structure between wild pea seeds and flour and between SBE-deficient pea seeds and flour.

Wild pea seeds and flour:

The results indicated a borderline increase when comparing plasma PYY iAUC₀₋₄₈₀ following wild pea consumption (4071.2 ± 2198.6 pmol/L x min) with that following wild flour consumption (134.5 ± 1974.1 pmol/L x min). However, this difference did not reach a statistical significant level ($p=0.07$). The same effect was observed when comparing plasma PYY iAUC₀₋₁₂₀ concentrations following wild pea consumption (1943.2 ± 515.9 pmol/L x min) to those following wild flour consumption (845.5 ± 520.0 pmol/L x min) ($p=0.05$).

No main effects of meal ($p=0.50$) and meal X time interaction ($p=0.71$) were found.

SBE-deficient pea seeds and flour:

The results indicated no effect when comparing plasma PYY iAUC₀₋₄₈₀ between SBE-deficient peas and flour ($p=0.10$). There was a borderline increase when comparing plasma PYY iAUC₀₋₁₂₀ following SBE-deficient peas consumption (1861.2 ± 386.7 pmol/L x min) to that following SBE-deficient flour (1147.4 ± 469.9 pmol/L x min). However, this difference did not reach a statistical significant level ($p=0.07$).

No main effects of meal ($p=0.28$) and meal X time interaction ($p=0.81$) were found.

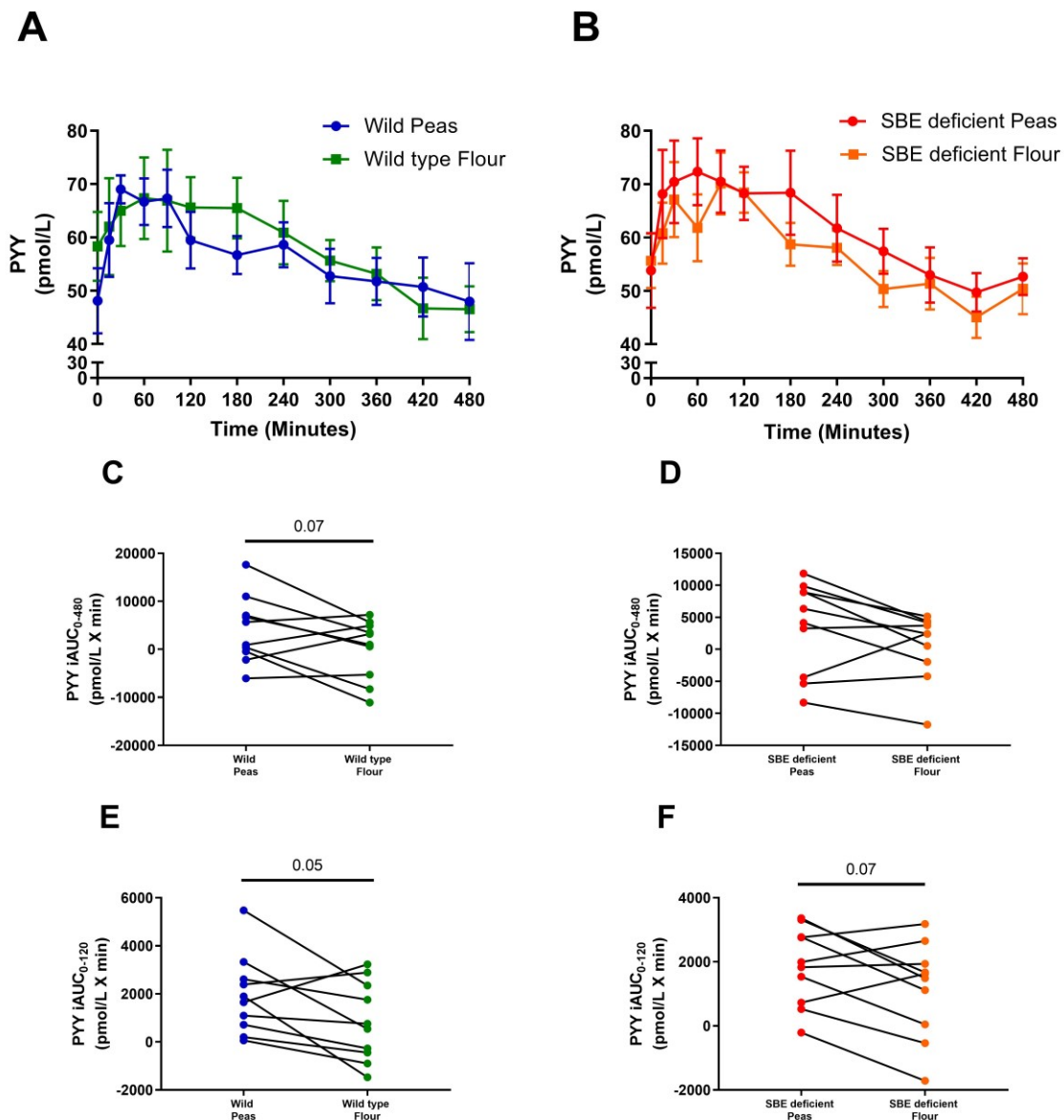


Figure 5.15: Postprandial plasma PYY in response to ingesting 50g dry weight of peas within a mixed meal test.

Data represent mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---). Insets represent (C) iAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (D) iAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●), (E) iAUC₀₋₁₂₀ for wild peas (●) and wild flour (●), (F) iAUC₀₋₁₂₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Repeated Measures ANOVA model was used for testing time course data with meal and time as factors. Fisher LSD post-hoc tests were performed between time-points. Paired t-tests were used for iAUCs calculations. Time-points and data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: iAUC, incremental area under the curve.

5.4.9 Visual analogue scales

5.4.9.1.1 Hunger

Figure 5.16 presents time course and tAUC data on subjective hunger, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective hunger scores between wild and SBE-deficient peas consumption ($p=0.77$).

Pea flours:

There were no differences in subjective hunger scores between wild and SBE-deficient flour consumption ($p=0.32$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective hunger scores between wild peas and wild flour consumption ($p=0.13$).

Mutant pea seeds:

There were no differences in subjective hunger scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.98$).

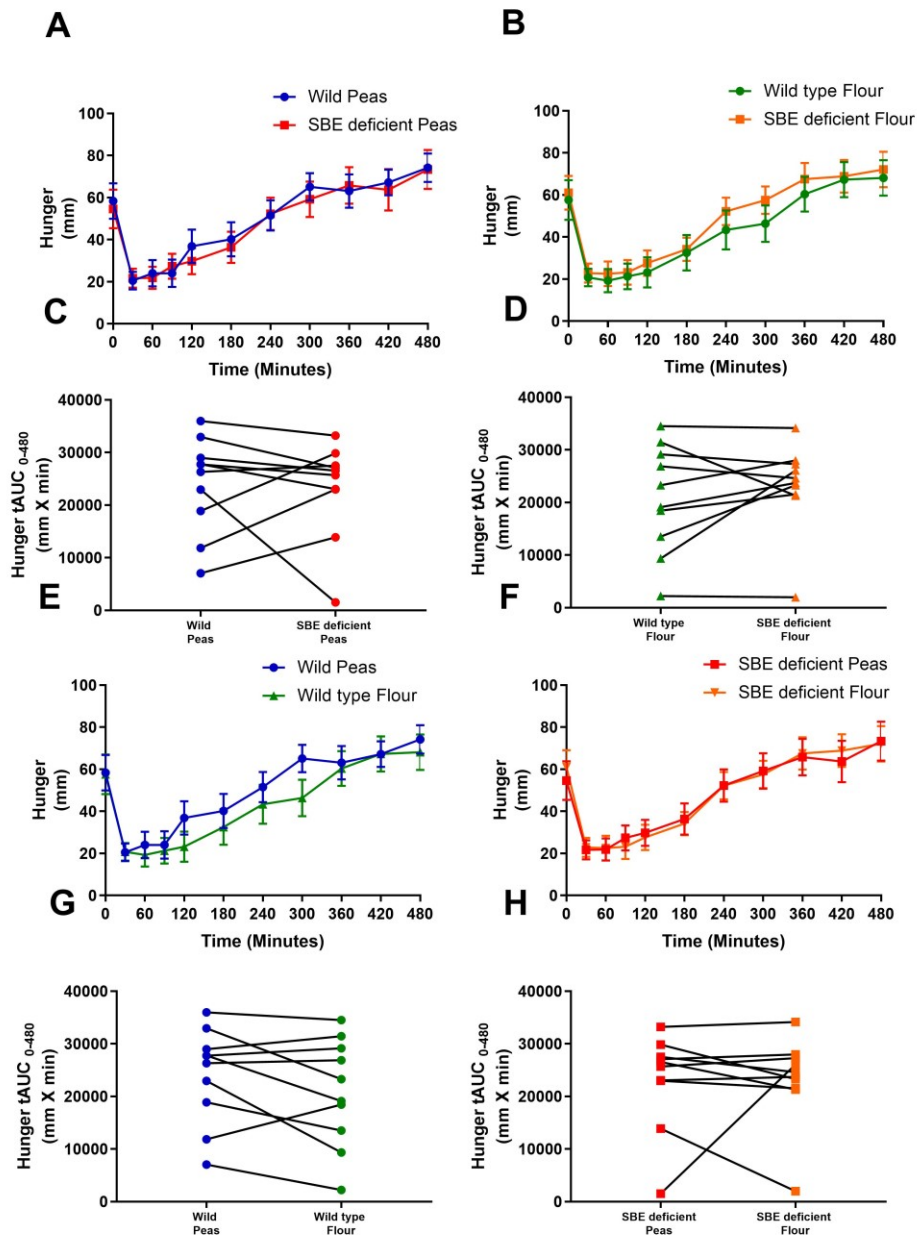


Figure 5.16 Subjective hunger score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

5.4.9.1.2 Fullness

Figure 5.17 shows time course and tAUC data on subjective fullness, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective fullness scores between wild and SBE-deficient peas consumption ($p=0.38$).

Pea flours:

There were no differences in subjective fullness scores between wild and SBE-deficient flour consumption ($p=0.17$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective fullness scores between wild peas and wild flour consumption ($p=0.52$).

Mutant pea seeds:

There were no differences in subjective fullness scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.62$).

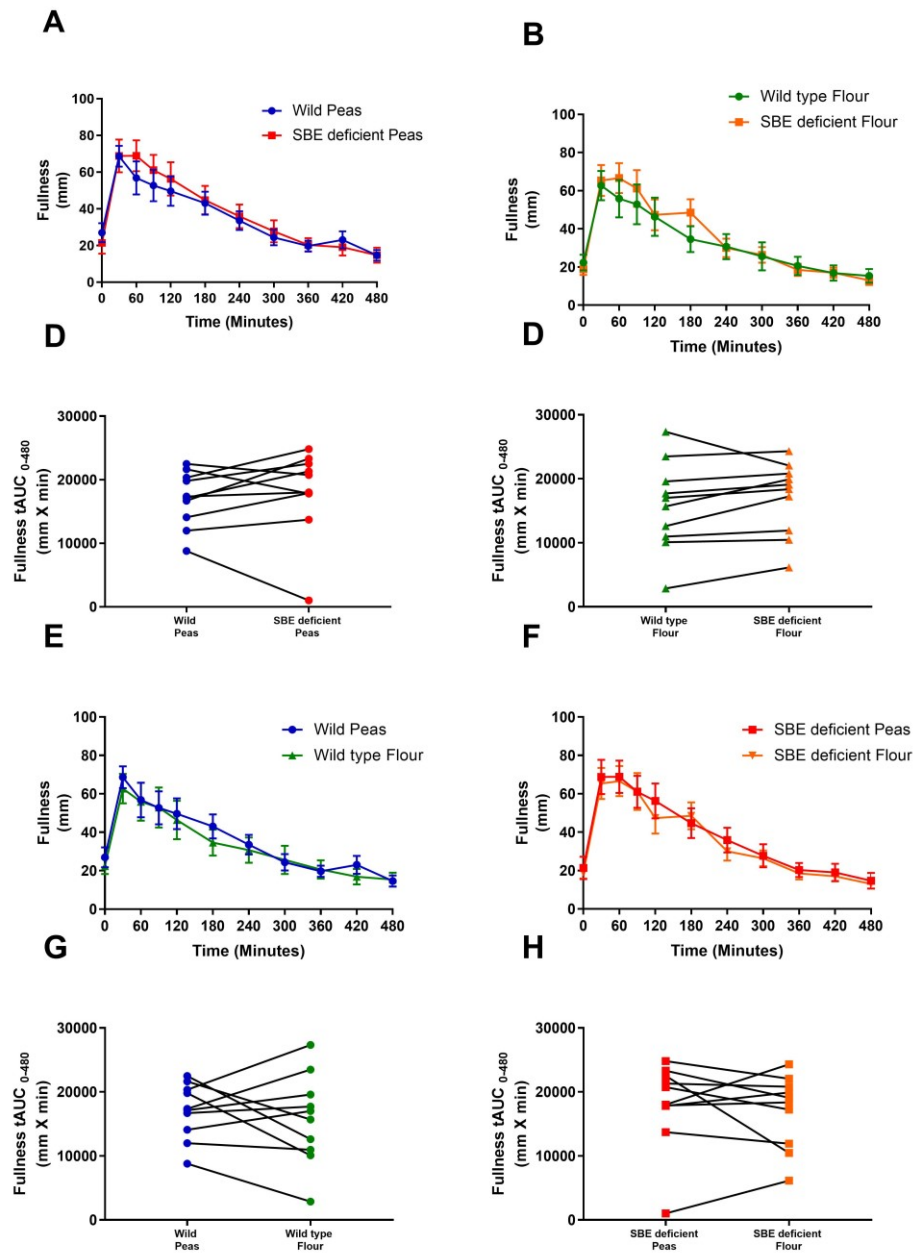


Figure 5.17 Subjective fullness score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

5.4.9.1.3 Desire to eat

Figure 5.18 presents time course and tAUC data on subjective desire to eat scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient peas consumption ($p>0.99$).

Flour pea seeds:

There were no differences in subjective desire to eat scores between wild and SBE-deficient flour consumption ($p=0.49$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective desire to eat scores between wild peas and wild flour consumption ($p=0.17$).

Mutant pea seeds:

There were no differences in subjective desire to eat scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.55$).

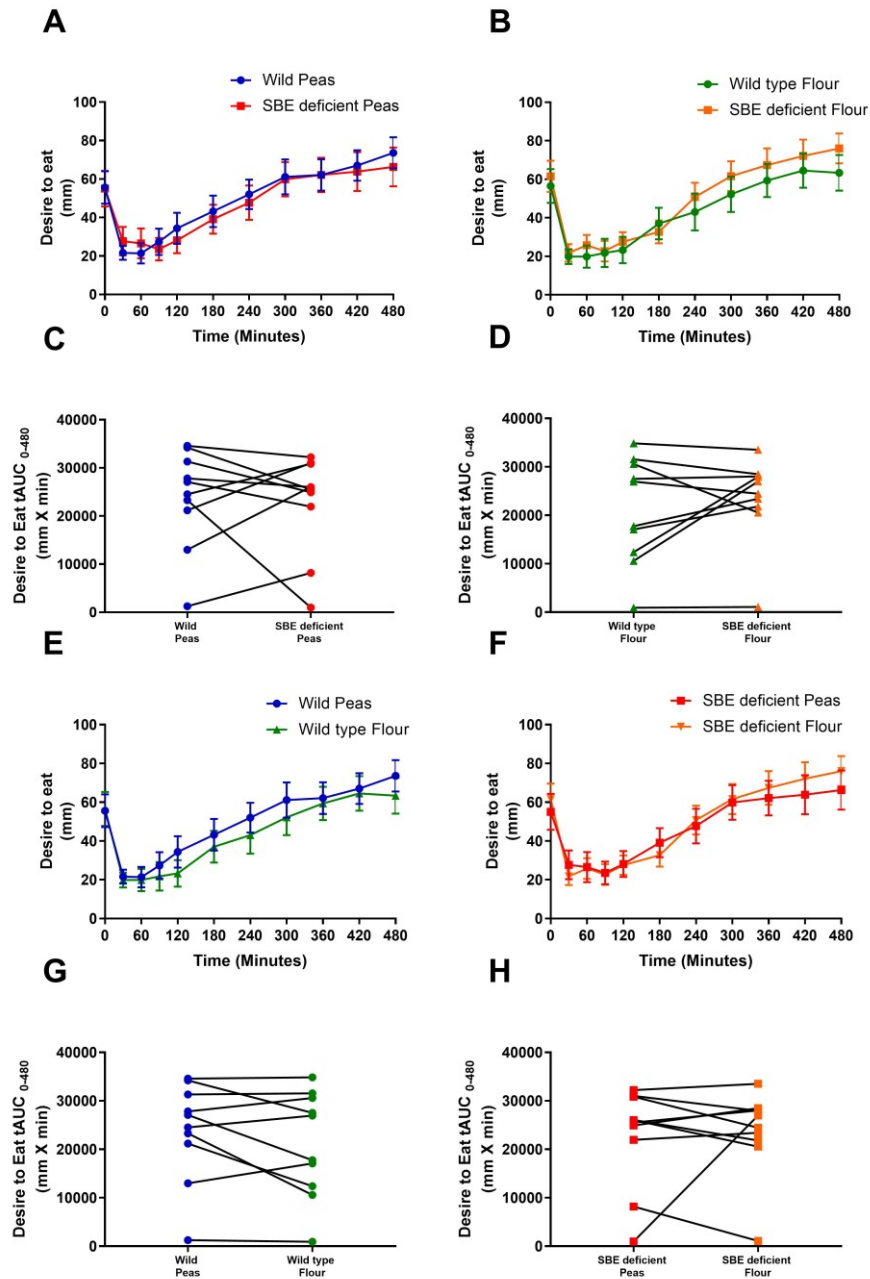


Figure 5.18 Subjective desire to eat score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve.

5.4.9.1.4 Appetite for a meal

Figure 3.16 shows time course and tAUC data on subjective appetite for a meal scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective appetite for a meal scores between wild and SBE-deficient peas consumption ($p=0.45$).

Flour pea seeds:

There were no differences in subjective appetite for a meal scores between wild and SBE-deficient flour consumption ($p=0.84$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There was a significant reduction in subjective appetite for a meal scores following the consumption of wild flour (43.0 ± 6.9 mm) when compared with wild pea consumption (50.9 ± 5.8 mm) ($p=0.04$).

Mutant pea seeds:

There were no differences in subjective appetite for a meal scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.37$).

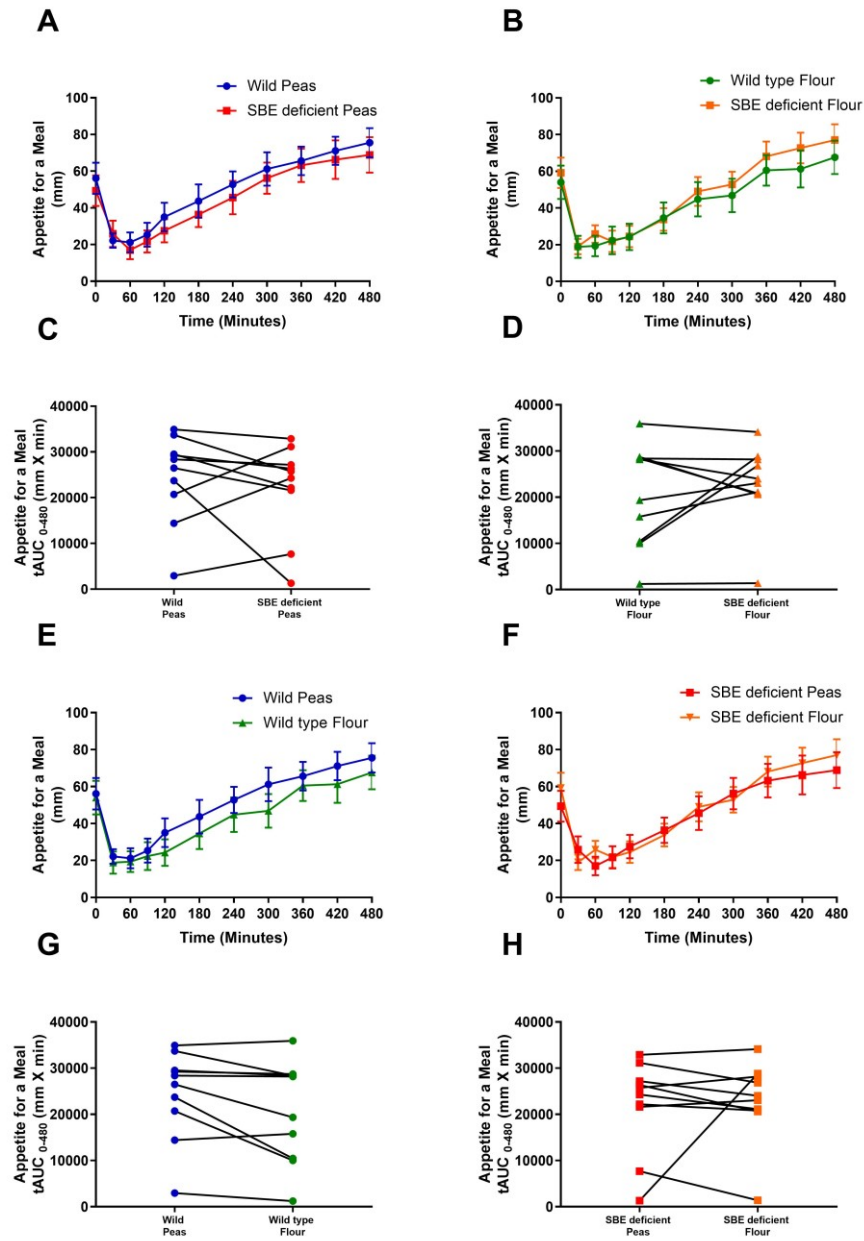


Figure 5.19 Subjective appetite for a meal score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

5.4.9.1.5 Composite appetite score

Figure 3.17 presents time course and tAUC data on composite appetite scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective composite appetite scores between wild and SBE-deficient peas consumption ($p=0.56$).

Flour pea seeds:

There were no differences in subjective composite appetite scores between wild and SBE-deficient flour consumption ($p=0.46$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective composite appetite scores between wild peas and wild flour consumption ($p=0.20$)

Mutant pea seeds:

There were no differences in subjective composite appetite scores between SBE-deficient peas and SBE-deficient flour consumption ($p=0.74$).

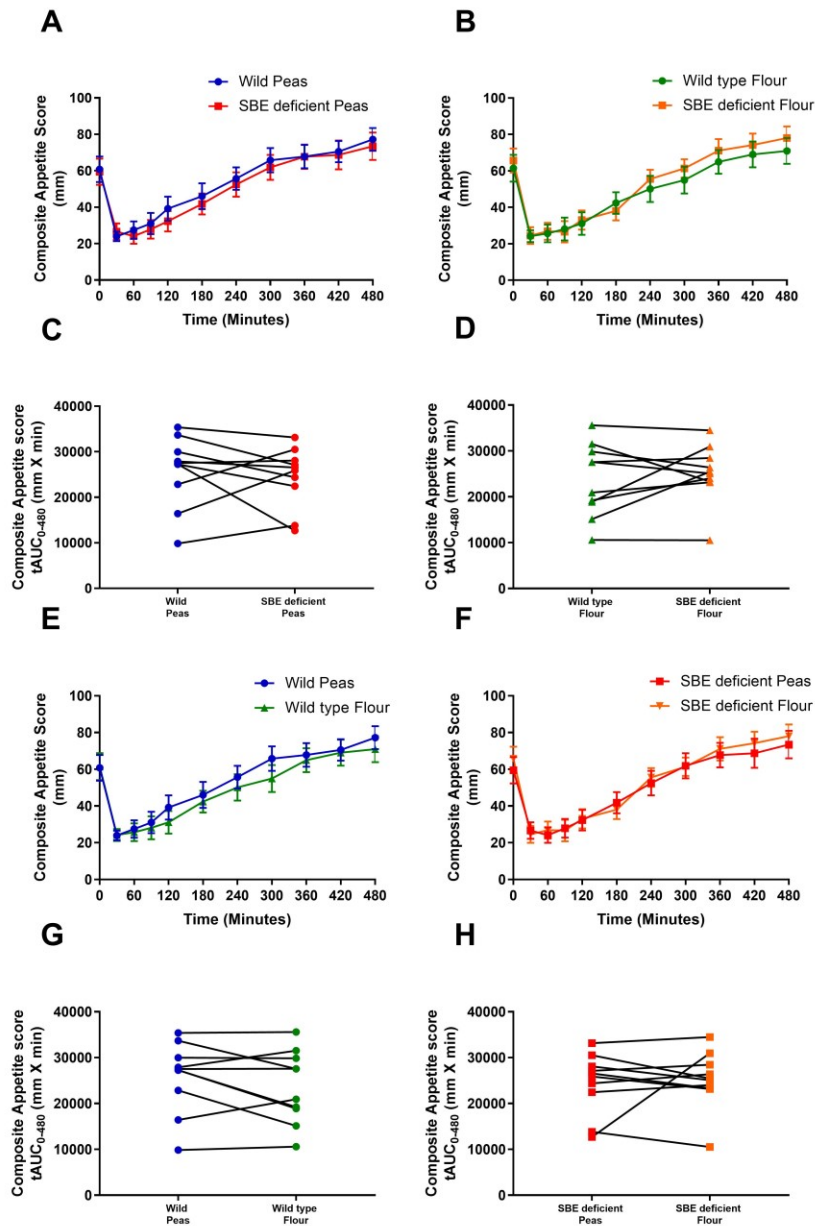


Figure 5.20 Subjective composite appetite score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM (n=10) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, *p<0.05, **p<0.01, ***p<0.001. Abbreviations: tAUC, total area under the curve.

5.4.9.1.6 Nausea

Figure 3.18 shows time course and tAUC data on nausea scores, comparing the effects of both mutation and structure between different pea seeds.

There were no differences in baseline values.

Primary comparisons looking at the effect of starch branching enzyme mutation

Whole pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient peas consumption ($p=0.35$).

Flour pea seeds:

There were no differences in subjective nausea scores between wild and SBE-deficient flour consumption ($p=0.84$).

Secondary comparisons looking at the effect of cellular structure

Wild pea seeds:

There were no differences in subjective nausea scores between wild peas and wild flour consumption ($p=0.84$).

Mutant pea seeds:

There were no differences in subjective nausea scores between SBE-deficient peas and SBE-deficient flour consumption ($p>0.99$).

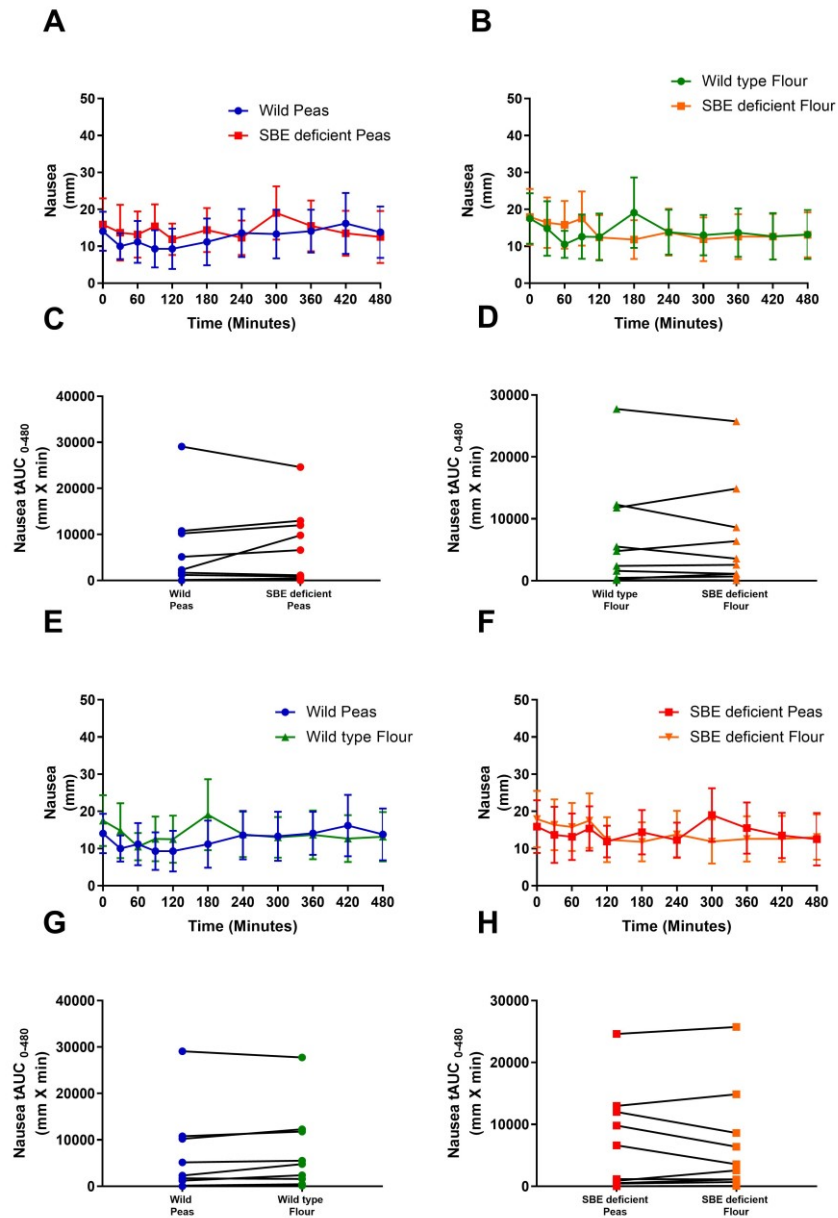


Figure 5.21 Subjective nausea score in response to ingesting 50g dry weight of pea seed within a mixed meal test.

Data represents mean $\pm$ SEM ($n=10$) of (A) wild peas (---) and SBE-deficient peas (---), (B) wild flour (---) and SBE-deficient flour (---), (C) tAUC₀₋₄₈₀ for wild peas (●) and SBE-deficient peas (●), (D) tAUC₀₋₄₈₀ for wild flour (●) and SBE-deficient flour (●), (E) wild peas (---) and wild flour (---), (F) SBE-deficient peas (---) and SBE-deficient flour (---), (G) tAUC₀₋₄₈₀ for wild peas (●) and wild flour (●), (H) tAUC₀₋₄₈₀ for SBE-deficient peas (●) and SBE-deficient flour (●). Paired t-tests were used for tAUCs calculations. Data at which values differed significantly, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve.

5.5 Discussion

5.5.1 Main findings

Primary findings looking at the effect of starch branching enzyme mutation in peas

- ^{13}C plasma glucose was significantly lower following SBE deficient pea consumption than following wild pea consumption. The same effect was not observed between pea flours.
- Fractional ^{13}C propionate and ^{13}C butyrate enrichment in 24-hour urine was significantly higher following SBE-deficient pea consumption than following wild pea consumption. The same effect was observed in fractional ^{13}C propionate and butyrate enrichment in 24-hour urine following SBE-deficient flour consumption when compared to wild flour consumption.
- Breath H_2 excretion was not affected when compared between whole pea seeds. The same effect was not observed between pea flours, as breath H_2 excretion was significantly increased following SBE deficient flour consumption when compared to wild flour consumption. The breath hydrogen marker results suggest that gut fermentation started to occur between three or four hours postprandial.
- Plasma glucose was not affected when compared between whole pea seeds. The same lack of effect was observed between pea flours.
- Serum insulin was significantly lower following SBE deficient pea consumption than following wild pea consumption. The same effect was not observed between pea flours.
- Plasma GLP-1 levels were not affected differently by whole pea seeds. The same effect was not observed between pea flours, where plasma GLP-1 during 0-120 minutes was significantly higher following SBE-deficient flour consumption when compared to wild flour consumption.
- Plasma PYY was not affected when compared between whole pea seeds. The same lack of effect was observed between pea flours.
- VAS was not affected when compared between whole pea seeds. The same lack of effect was observed between pea flours.

Secondary findings looking at the effect of cellular structure in peas

- ^{13}C plasma glucose was significantly lower following wild pea consumption than following wild flour consumption. The same reduction was observed following SBE-deficient pea consumption when compared to SBE-deficient flour consumption.
- Fractional ^{13}C propionate enrichment in 24-hour urine was significantly higher following wild flour consumption than following wild pea consumption. The same effect was not observed in the case of SBE-deficient pea seeds and flour.
- Breath H_2 excretion was borderline increased following SBE-deficient flour consumption compared to SBE-deficient pea consumption. The same effect was not observed in the case of wild pea seeds and flour.
- Plasma glucose was borderline reduced following SBE-deficient pea consumption compared to SBE-deficient flour consumption. The same effect was not observed in the case of wild pea seeds and flour.
- Serum insulin was not affected by wild pea seeds and flour. The same lack of effect was observed for SBE-deficient pea seeds and flour.
- Plasma GLP-1 was not affected by wild pea seeds and flour. The same lack of effect was observed for SBE-deficient pea seeds and flour.
- Plasma PYY was increased following wild pea consumption when compared to wild flour consumption. The same effect was observed between SBE-deficient pea seeds and flour, as there was a borderline increase in plasma PYY following SBE-deficient pea consumption when compared to SBE-deficient flour consumption.
- Subjective appetite for a meal scores were reduced following wild flour consumption when compared to those following wild pea consumption. The same effect was not observed between SBE-deficient pea seeds and flour.

5.5.2 Detailed discussion

From experimental Chapter 4, we were able to conclude that SBE-deficient peas are more resistant to digestion, which is further reflected in the metabolic profile response. Thus, the

assumption in the current study was that a higher fermentation profile is associated with reduced digestion in the upper GI tract. This speculation will allow the researcher to differentiate between 'digestible' peas (by producing more glucose as a marker of starch digestion) and 'fermentable' peas (by producing more H₂ and SCFAs as markers of anaerobic microbial activity).

5.5.2.1 Primary findings looking at the effect of starch branching enzyme mutation in peas

5.5.2.1.1 Exogenous ¹³C plasma glucose

Whole pea seeds

¹³C plasma glucose was significantly lower following SBE deficient pea seed consumption than following wild pea seed consumption.

Pea flours

¹³C plasma glucose was not affected by the pea flours.

As naturally enriched ¹³C pea seeds were used in this study, the amount of ¹³C glucose in the circulation was certainly due to the metabolism of the peas' carbohydrate content. These data suggest that wild peas were digested at higher rates, while SBE-deficient peas were less prone to digestion. Escaping small intestinal digestion means that SBE-deficient peas may be subjected to fermentation and/or excreted in faeces. Alternatively, a rapid, early release of ¹³C glucose following the consumption of both wild and SBE-deficient pea flours was shown between 30 and 90 minutes. This is logical when considering the effect of milling. Known for its shearing action, milling can greatly facilitate starch digestion, as it involves the removal of the seed coat and might destroy plant CW and thus increase starch bioavailability (Anguita *et al.*, 2006), causing legumes to lose their 'slow digestibility' feature (Tovar *et al.*, 1991). These data are in line with the small intestinal glucose availability from experimental Chapter 4. These results imply that the structural barrier of the whole pea seed, which is more pronounced with the SBE-deficient mutation, affects glucose absorption by decreasing starch digestibility.

5.5.2.1.2 Fractional ^{13}C SCFAs enrichment in 24-hour urine

Whole pea seeds

Fractional ^{13}C propionate and ^{13}C butyrate enrichment in 24-hour urine was significantly higher following SBE-deficient peas consumption than following wild pea consumption.

Pea flours

Fractional ^{13}C propionate and ^{13}C butyrate enrichment in 24-hour urine was significantly higher following SBE-deficient flour consumption than following wild flour consumption.

These findings suggest that more resistant structures from ^{13}C peas have reached the colon from the SBE-deficient line, leading to more ^{13}C SCFAs being produced as a result of the fermentation process in comparison to wild peas. This is in line with the plasma ^{13}C glucose results, which suggest that SBE-deficient peas escaped upper GI digestion and were thus fermented by the gut microbiota, resulting in higher propionate and butyrate concentrations within 24 hours of consumption. Interestingly, the same effect was seen in the case of SBE-deficient flour when compared to wild flour. These findings suggest that, regardless of the effect of milling, both SBE-deficient peas and flour have retained a considerable amount of their resistant structures and were therefore available for fermentation in the lower gut. Accordingly, they both serve as ideal candidates for microbiota fermentation. This further highlights the importance of intrinsic starch properties in limiting digestibility and increasing fermentation activity, such as in the SBE-deficient treatments compared to the WT controls, regardless of processing and food matrix.

As these urinary samples were cumulatively collected during the study visit and after participants had left the research facility, it must be noted that analysis of the samples during the first 8 hours (i.e. study visit period) showed no statistical significant differences (data not shown), suggesting that fermentation began after the study visit was completed. In addition, these data reflect the prolonged time (more than 480 minutes postprandial) needed for the SBE-deficient whole pea seeds to be fermented, which was not examined in blood sampling in this acute study. However, 24-hour urine analysis offers few indications regarding the specific timing required to generate

and excrete SCFAs. Thus, it cannot be used to confirm the timing of starting and/or finishing the fermentation process.

5.5.2.1.3 Breath H₂ excretion as a fermentation marker

Whole pea seeds

Breath H₂ excretion was not affected by whole pea seeds.

Pea flours

Breath H₂ excretion was significantly increased following SBE-deficient flour consumption when compared to wild flour consumption. Furthermore, the results suggest that gut fermentation started to occur between three or four hours postprandial.

The difference between pea flours is in line with the results from fractional ¹³C SCFA-enriched urine, as it again suggests that SBE-deficient flour retains a considerable amount of RS and is thus available for fermentation in the lower gut.

However, the same effect was not apparent between whole pea seeds. According to the findings from experimental Chapters 3 and 4, a lower glycaemic response was found following SBE-deficient peas consumption, and thus a higher fermentation profile was expected. Indeed, the lower breakdown of SBE-deficient peas was confirmed by the ¹³C glucose results. However, although the H₂ breath level was higher following the consumption of SBE-deficient peas, this increase did not reach a significant level when compared to wild peas. It is possible that the SBE-deficient whole pea seeds required more time to be fermented. Thus, in order to track their fermentation, more time was needed to express the hydrogen marker in breath. Indeed, data from fractional ¹³C SCFA-enriched urine, which is a more reliable quantitative method for tracking macronutrient digestion (Robertson, Livesey and Mathers, 2002; Umpleby, 2015), confirmed the fermentation effect and SCFA production in response to SBE-deficient pea and flour consumption in a 24-hour period.

5.5.2.1.4 Glucose, insulin, and GLP-1 responses

Whole pea seeds

Plasma glucose and GLP-1 levels were not affected by whole pea seeds. However, serum insulin was significantly increased following wild pea consumption when compared to SBE-deficient pea consumption.

Pea flours

Plasma glucose and serum insulin were not affected by the pea flours. However, plasma GLP-1 was significantly higher 0–120 minutes following SBE-deficient flour consumption than following wild flour consumption.

Unlike the plasma ^{13}C glucose results, which only reflect the ^{13}C peas ingested, total plasma glucose reflects the total carbohydrate content of the test meal consumed. This can explain the difference in the pattern of plasma glucose compared to that of plasma ^{13}C glucose. The total plasma glucose curve represents the normal postprandial response in healthy individuals, returning to baseline within the first 2 hours. This is the same period in which serum insulin was increased following wild pea consumption compared to SBE-deficient pea consumption, suggesting stimulation by glucose early in the digestive process and thus reflecting the normal glucose curve observed. This result is in line with the other findings supporting the effect of the mutation and its impact on the CW, entrapping starch granules due to the limited availability of readily digestible starches and consequently stimulating the secretion of insulin. These positive glucose and insulin responses would benefit patients with T2D, as changes in glucose might be more pronounced, as in the study of Golay and colleagues, in which it was found that adding legumes to mixed meals significantly lowered the postprandial incremental blood glucose and insulin responses in patients with T2D but led to no changes in blood glucose in healthy participants (Golay *et al.*, 1986).

However, no differences were observed in plasma glucose and serum insulin between pea flours. These results are in line with the concept of milled plant-based foods having increased bioavailability (Tovar *et al.*, 1991; Berg *et al.*, 2012). Interestingly, higher GLP-1 was shown 0–120

minutes following SBE-deficient flour consumption. This result could be explained by the pleotropic effects of the SBE mutation on pea seeds, resulting in a higher sucrose content, which might have stimulated the incretin release.

5.5.2.1.5 Gut hormones and appetite measures

Whole pea seeds

Plasma GLP-1 and PYY were not affected by whole pea seeds. VAS was also not affected by whole pea seeds.

Pea flours

Plasma GLP-1 and PYY were not affected when compared between pea flours. VAS was also not affected by pea flours.

Despite the fermentation effects and the subsequent SCFA production in response to SBE-deficient pea seed and flour consumption, when compared to wild pea seeds and flour, gut hormones were not affected. This might suggest that the amount of SCFAs was not sufficient to trigger the release of gut hormones (Psichas *et al.*, 2015). Considering that SCFAs act as signalling molecules to affect appetite, this might explain the non-significant effects on subjective appetite as well.

5.5.2.2 Secondary findings looking at the effect of cellular structure in peas

5.5.2.2.1 Exogenous ¹³C plasma glucose

Wild pea seeds and flour:

¹³C plasma glucose was significantly lower following wild peas consumption than following wild flour consumption.

SBE-deficient pea seeds and flour:

¹³C plasma glucose was significantly lower following SBE-deficient peas consumption than following SBE-deficient flour consumption.

These results highlight the importance of the food matrix CWs of both lines of peas in entrapping starchy nutrients within and thus delaying the peak and decreasing plasma glucose concentrations. Milling can greatly facilitate starch digestion, as it involves the removal of the seed coat and might destroy plant CW and thus increase starch bioavailability (Anguita *et al.*, 2006), causing legumes to lose their 'slow digestibility' feature (Tovar *et al.*, 1991). These effects were seen previously in navy beans, where in vitro starch hydrolysis significantly increased in the case of bean flour when compared to whole beans (Berg *et al.*, 2012). These findings suggest the beneficial effect of whole food structure in terms of delivering a fairly intact substrate to the lower gut for fermentation.

5.5.2.2.2 Fractional ^{13}C SCFAs enrichment in 24-hour urine

Wild pea seeds and flour:

Fractional ^{13}C propionate enrichment in 24-hour urine was significantly higher following wild flour consumption than following wild pea consumption.

SBE-deficient pea seeds and flour:

Fractional ^{13}C SCFA enrichment in 24-hour urine was not affected by SBE-deficient pea seeds and flour.

These findings are unexpected, as they suggest that a considerable amount of RS from wild pea flour reached the lower GI to be subjected to fermentation when compared with wild pea seeds. However, these observations are not in line with the rest of findings and thus warrant further investigations.

Meanwhile, the comparable SCFA production resulting from SBE-deficient pea seed and flour might imply that both SBE-deficient pea seed and flour have retained a considerable amount of their RS, and thus both were available, to a similar extent, for fermentation in the lower GI tract. Hence, they both serve as ideal candidates for microbiota fermentation in the total 24-hour period following consumption.

5.5.2.2.3 Breath H₂ excretion as a fermentation marker

Wild pea seeds and flour:

Breath H₂ excretion was not affected by wild pea seeds or flour.

SBE-deficient pea seeds and flour:

Breath H₂ excretion was borderline increased following SBE-deficient flour consumption compared to SBE-deficient pea consumption.

These results suggest that, despite the effect of processing, SBE-deficient flour retained considerable amount of RS granules and was thus available for fermentation in the lower gut. Moreover, this highlights the importance of intrinsic starch properties in limiting digestibility and increasing fermentation activity.

5.5.2.2.4 Glucose, insulin, and GLP-1 responses

Wild pea seeds and flour:

Plasma glucose, serum insulin, and plasma GLP-1 were not affected by wild pea seeds or flour.

SBE-deficient pea seeds and flour:

Plasma glucose was borderline reduced following SBE-deficient peas consumption when compared to SBE-deficient flour consumption. However, serum insulin and plasma GLP-1 levels were not affected by SBE-deficient pea seeds or flour.

These results are in line with the ¹³C plasma glucose levels of SBE-deficient pea seeds and flour, which highlight the importance of the CW matrix (more pronounced in the case of SBE-deficient line) in entrapping starchy nutrients and thereby limiting starch digestibility and ensuring fairly intact delivery to the colon. However, there were no differences in insulin and incretin between the same interventions. The lower plasma glucose levels could lead to the speculation that the amount of serum insulin needed to clear that amount of glucose is also low. It is important to

highlight the variability of insulin when compared to the tightly regulated glucose in this healthy data set of participants, which could explain the insulin results.

5.5.2.2.5 Gut hormones and appetite measures

Wild pea seeds and flour:

Plasma GLP-1 was not affected by wild pea seeds or flour. However, plasma PYY was borderline higher following wild pea seed consumption than following wild pea flour consumption. Moreover, subjective appetite for a meal scores were reduced following wild pea flour consumption when compared to wild pea seed consumption.

SBE-deficient pea seeds and flour:

Plasma GLP-1 and VAS were not affected by SBE-deficient pea seeds or flour. Plasma PYY was borderline higher following SBE-deficient pea seed consumption when compared to SBE-deficient pea flour consumption.

Looking at the individual analyses with regards to the changes in these parameters, the data highlight the inter-individual variability in this data set of participants. This could be driven by the low power of the participants to confirm the differences in these parameters and thus warrants further investigation taking into consideration the current findings to calculate the sample size required to detect a significant difference in those appetite parameters between pea groups ($p=0.05$, 0.80 power). For instance, to detect a significant difference in PYY levels between wild and SBE-deficient pea seed (mean difference of 0.88 ± 3.08 pmol/L), a power calculation based on the results of the current study showed that 89 volunteers would be needed in a crossover manner to detect a significant difference between groups.

5.5.3 Strengths, weaknesses and future recommendations

Designing the study day to last up to 480 minutes allowed quantification of the acute differences between the interventions. However, gut hormone and appetite measures were inconsistent in the first three studies. This might highlight the length of supplementation period needed to exert a significant effect on gut hormones and subjective appetite and/or the insufficient number of

participants to detect significant differences. Future studies should include measuring GIP in and to test the effect of long-term supplementation of NDCs of peas and/or SCFAs on appetite measures. It would also be more informative to analyse in vitro digestion kinetics of wild and SBE-deficient pea seeds and flours, along with structural microscopy imaging.

5.6 Conclusion

A single starch assembly mutation in pea seeds can cause the human body to respond to glucose homeostasis and body metabolism in a way that is different from that with wild pea seeds. Tracking the ^{13}C in pea treatments allowed for better understanding of the bioavailability of starch-based foods for digestion and fermentation and showed a resistance to digestion feature of SBE-deficient pea seeds and flour in the upper GI tract, regardless of processing and food matrix, causing greater availability for fermentation than wild pea seeds and flour. In addition, processing and the subsequent increase in substrate surface area does not always result in readily digestible nutrients, especially in foods with such starch mutations. In the current study, both SBE-deficient pea seeds and flour were more available for fermentation and SCFA production. However, taking all of the pea studies together, there is no solid conclusion that can be drawn regarding the impact of acute pea ingestion on appetite regulation. This could be due to the small amount of SCFAs needed to trigger any appetite response. The next experimental chapter will be aimed to investigate the effect of long-term SCFA supplementation on appetite regulation while following a weight loss diet.

Chapter 6

6. The effect of increased colonic propionate on weight loss while following a lower energy diet

6.1 Background:

Although data from Chapters 4 and 5 clearly show that the consumption of a food system (peas) high in RS impacts postprandial glycaemia, there was no evidence of effect on appetite. However, previous work from our lab has demonstrated that enriching the colon with propionate reduced appetite in humans. The prevention of non-communicable diseases such as type 2 diabetes relies on weight loss. To gain insight into the potential impact of fermentation on weight loss, I explored the use of IPE to enrich the colonic propionate and enhance weight loss in overweight volunteers.

6.1.1 Propionate

Propionate is an SCFA with three carbon atoms, and is the second most abundant SCFA produced in the colon after acetate. Propionate is endogenously produced through the bacterial fermentation dietary fibre, such as RS, inulin, and L-rhamnose. In addition to endogenous propionate production, certain foods naturally contain small amounts of propionate that have been produced from the fermentation effect of propionibacteria, including sourdough bread, yoghurt, milk, and cheese (Fernandez-Garcia and McGregor, 1994). Due to its bacterial and fungal growth-suppressive factor, propionate has also been used as a preservative factor of E280 (Levison, 1973). Propionate is absorbed by colonocyte favourably by facilitated diffusion. Ninety per cent of propionate is transported to the liver, while the remainder is circulated to the periphery for further metabolism (Wong *et al.*, 2006).

Besides the natural appearance of propionate in some foods, previous experiments have supplemented propionate into certain food products to increase its endogenous production. These products included beverages (Ruijschop, Boelrijk and te Giffel, 2008), pasta (Frost *et al.*, 2003), and breads (Todesco *et al.*, 1991; Liljeberg, Lönner and Björck, 1995; Liljeberg and Björck, 1996; Dakin *et al.*, 2004; Darzi, Frost and Robertson, 2012). This method helped in defining the metabolic profile in response to a specific amount of propionate. However, propionate in these fortified foods has poor organoleptic properties and is absorbed early in the small intestine, which can lead to different metabolic effects compared to those of colonic-fermentation-generated propionate. Thus, methods that promote the absorption of propionate from the colon

are encouraged to enhance its tolerability and selectively induce colonic-derived metabolic outcomes.

6.1.2 Propionate metabolic effects

6.1.2.1 Role of propionate in appetite regulation

It has been reported that propionate stimulates the secretion of GLP-1 and PYY gut hormones from WT primary murine colonic crypt cultures when compared with FFAR2 knock-out (KO) mouse cultures (Psichas *et al.*, 2015). Moreover, in vivo infusions of intra-colonic propionate in mice increased the circulating concentration of both gut hormones when compared with FFAR2 KO mice (Psichas *et al.*, 2015). These data highlight the role of FFARs in SCFA-mediated gut hormone secretion. In support of this, human feeding studies also suggest a primary role of propionate in appetite regulation. Chambers *et al.* demonstrated that selectively increasing colonic propionate delivery via IPE supplementation significantly increased plasma GLP-1 and PYY levels and reduced ad libitum intake acutely. Interestingly, the long-term (24 weeks) supplementation of IPE significantly reduced body weight gain and the development of abdominal adipose tissue, without significant changes in circulating gut hormone concentrations (Chambers *et al.*, 2014). This indicates that the long-term beneficial effects associated with elevated colonic propionate are not mediated by the stimulation of gut hormone secretion. However, the exact underlying mechanism is not yet known. Indeed, Byrne *et al.* (2016) demonstrated a significant reduction in the activation of brain reward-based eating behaviours (i.e. caudate and nucleus accumbens) following acute IPE intake, again without changes in gut hormone levels, suggesting other independent mechanisms.

Alterations to hepatic metabolism could be a mechanism by which propionate exerts its appetite suppressing effects, as more than 90% of the propionate available in the portal vein is metabolised by the liver as a precursor for gluconeogenesis (Cummings *et al.*, 1987). Moreover, it was suggested earlier that increasing hepatic energy levels could regulate appetitive behaviour via stimulating hepatic–vagal afferent and thus signalling the nucleus of the solitary tract within the brainstem (Allen, Bradford and Oba, 2009). Ruminant studies have also linked propionate with the reduction in energy intake following portal propionate infusion, an effect that was

abolished with total liver denervation resulting from hepatic vagotomy (Anil and Forbes, 1980, 1988). Indeed, although the results of ruminant studies cannot be directly translated to humans, evidence suggests that an increase in propionate supply to the liver causes an increase in hepatic glucose production by 30% (Befroy *et al.*, 2014), indicating that hepatic propionate metabolism may act as a satiety signal.

6.1.2.2 Role of propionate in glucose metabolism and homeostasis

Propionate has been identified as the main gluconeogenic substrate in ruminants (Wiltout and Satter, 1972; Lin *et al.*, 1995) and as the only gluconeogenic SCFA in humans (Al-Lahham, Peppelenbosch, *et al.*, 2010). In gluconeogenesis, propionate acts as a substrate for this process by entering the Krebs cycle after its conversion to succinyl-CoA, the latter being converted subsequently to oxaloacetate to produce glucose (Wolever, Spadafora and Eshuis, 1991). Moreover, propionate can also play a role in reducing glucose metabolism by inhibiting the activation of the enzyme responsible for converting pyruvate to oxaloacetate (pyruvate carboxylase enzyme), thus suppressing glycolysis. In addition, the propionate metabolites methyl malonyl-CoA and propionyl-CoA also play a role in inhibiting pyruvate carboxylase and thereby further reducing glucose metabolism (Chan and Freedland, 1972; Wong *et al.*, 2006). However, inconsistent findings regarding the effect of propionate in regulating glucose in humans implies the need for future work to clarify this effect (Todesco *et al.*, 1991; Laurent *et al.*, 1995).

IGN is considered to be a beneficial regulator of glucose and energy homeostasis (Mithieux *et al.*, 2005; Troy *et al.*, 2008). Of the main SCFAs, propionate was shown to be a substrate for IGN, and butyrate was shown to directly activate IGN-related gene expression (De Vadder *et al.*, 2014). A study in mice showed a reduction in hepatic glucose production and improvements in glucose control and body composition in SCFA-supplemented animals. These beneficial effects were absent in intestinal glucose-6-phosphatase KO mice (the catalytic subunit of a key enzyme involved in IGN) despite a similar shift in gut microbiota composition. It was proposed that IGN causes activation of portal glucose sensors, which signals the brain to mediate these beneficial effects (Delaere *et al.*, 2013). These data suggest the importance of IGN in mediating a number

of the beneficial effects following the consumption of NDCs. Thus, propionate can serve as both substrate and signalling molecules in glucose homeostasis.

6.1.2.3 Role of propionate in insulin sensitivity and β -cell function

Free fatty acids are generated from lipolysis in adipose tissue, and their release is positively related to insulin resistance (Sharma and Chetty, 2005; Kennedy *et al.*, 2008; Micha and Mozaffarian, 2009). Evidence gathered regarding the positive role of propionate in modulating lipid metabolism by reducing free fatty acid and cholesterol levels suggest that propionate might improve insulin sensitivity. Specifically, studies have shown improvements in insulin sensitivity following the reduction of free fatty acid levels (Boden, 2002; Boden and Shulman, 2002). Looking closer at the mechanism of action, theories have linked the positive role of propionate on insulin sensitivity to SCFA receptors FFAR2 and FFAR3 found on adipocytes to be responsible for stimulating adipogenesis and lipogenesis and suppressing lipolysis, as will be discussed in the following section. Furthermore, supplementation of propionate led to reductions in circulating insulin levels in rodents (Ximenes *et al.*, 2007) and humans (Liljeberg, Lönner and Björck, 1995; Liljeberg and Björck, 1996; Darwiche *et al.*, 2001).

Supplementation of IPE for 24 weeks in healthy humans resulted in an acute increase in circulating insulin levels in the first 30 minutes postprandial, independent of elevations in the early postprandial glucose levels. This was accompanied with a decrease in circulating non-esterified fatty acid (NEFA) levels and improvement in β -cell function. Similarly, incubating human islets with propionate directly potentiated glucose-stimulated insulin release in vitro. Upon closer investigation, it was found that FFAR2 was expressed by human islets β -cells, which suggests that the associated improvements in β -cell function are a consequence of circulating propionate potentiating insulin secretion directly through FFAR2 activation on human islets β -cells (Pingitore *et al.*, 2017).

6.1.2.4 Role of propionate in adiposity and energy expenditure

The discovery of FFAR2 and FFAR3 receptors' availability to adipocyte cells and peripheral afferents has led to the proposal that SCFAs play a potentiate role in regulating adiposity and

energy expenditure (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Xiong *et al.*, 2004; Hong *et al.*, 2005; Ge *et al.*, 2008; Al-Lahham, Roelofsen, *et al.*, 2010; De Vadder *et al.*, 2014) (for more information on the physiological role and the characteristics of these receptors, please refer to Chapter 1). In vitro studies have suggested the beneficial effects of propionate and acetate on inhibiting lipolysis and inducing lipogenesis through their action on FFAR2. The mechanism behind this effect involves stimulation of Peroxisome proliferator activated receptor (PPAR- γ 2), a pre-adipocyte differentiation marker and a known regulator of uncoupling protein 2 (UCP2) expression, fatty acid oxidation, and whole-body lipid metabolism (Hong *et al.*, 2005; Ge *et al.*, 2008). In addition, den Besten *et al.* demonstrated that supplementing a high-fat diet with 5% propionate for 12 weeks protected mice from diet-induced obesity through increased expression of mitochondrial UCP2 and increased AMP:ATP ratio, thus inhibiting lipogenesis and inducing a shift from carbohydrate to lipid oxidation, as marked by the lower resting energy expenditure (Den Besten *et al.*, 2015). Other impacts of propionate on energy metabolism following intraperitoneal infusion at a rate of 1 g/kg body weight include increased oxygen consumption and promotion of the expression of UCP1, which is known to induce thermogenesis in brown adipose tissue, suggesting the important role of SCFAs in the sympathetic nervous system and adiposity (Kimura *et al.*, 2011). Moreover, oral propionate supplementation in humans, in the form of sodium propionate, increased both lipid oxidation and resting energy expenditure (Chambers *et al.*, 2018). Meanwhile, rectal infusion of an SCFA mixture with high propionate levels in 12 overnight-fasted normoglycemic men enhanced resting energy expenditure (Canfora *et al.*, 2017). Together, these data highlight the role of propionate in inducing adiposity and energy expenditure and helping to control body weight.

Collectively, these data indicate that there are numerous beneficial effects associated with propionate, similar to that of NDC fermentation. As explained earlier, previous work from our lab has demonstrated that enriching the colon with propionate reduced appetite in humans (Chambers *et al.*, 2014; Byrne *et al.*, 2016). However, to date, there is insufficient evidence regarding whether long-term selectively increased propionate delivery to the colon can reduce long-term energy intake and body weight in humans. Nevertheless, by modulating PYY and GLP-1 levels, propionate supplementation to the colon might overcome the negative effects of weight

loss diets, where appetite might increase over time to adapt to the shortage in energy supply and lead to restoration of the weight lost through several physiological pathways, which are summarised in table 6.1 below (Sumithran *et al.*, 2011). For instance, a direct proportionate relationship has been shown between diet-induced weight loss and a decrease in total energy expenditure (TEE), resembling physical activity. This accounts for the alteration in body composition and body mass, as skeletal muscle demonstrated increased efficiency with small work loads, which can persist for up to one year while maintaining reduced body weight (Doucet *et al.*, 2003; Rosenbaum *et al.*, 2003, 2008). Moreover, the shift from carbohydrate metabolism to lipid metabolism by the body during periods of weight loss maintenance and weight regain can lead to the sparing of dietary fat for deposition and impair the body's ability to increase fat oxidation in response to a high-fat diet, leading to a positive fat and negative carbohydrate balance and stimulating appetite and energy intake to restore glycogen stores (Flatt, 1987; Astrup *et al.*, 1994; Ballor *et al.*, 1996; MacLean *et al.*, 2004). Additionally, following weight loss, the concentration of several appetite-regulating hormones changes to collectively promote restoration of the body's energy balance and induces weight regain, and this can persist for 12 months after weight regain (Sumithran *et al.*, 2011). These effects can all lead to a sustained increase in subjective appetite following diet-induced weight loss, with an increased preference for high-calorie foods. It was shown previously that while a normal-weight group found the taste of a solution containing 20% lipid and <10% sucrose optimal, another group with obesity, who had recently lost around 13.6 kg and maintained the weight loss for 1 year, preferred a solution higher in both fat and sugar (Drewnowski *et al.*, 1985).

Table 6.1: Physiological changes induced by following a weight loss diet

Factor	Expected effect
↓Fat oxidation	Increase energy storage
↓Energy expenditure	
↓PYY	Increase food intake
↑Appetite	
↓Insulin	
↑GIP	
↑Ghrelin	
↓Leptin	
↓Amylin	
Altered neural activation	

Table adapted from (Sumithran and Proietto, 2013)

The pilot study presented in this chapter aimed to selectively increase long-term propionate enrichment in the colon via IPE supplementation, to answer the question of whether propionate can reduce energy intake in the long term by modulating subjective and objective appetite (i.e. VAS and gut hormones, respectively) and body weight in humans following a lower energy diet.

6.1.3 Rational behind the study

Targeting small molecule delivery to the large intestine is challenging, as the vector needs to avoid digestion and absorption in the small intestine, resist brush border and exocrine enzymes, and thus selectively release the small molecule in the lower gut. Techniques such as direct instillation using an SCFA enema (Wolever *et al.*, 1989), time and/or pH-dependent release (Maurer *et al.*, 2015), and encapsulation using a fermentable polymer coating (Tuleu *et al.*, 2002) are all aimed at small molecule delivery. However, each of these methods is unsuitable for population-level interventions or limited to relatively small dosing regimens. The binding affinity between FFAR and SCFAs in the colonic environment appears adapted for the relatively high concentration of SCFAs, leading to a key specification of any colonic delivery system for SCFAs: inducing significant change in the large colonic SCFA pool. Observational studies from sudden death victims have estimated that the caecum alone has a propionate volume of 1.87 g/kg (Cummings *et al.*, 1987). Therefore, a threshold was set to deliver >2 g/day of propionate to induce a fivefold increase in daily propionate production, broadly equivalent to 90 g of fibre fermentation per day. Previous colon-specific drug delivery methods have used SCFA functionalised starches as an enteric coating (Pu *et al.*, 2011). Subsequently, the concept was extended to starch SCFA esters as a prodrug delivery system for SCFAs to the colon (Clarke *et al.*, 2011). However, having a starch as a starting material for this delivery system was rather challenging due to complexities in the synthesis, characterisation, and determination of SCFA delivery efficacy because of the nature and digestibility of the starch itself.

In partnership with the University of Glasgow, our group has developed a propionate carrier system based on inulin (β -2,1-linked polymer of fructose) propionate esters, which have been previously optimised with favourable organoleptic properties and are designed to release propionate within the large intestine, thus serving as a colonic delivery vehicle. The choice of inulin was predicated on the fact that inulin is water soluble and amenable to aqueous-based functionalisation, and its gastrointestinal handling has been well characterised while escaping upper GI digestion (Nilsson and Björck, 1988; Andersson, Ellegård and Bosaeus, 2018). Supplementation with IPE was demonstrated to selectively, reliably, and reproducibly increase

colonic propionate levels (Chambers *et al.*, 2015). Using this carrier, propionate is only released to the colon when the inulin is fermented by gut microflora. Using stable-isotope-labelled (^{13}C) propionate bound to a variant of that inulin ester, it was confirmed that over 80% of the ^{13}C was recovered in breath, after an initial increase in breath H_2 (a marker of gut fermentation) at 240 minutes post ingestion. A small gradual release of ^{13}C was apparent between 0–240 minutes, suggesting that the majority of conjugated propionate was released through fermentation in the lower gut (Chambers *et al.*, 2014). Isotopic data from tracking ^{13}C -IPE following acute supplementation demonstrated that, where circulating plasma propionate was detectable, significant increases in ^{13}C propionate enrichment and total concentration were significantly higher in peripheral blood 360 minutes post ingestion when compared with baseline, suggesting the availability of propionate systemically (Polyviou *et al.*, 2016). The same group also showed that, after accounting for the small intestinal losses of 0.49 g (less than 30% of the bound propionate), 10 g of IPE has the ability to deliver 2.4 g of propionate to the colon (i.e. a 2.5-fold increase of propionate production in the colon). This level of propionate could only be achieved through traditional dietary fibre supplementation in excess of 60 g per day. Interestingly, the participants reported limited side effects from the long-term intake of propionate esters, similar to the inulin control intervention (Chambers *et al.*, 2014).

Propionate was the SCFA chosen for the following reasons:

- It has the highest affinity for FFAR2 receptor (Le Poul *et al.*, 2003). As this receptor co-expresses with GLP-1- and PYY-secreting L-cells in the GI tract, it is suggested that propionate might be a potent SCFA in stimulating the release of these anorexigenic gut hormones.
- It is a known substrate for the gluconeogenic process in both the liver and gut epithelium, and evidence has linked the beneficial metabolic effects with propionate to IGN mediation (De Vadder *et al.*, 2014).
- It was shown to act as a signalling molecule to stimulate sympathetic nervous system activity through the induction of the vagus nerve in the gut or portal vein via FFAR3 (Lal

et al., 2001; Kimura *et al.*, 2011; De Vadder *et al.*, 2014; Kentish and Page, 2015). Propionate therefore stimulates direct gut–brain neural pathways.

- Unlike acetate, propionate does not undergo inter-conversion to other SCFAs (den Besten, van Eunen, *et al.*, 2013), which an important consideration when designing a study as well as in the interpretation of future results.
- Following the RYGB surgery, rodents were found to have higher concentrations of caecal propionate, suggesting a partial role of propionate in mediating the benefits associated with the surgery (Liou *et al.*, 2013).

6.2 Purpose of the pilot study

6.2.1 Hypothesis

Increasing colonic propionate through IPE supplementation will increase satiety while following a lower energy diet by stimulating GLP-1 and PYY and decreasing subsequent energy intake.

6.2.2 Aim

This study aims to explore the effect of colonic propionate enrichment on appetite regulation while following a lower energy diet.

6.2.3 Outcome measure

The primary outcome was to measure the differences in subjective appetite through VAS.

Secondary outcome measurements were:

- Weight loss between and within groups
- Energy intake following an ad libitum meal
- Serum insulin, and plasma glucose, GLP-1, and PYY levels
- Insulin sensitivity and β -cell function

6.3 Materials and methods

6.3.1 Randomisation

Both the researcher and volunteers were blinded to the type of product consumed by both of the groups during the whole study length. Randomisation was performed using an independent telephone and internet company (sealedenvelope.com).

6.3.2 Sample size

Ten volunteers were recruited for this pilot study. Males and females were evenly distributed between the two groups (4 females to 1 male).

6.3.3 Volunteer recruitment and eligibility

Healthy male and female volunteers with a BMI of 25–35 kg/m² were recruited to take part in this long-term, randomised, controlled, paralleled, double-blind study (subjects' characteristics are described in section 6.4.1). Volunteers were recruited through the NIHR/Wellcome Trust Imperial CRF at Hammersmith Hospital healthy volunteers' panel database and via poster advertisements. During the first stage, volunteer eligibility was determined through a brief phone interview. The exclusion criteria were as follows: current smoker; substance abuser; excess alcohol intake; undergone any weight loss surgery or gastric procedure to lose weight; pregnant; diabetic; cardiovascular disease; cancer; GI disease (e.g. inflammatory bowel disease or irritable bowel syndrome); kidney disease; liver disease; pancreatitis; using medications likely to interfere with energy metabolism, appetite regulation, or hormonal balance, including L anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin, or thyroid hormones; weight change of ≥3kg in the preceding 2 months; recently donated blood within the last three months. PIS was provided to participants who expressed an interest in taking part in the study (Appendix 7). Before volunteers were able to participate in the study, they had to attend a screening visit at the NIHR/ Wellcome Trust Imperial CRF at Hammersmith Hospital to further assess their eligibility. During the screening visit, the study protocol was explained in full, and participant provided full written informed consent before commencing any assessment (Appendix 8). The following measurements were collected: height, weight, blood pressure, electrocardiogram, a

standard screening blood sample, and a pregnancy test for all women of childbearing age. A research doctor had to check all of the volunteers' results before they were enrolled. Participants were asked not to start any intensive exercise regimen or new diet during the study period as it could impact the results. This study received ethical approval from the London Bridge REC (project reference number 16/LO/2021) and was performed in accordance with the Declaration of Helsinki.

6.3.4 Study design

This was a long-term, randomised, controlled, paralleled, double-blinded study (Figure 6.1). Participants were randomised to consume ready-to-use 10 g sachets of either inulin (positive control) or IPE (intervention group). These were to be taken once a day, added to their low-energy diet for 12 weeks. Participants were required to attend six study visits held at the NIHR/Wellcome Trust Imperial Clinical Research Facility. These included a screening visit, acclimatisation visit, baseline and end-of-study visits, and two follow-up visits. The acclimatisation visit was required once for every participant to allow him or her to become familiar with the taste of the breakfast and *ad libitum* meals (Chandarana *et al.*, 2009). Follow-up visits were conducted to ensure adherence to the supplement and diet plan, scheduled every four weeks.

The same standard meal of the participants' choice was consumed the evening before every study visit to insure consistency between study visits. Participants were also required to refrain from alcohol and strenuous exercise. Following overnight fasting from 09:00 p.m., volunteers were asked to attend the CRF at 09:00 a.m. Prior to consuming the test breakfast, participants were asked to empty their bladder in order to measure their weight and body composition using BIA. To promote consistency, participants were asked to change into a set of hospital scrubs and stand barefoot on a Tanita segmental body composition analyser (Tanita MC-780MA analyser; Tanita Corporation, Japan). The BIA scale is used to evaluate total body water content with an electrical current. Then, TBW is used to estimate FFM, and the fat mass is calculated by subtracting FFM from total body weight. A standardised breakfast was served at 0 minutes.



Figure 6.1: Overview of study timeline.

Participants first attended an acclimatisation study visit and then a baseline study visit. They started their first supplement with either IPE or inulin in a randomised order following their baseline visit. Participants received their final supplement dose on the morning of the post-supplementation study visit.

A standardised breakfast was served at 0 minutes, which contained 358 kcal (6 g of protein, 61.6 g of carbohydrates, 9.4 g of fibre, 6.7 g of fat). Participants were asked to finish their breakfast in 15 minutes. After the 240 minutes of the study day, an ad libitum meal, a quantitative way of measuring appetite, was served to assess the effectiveness of supplementation on appetite control. The ad libitum meal was included at both baseline and after the supplementation period visits and contained 246 kcal/100 g (11.9 g of protein, 29.3 g of carbohydrates, 2.3 g of fibre, 8.5 g of fat). To control external appetite stimuli in restraint eaters, the test meal was served to all of the participants under standardised conditions: same room conditions; controlled temperatures and constant light; all rooms were kept odour-free; and participants were kept in separate rooms to prevent the effect of social interaction on food intake and reduce any external distractions while eating (Bellisle *et al.*, 1995; Fedoroff, Polivy and Herman, 1997). Volunteers were kept on their own while consuming their meal to avoid any impact of public attention (Polivy *et al.*, 1986). Participants were told to eat until they felt comfortably full. The meal was quantified before serving and after consumption. Energy density was multiplied by the meal weight (g) to calculate the energy intake in calories.

Participants were discharged from the CRF following the ad libitum meal.

6.3.5 Free-living dieting and supplementation period

6.3.5.1 Dietary supplements

IPE was the interventional supplement used in this study and was produced at the University of Glasgow, as previously described (Chambers *et al.*, 2014; Polyviou *et al.*, 2016). Inulin (Beneo HP; DKSH, London, UK) was used as a positive control. In vitro fermentations of inulin and IPE suggest comparable increases in acetate and butyrate production, and thus any differences in outcome measures could be attributed to the preferential increase in propionate production with IPE (Chambers *et al.*, 2014). The time point where > 80% of propionate had entered the colon was 240 minutes (Chambers *et al.*, 2014). Furthermore, 10 g/day was the chosen dose of both supplements, as it has been shown to be tolerated by participants and is an effective amount to increase the release of gut hormones and decrease ad libitum energy intake (Chambers *et al.*, 2014; Polyviou *et al.*, 2016).

Participants were instructed to start consuming their supplements on the day following the baseline appetite study day (visit 3) for 12 weeks. During this period, they lived freely in their own homes and prepared their own oral intake according to the dietary guidelines given to them and supplemented with IPE or inulin. Participants in both groups were required to consume 10 g/day (one sachet). They were allowed to consume their supplements at any time of day. They were given the choice of either mixing it with a cold drink or mixing into their habitual food. Participants were asked to avoid cooking the supplements or exposing them to high temperatures.

6.3.5.2 Lower energy diet plan

Basal metabolic rate (BMR) was quantified using Schofield equations based on gender, age, and weight (Policy Committee on Medical Aspects of Food, 1991), as follows in table 6.2:

Table 6.2: Schofield equation used in the current study	
Gender/ Age (years)	BMR (kcal/d)
Males	
18-29	$15.0 \times Wt + 692.1$
30-59	$11.5 \times Wt + 873.0$
Females	
18-29	$14.8 \times Wt + 486.6$
30-59	$8.1 \times Wt + 845.6$

The estimated BMR is then multiplied by physical activity level (PAL), which is adjustable individually (Robert-McComb, Carnero and Iglesias-Gutiérrez, 2014). Most of the participants had a factor of 1. , indicating that they were lightly active.

Based on the estimated total energy expenditure (TEE), a daily energy requirement was prescribed to all participants in both groups that was 20% below their individual needs, aiming for a weight loss of approximately 5%. This diet plan was based on healthy general guidelines and calorie distribution per day.

6.3.5.3 Compliance

Weekly electronic mails were used to ensure the instructions were followed and to address any side-effects. Assessing supplement compliance was done by monitoring the supplementation diary and counting the number of returned unused sachets after the supplementation period.

Compliance with the diet plan was monitored by measuring the volunteers' actual weight at every visit and comparing it with the estimated weight lost as well as by measuring their actual

energy intake through collected and completed 24-hour food diaries, using DietPlan 7.0 software (Forestfield Software, Horsham, UK, McCance and Widdowson's 6th Edition) and specifying food choices from the Composition of Foods UK Nutritional Data set (UKN). The reported energy intakes from the food diaries were tested against the bias of the participants' energy misreporting, based on the assumption that participants were following the recommended lower energy allowance to lose weight, and thus energy intake (EI) should be equal to 20% less calories than their estimated TEE. The Goldberg equation was then applied, which takes into account the estimated variation in daily EI, PAL, BMR, and the number of days of food diaries (Black, 2000; European Food Safety Authority, 2014). Confidence intervals (CI) were then calculated based on individual PAL and days of food diaries completed and used as cut-off values to classify under- and over-reporting for below and above 95% CI cut-offs, respectively.

6.3.6 Subjective appetite scores

Please refer to Chapter 2. This questionnaire was used in the current study for each study visit every 30 minutes for 240 minutes.

6.3.7 Blood sampling

The blood sampling protocol was the same as explained in Chapter 2. Postprandial blood collection points were at 15, 30, 60, 90, 120, 180, and 240 minutes. A total of 72 ml (8 X 9 samples) of blood were collected during each study visit. Cannulae were removed after 240 minutes of each study day, and volunteers were discharged from the CRF.

6.3.7.1 Glucose analysis

Please refer to Chapter 2. The % CV of this assay was 2.4 %. This CV is the average of five assays.

6.3.7.2 Radioimmunoassays

The principals were explained in Chapter 2.

6.3.7.2.1 Insulin analysis

Please refer to Chapter 2. The % CV of this assay was 2.0%.

6.3.7.2.2 Glucagon like peptide-1 analysis

Please refer to Chapter 2. The % CV of this assay was 2.6%.

6.3.7.2.3 Peptide tyrosine tyrosine analysis

Please refer to Chapter 2. The % CV of this assay was 2.4%.

6.3.7.3 Estimation of insulin sensitivity and β -cell function

Insulin resistance and β -cell function can both be estimated through numerous simple indices found in the literature. Investigating the changes in insulin resistance and β -cell output in response to a test meal over a term of 12 weeks was a point of interest in the current study, and thus two indices were used for these estimates: the Homeostatic model assessment of insulin resistance (HOMA-IR) and the insulinogenic index (IGI).

HOMA-IR is an absolute measure of hepatic insulin resistance. It is based on fasting levels of both insulin and glucose (Matthews *et al.*, 1985), as shown below:

$$\text{HOMA-IR} = (\text{fasting glucose} \times \text{fasting insulin}) / 22.5$$

Fasting glucose is listed as mmol/L and fasting insulin as $\mu\text{U/ml}$. The outcome of this simple index has been correlated well with estimates from the euglycaemic clamp method ($r=0.88$, $p<0.0001$) (Matthews *et al.*, 1985). Moreover, this simple test has been validated to measure insulin resistance in both patients with diabetes and healthy volunteers (Hermans *et al.*, 1999).

Estimating the improvements in β -cell output was done using IGI. This index is a ratio of the increase in insulin release to the correspondent magnitude of glucose response. It is calculated as shown below (Seltzer *et al.*, 1967):

$$\text{IGI} = \text{increment in insulin at 30 min} / \text{increment in glucose at 30 min}$$

Insulin is listed as $\mu\text{U/ml}$, glucose as mmol/L, and increments are calculated from basal values using the trapezoid rule. An increase in insulin release is expected in healthy volunteers in response to oral glucose ingestion, which is followed by a fast clearance of blood glucose. The IGI

has been validated against both intra-venous glucose tolerance test techniques and hyperinsulinemic euglycaemic clamps (Hanson *et al.*, 2000; Tura, Kautzky-Willer and Pacini, 2006).

6.3.8 Statistical analysis

Data were checked for normality using a Shapiro–Wilk test (defined as $P \geq 0.01$). In the case of non-normal distribution, data were normalised using log-transformation (the function LN or LN+1 where applicable). If the data were still not normally distributed, non-parametric tests were used. Percentage of weight loss was calculated using the weight at the end of the intervention divided by the baseline weight multiplied by 100. All figures are presented as raw data. Results are expressed as mean difference $\pm$ standard error of the mean (SEM). The average pre and post data points were calculated and used to replace any missing data values. The two baseline samples for blood and VAS data were averaged and used as the 0 min measurement. The tAUC was calculated for all parameters using the trapezoidal rule. A mixed between-within subjects ANOVA was used to assess the impact of two different interventions (IPE and Inulin) on participant parameters across the 12-week supplementation period (baseline and week 12), with the supplementation group used to determine between-subjects differences and the Fisher LSD method was used to correct for multiple comparisons. A p-value ≤ 0.05 was considered to be significant, with the significance level indicated as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Statistical analysis was performed using IBM SPSS version 23. Graphs were prepared using GraphPad Prism version 7.04.

6.4 Results

6.4.1 Baseline characteristics

Ten adults were randomised to participate in the study. The baseline characteristics of these participants are described in Table 6.3.

Table 6.3: Participants demographics and baseline dietary intake of both supplementation groups.

Variable	IPE	Inulin	p-value
Male: female (n)	1:4	1:4	--
Age (Y)	30.6 ± 2.7	35.2 ± 5.4	0.46
Weight (Kg)	79.4 ± 5.7	77.6 ± 5.9	0.83
BMI (Kg/m ²)	29.0 ± 1.8	28.3 ± 1.1	0.74
Body Fat (%)	35.1 ± 3.5	32.1 ± 2.8	0.51
Energy intake (Kcal)	2122.02 ± 370.13	1754.84 ± 157.02	0.38
CHO intake (%)	50.67 ± 3.34	42.40 ± 4.15	0.15
Fibre intake (%)	6.77 ± 2.21	9.88 ± 1.33	0.26
Protein intake (%)	15.81 ± 0.59	15.43 ± 1.45	0.81
Fat intake (%)	33.50 ± 3.15	42.16 ± 3.26	0.93

Data are means ± SEM, (n) or (%)

Un-paired t-test was used between groups to detect differences.

IPE; inulin propionate ester, CHO; Carbohydrate

6.4.2 Visual analogue scales

6.4.2.1 Composite appetite score

The appetite composite score was calculated as in methods section 2.1.3.2.5. Time course data for composite appetite score and tAUC results are shown in Figure 6.2.

There was no significant interaction between supplement type and supplementation period ($p=0.44$). There was a significant difference in the main within-subjects effect of supplementation period on the composite appetite score ($p=0.03$). Post hoc comparisons showed a significant mean reduction in the IPE group ($51.3 \pm 9.6\text{mm}$) when compared with their baseline ($61.2 \pm 7.7\text{mm}$) ($p=0.01$). However, the inulin group showed no effect on composite appetite score when compared with their baseline ($p=0.39$).

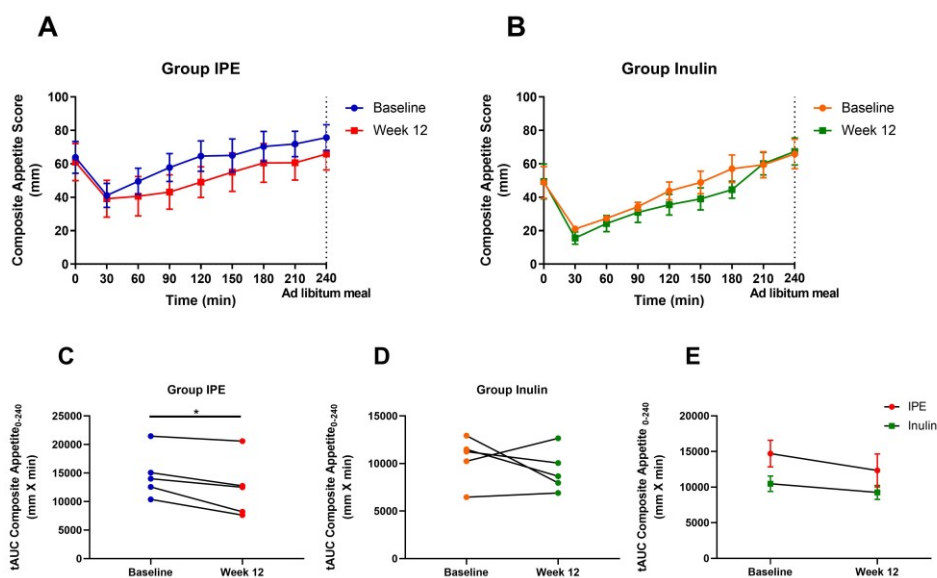


Figure 6.2: Subjective composite appetite score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.2.2 Hunger

Time course and tAUC data for subjective hunger score are shown in Figure 6.3.

There was no significant interaction between supplement type and supplementation period ($p=0.32$). There was a significant reduction in the main within-subjects effect of supplementation period on the hunger score ($p=0.02$). Post hoc comparisons showed a significant mean reduction in the IPE group ($43.0 \pm 12.2\text{mm}$) when compared with their baseline ($56.4 \pm 10.8\text{mm}$) ($p \leq 0.01$). However, the inulin group showed no effect on hunger score when compared with their baseline ($p=0.44$).

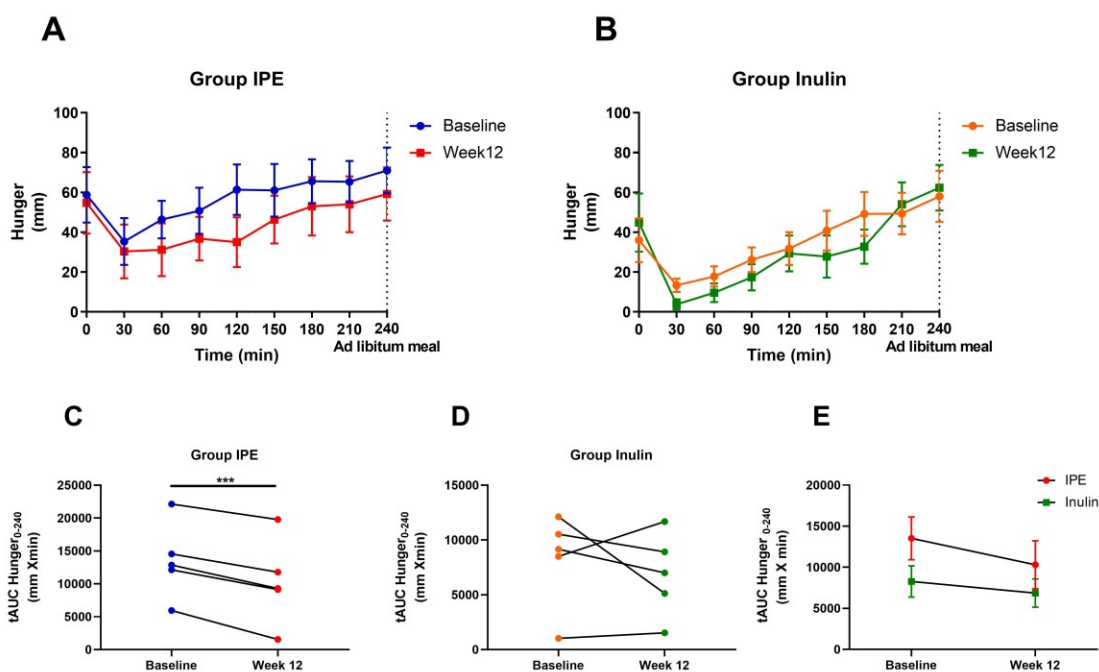


Figure 6.3: Subjective hunger score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.2.3 Fullness

Time course and tAUC data for subjective fullness score are shown in Figure 6.4.

There was no significant interaction between supplement type and supplementation period ($p=0.82$). There was no significant reduction in the main within-subjects effect of supplementation period on the fullness score ($p=0.19$).

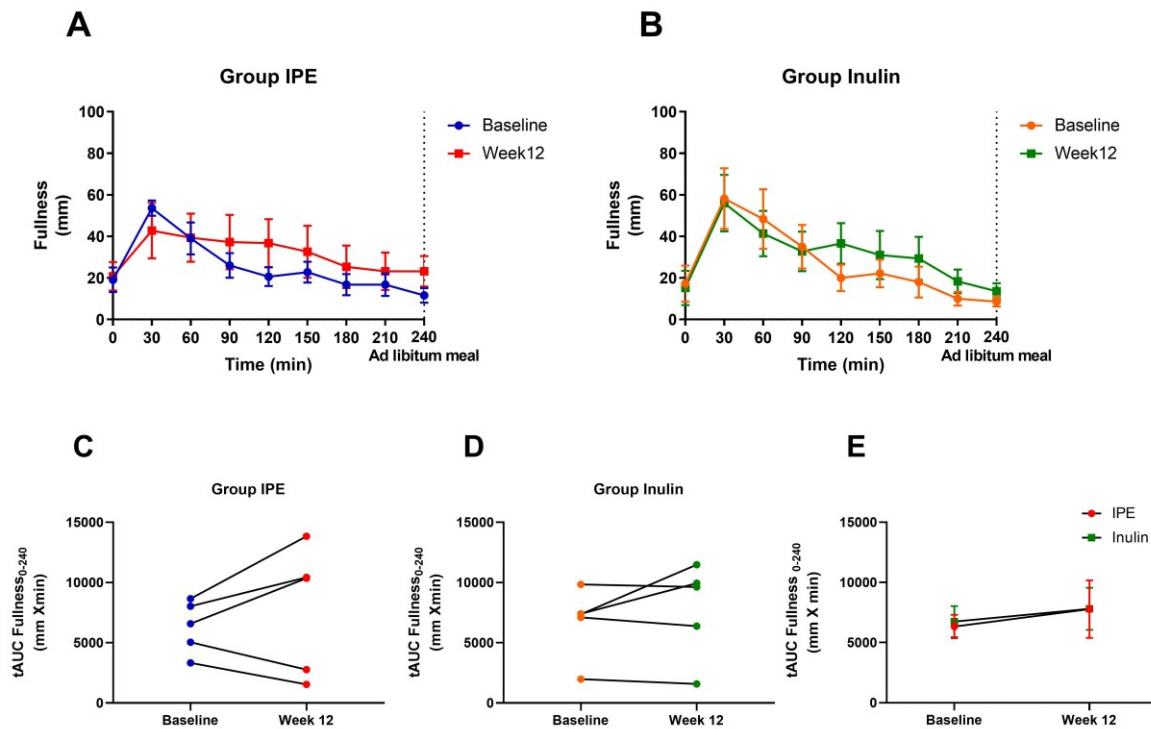


Figure 6.4: Subjective fullness score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.2.4 Desire to eat

Time course and tAUC data for subjective desire to eat score are shown in Figure 6.5.

There was no significant interaction between supplement type and supplementation period ($p=0.33$). There was no significant reduction in the main within-subjects effect of supplementation period on the desire to eat score ($p=0.14$).

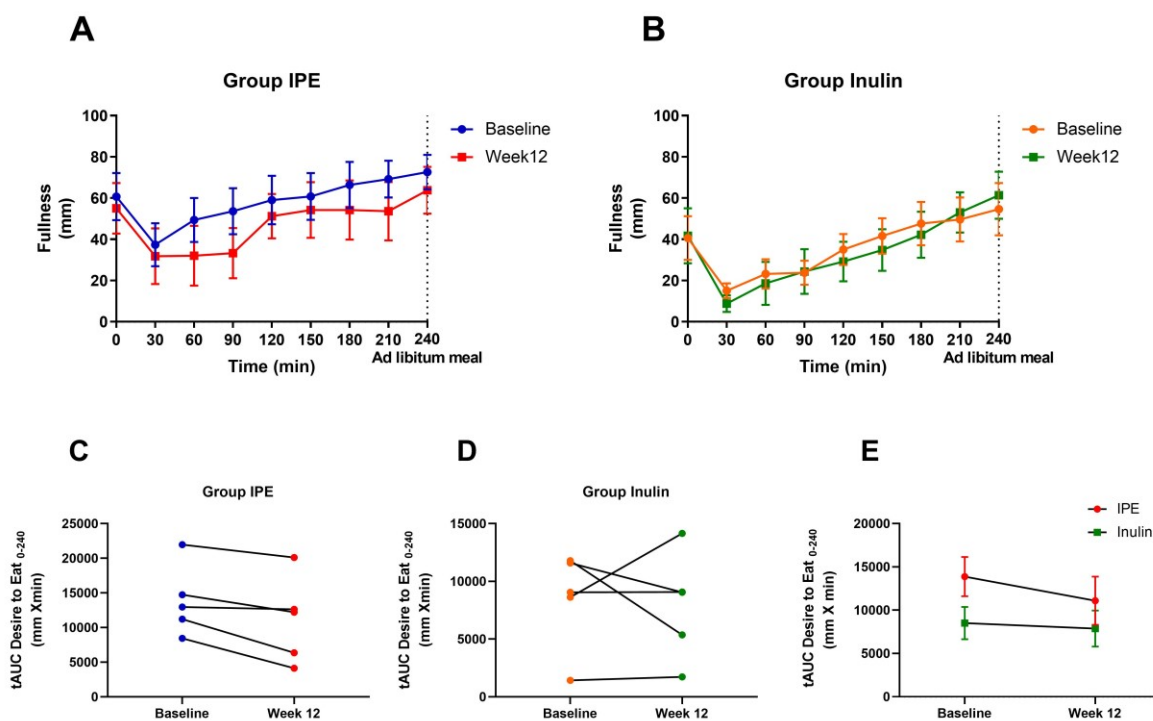


Figure 6.5: Subjective desire to eat score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.2.5 Appetite for a meal

Time course and tAUC data for subjective appetite for a meal score are shown in Figure 6.6.

There was no significant interaction between supplement type and supplementation period ($p=0.92$). There was no difference in the main within-subjects effect of supplementation period on appetite for a meal score ($p=0.60$).

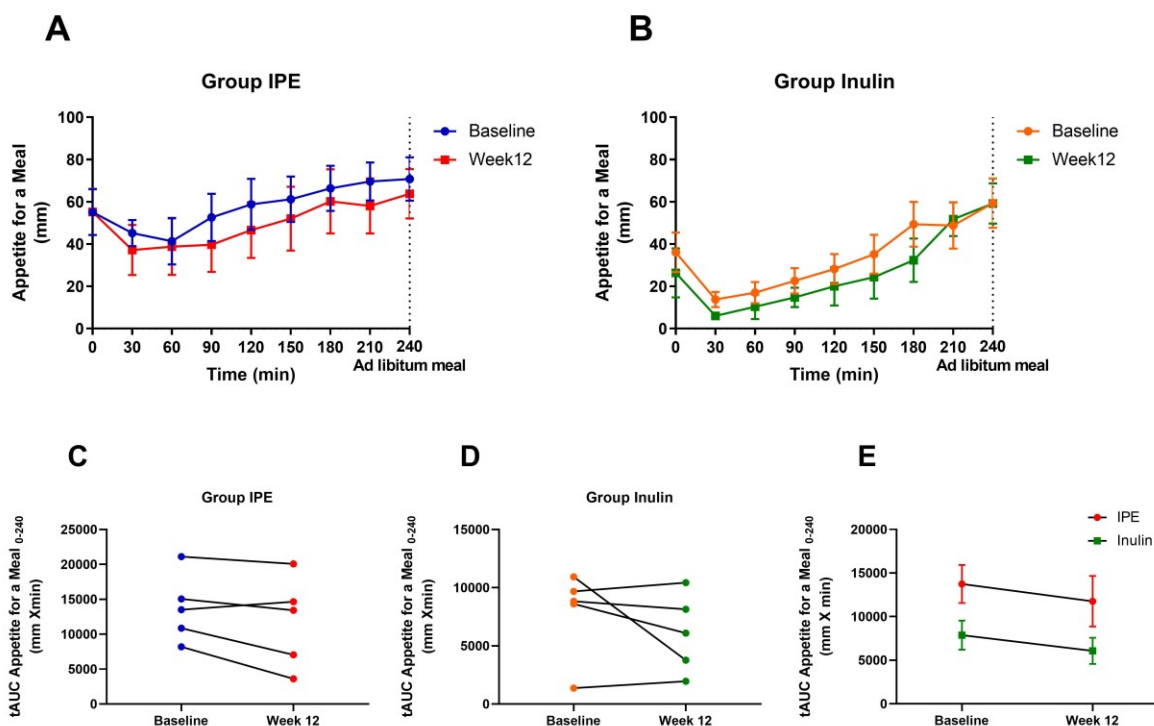


Figure 6.6: Subjective appetite for a meal score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.2.6 Nausea

Time course and AUC data for nausea score are shown in Figure 6.7.

There was no significant interaction between supplement type and supplementation period ($p=0.14$). There was no significant reduction in the main within-subjects effect of supplementation period on the sickness score ($p=0.71$).

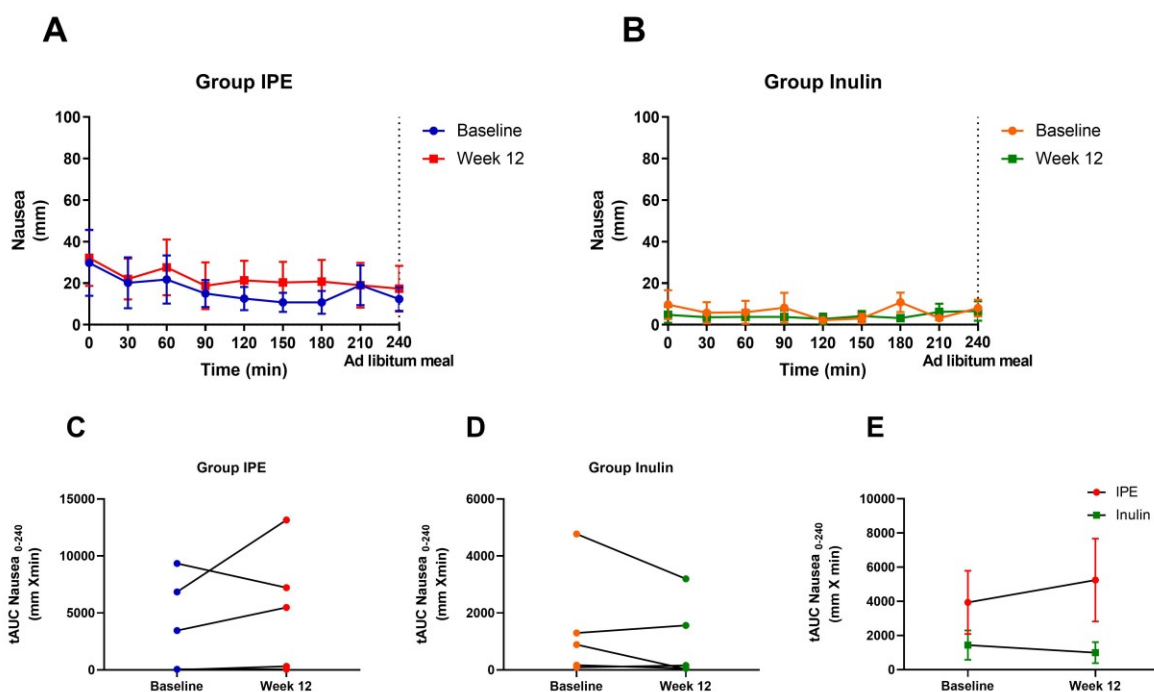


Figure 6.7: Subjective nausea score in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) $tAUC_{0-240}$ of within IPE group differences, (D) $tAUC_{0-240}$ of within Inulin group differences, (E) mean $\pm$ SEM $tAUC_{0-240}$ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.3 Weight loss

Percentage of weight loss data for both of the study groups are shown in Figure 6.8.

There was no significant interaction between supplement type and supplementation period ($p=0.88$). There was a trend difference in the main within-subjects effect of supplementation period on the percentage of weight lost ($p=0.06$), with both groups showing a reduction in percentage of weight loss across the four-study time frame (Table 6.4). Post hoc comparisons showed a significant reduction in IPE group weight ($77.2\pm5.4\text{kg}$) when compared with their baseline ($79.4\pm5.8\text{kg}$) ($p=0.04$). However, the inulin group showed no effect on weight loss when compared with their baseline ($p=0.18$).

Table 6.4: Weight loss in both of the study groups throughout the course of 12 weeks

group	Week	Mean (kg)	Std. Error	P-value
IPE	0	79.440	5.75	0.04
	4	78.320	5.36	
	8	77.420	5.35	
	12	77.240	5.42	
Inulin	0	77.540	5.97	0.18
	4	76.840	6.24	
	8	75.520	5.96	
	12	75.240	5.95	

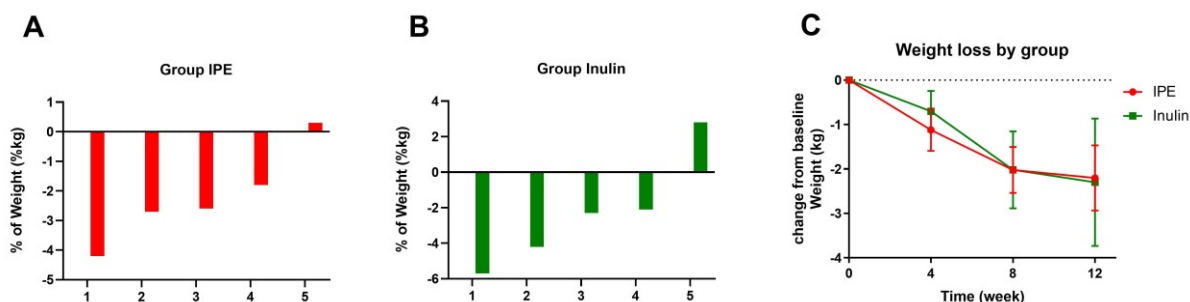


Figure 6.8: Weight loss in both of the groups following the supplementation of IPE (●) and Inulin (●) for 12 weeks.

Data represent mean $\pm$ SEM (IPE n=5, Inulin n=5) of (A) % of individual weight loss within IPE group, (B) % of individual weight loss within Inulin group, (C) % weight lost for both of the groups among the study period. Percentage of weight loss was calculated using the weight at the end of the intervention divided by the baseline weight multiplied by 100. Percentage of weight loss was calculated using the weight at the end of the intervention divided by the baseline weight multiplied by 100. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations (IPE) Inulin propionate ester, (%) percentage.

6.4.4 Actual energy intake from food diaries

The mean differences data for energy intake during the study course of 12 weeks are shown in Figure 6.9.

Total energy intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.55$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.63$).

Percentage of change in energy intake over 12 weeks:

Although the prescribed diet for both of the groups was set around 20% less than TEE, the percentage of energy intake was kept at around $5.5 \pm 12.0\%$ and $5.9 \pm 12.0\%$ less energy for the IPE and inulin groups, respectively. There was no significant interaction between supplement type and supplementation period ($p=0.58$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.70$).

Total carbohydrate (CHO) intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.37$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.42$).

Total fat intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.44$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.86$).

Protein intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.56$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.98$).

Alcohol intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.66$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.41$).

Fibre intake over 12 weeks:

There was no significant interaction between supplement type and supplementation period ($p=0.52$). There were no significant differences in the main within-subjects effect of supplementation period on energy intake ($p=0.17$).

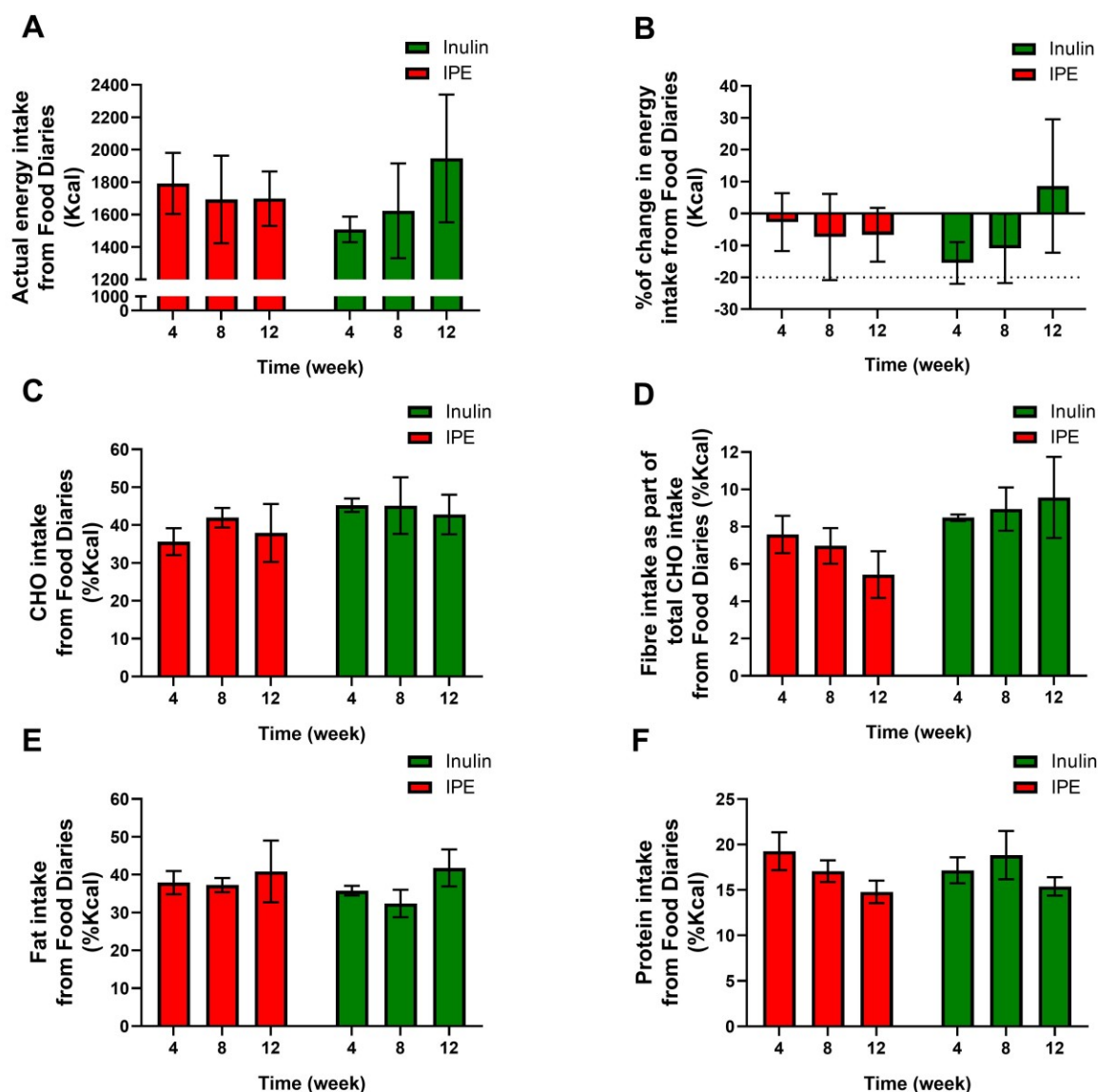


Figure 6.9: Actual dietary intake from food diaries in both of the study groups following the supplementation of IPE or inulin for 12 weeks.

Data represent mean $\pm$ SEM of (A) total energy intake, (B) change in energy intake as a percentage from total energy intake, (C) total CHO intake, (D) fibre intake as a percentage from total CHO intake, (E) total fat intake, (F) protein intake. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations: CHO, carbohydrate.

6.4.5 Energy intake from ad libitum meal

The mean differences data in energy intake in both visits are shown in Figure 6.10.

There was no significant interaction between supplement type and supplementation period ($p=0.31$). There were no significant differences in the main within-subjects effect of supplementation period on appetite for a meal score ($p=0.12$).

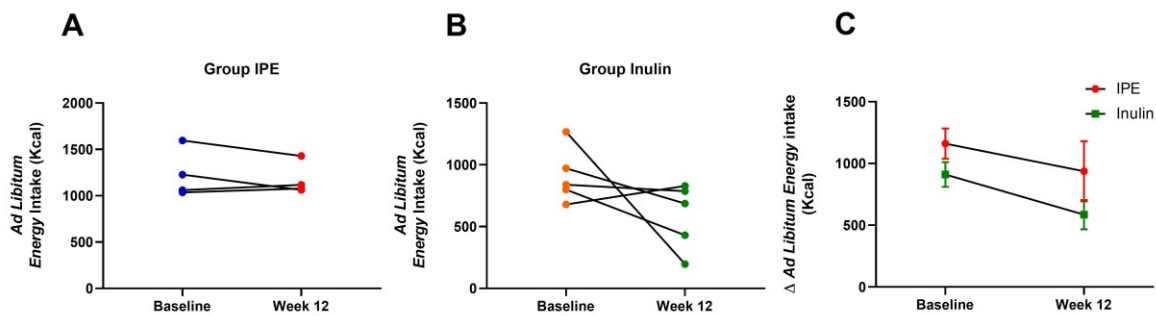


Figure 6.10: Ad libitum energy intake in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) individuals baseline IPE (●) and post intervention IPE (●), (B) individuals baseline Inulin (●) and post intervention Inulin (●), (C) mean $\pm$ SEM of differences between IPE (●) and Inulin (●) groups. Paired t-test was used within groups and un-paired t-test was used between groups. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.6 Plasma glucose

Time course and tAUC data for plasma glucose concentrations are shown in Figure 6.11.

There was no significant interaction between supplement type and supplementation period on plasma glucose ($p=0.81$). There was no significant effect of the main within-subjects effect of supplementation period on plasma glucose ($p=0.20$).

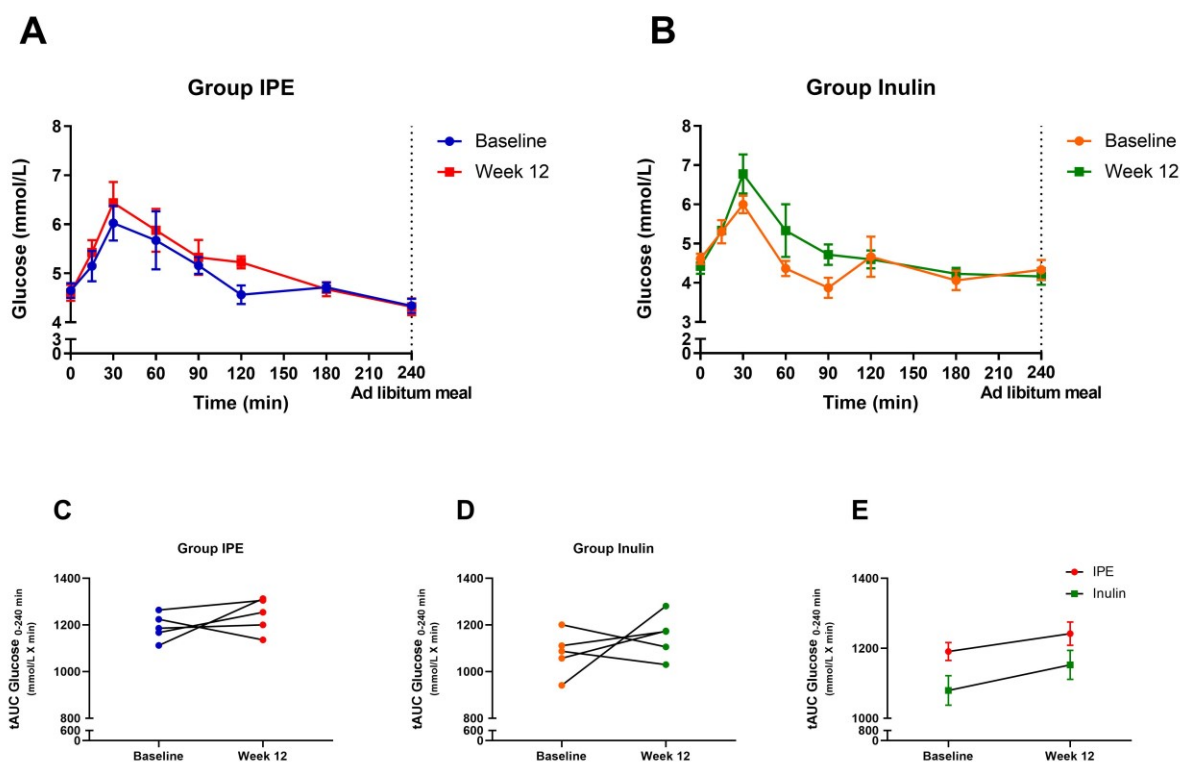


Figure 6.11: Postprandial plasma glucose in response to mixed meal in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.7 Serum insulin

Time course and tAUC data for serum insulin concentrations are shown in Figure 6.12.

There was no significant interaction between supplement type and supplementation period on total insulin concentration ($p=0.94$). There was no significant effect of the main within-subjects effect of supplementation period on the total serum insulin score ($p=0.40$).

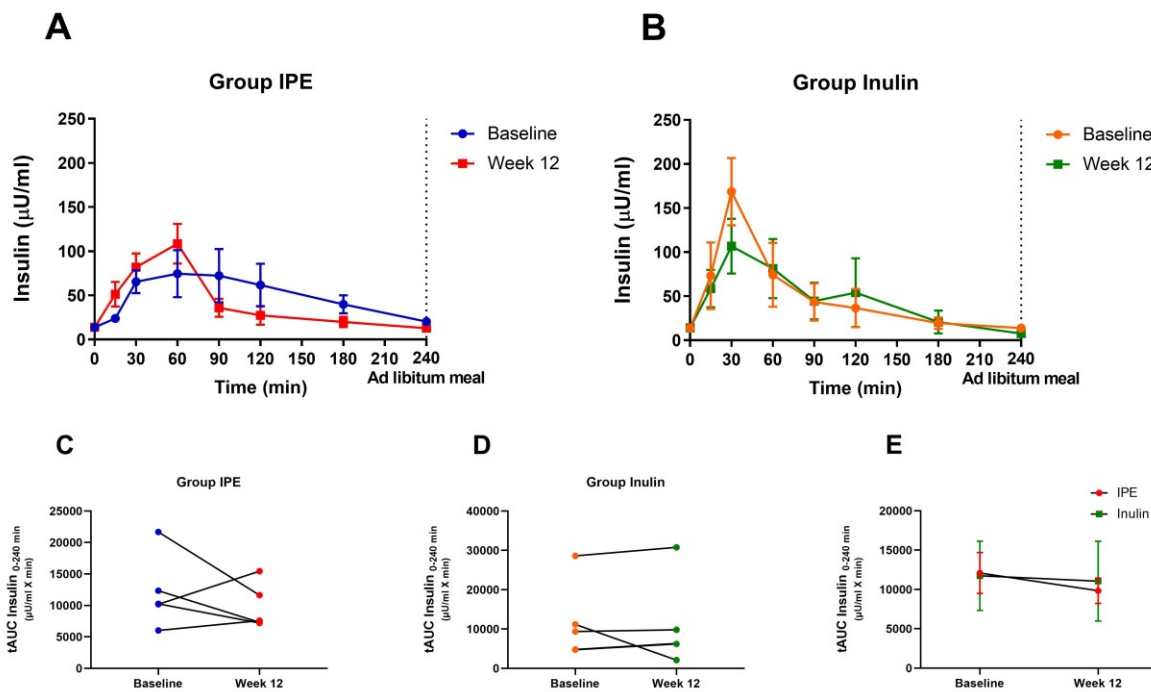


Figure 6.12: Postprandial serum insulin in response to mixed meal in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.8 Plasma GLP-1

Time course, AUC, and tAUC data for plasma GLP-1 concentrations are shown in Figure 6.13.

There was no significant interaction between supplement type and supplementation period ($p=0.88$). There was no significant effect of the main effect of supplementation period on plasma GLP-1 level ($p=0.10$).

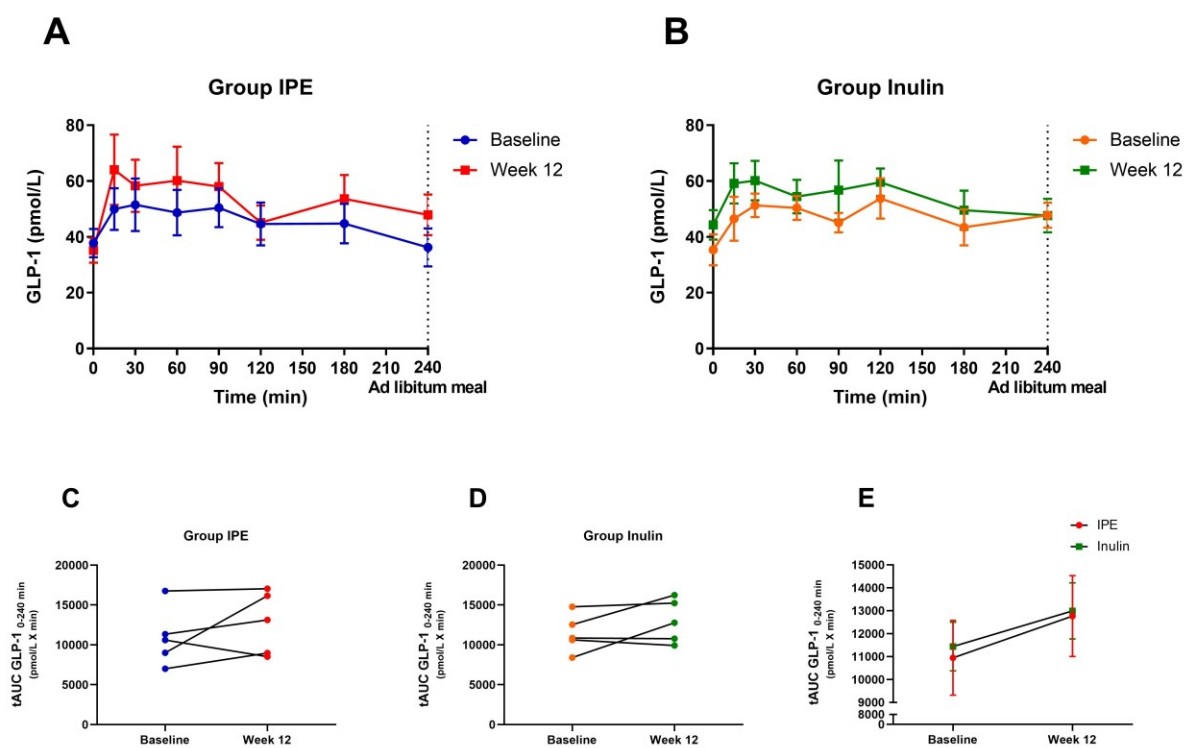


Figure 6.13: Postprandial plasma GLP-1 in response to mixed meal in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.9 Plasma PYY

Time course, AUC, and iAUC data for plasma PYY concentrations are shown in Figure 6.14.

There was no significant interaction between supplement type and supplementation period ($p=0.34$). There was no significant effect of the main within-subjects effect of supplementation period on plasma PYY levels ($p=0.99$).

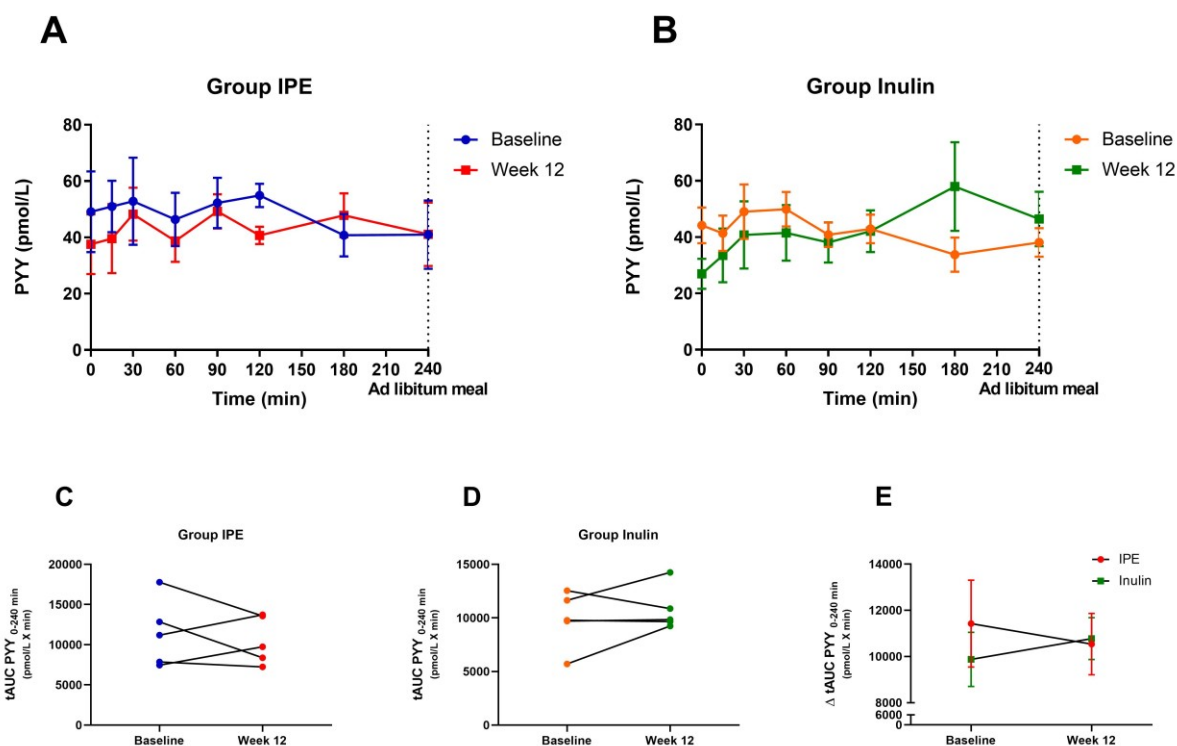


Figure 6.14: Postprandial plasma PYY in response to mixed meal in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) mean $\pm$ SEM baseline IPE (---) and post intervention IPE (---), (B) mean $\pm$ SEM baseline Inulin (---) and post intervention Inulin (---), (C) tAUC₀₋₂₄₀ of within IPE group differences, (D) tAUC₀₋₂₄₀ of within Inulin group differences, (E) mean $\pm$ SEM tAUC₀₋₂₄₀ of differences between IPE (●) and Inulin (●) groups. A mixed between-within subjects' analysis of variance was used, with supplement type as a between-subjects factor. Fisher LSD post-hoc tests were performed between time-points. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.10 Insulin resistance

HOMA-I was used as a measurement of insulin resistance (see Figure 6.15).

It was calculated as follows:

$$(\text{fasting insulin} \times \text{fasting glucose}) / 22.5$$

There was no significant interaction between supplement type and supplementation period ($p=0.95$). There was no significant effect of the main within-subjects effect of supplementation period on insulin resistance score ($p=0.92$).

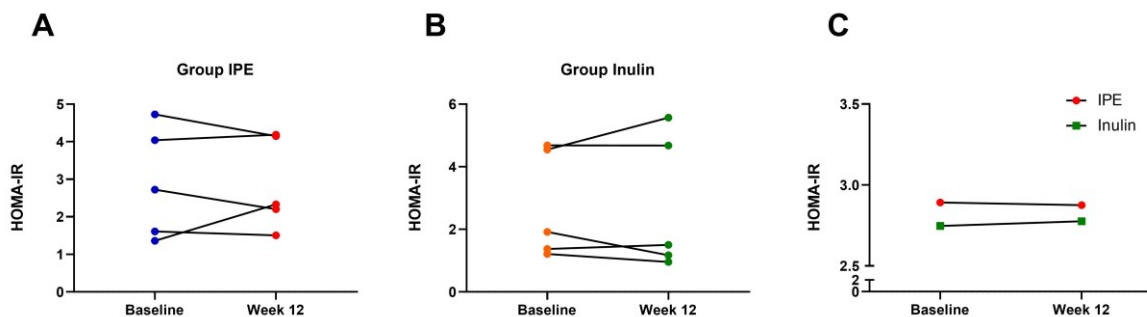


Figure 6.15: HOMA-IR in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) individuals baseline IPE (●) and post intervention IPE (●), (B) individuals baseline Inulin (●) and post intervention Inulin (●), (C) mean $\pm$ SEM of differences between IPE (●) and Inulin (●) groups. Paired t-test was used within groups and un-paired t-test was used between groups. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.4.11 β -cell function

Measurement of insulin secretion, to estimate β -cell function, is shown in Figure 6.16.

It was calculated as follows:

$$\text{AUC insulin}_{0-60\text{min}} / \text{AUC glucose}_{0-60\text{min}}$$

There was a significant interaction between supplement type and supplementation period ($p=0.02$). There was a borderline difference in the main effect of within-subjects supplementation period on insulin secretion ($p=0.06$). Post hoc comparisons showed that there was a significant mean increase in the IPE group (12.4 ± 1.3) when compared with their baseline (9.1 ± 1.3) ($p=0.02$).

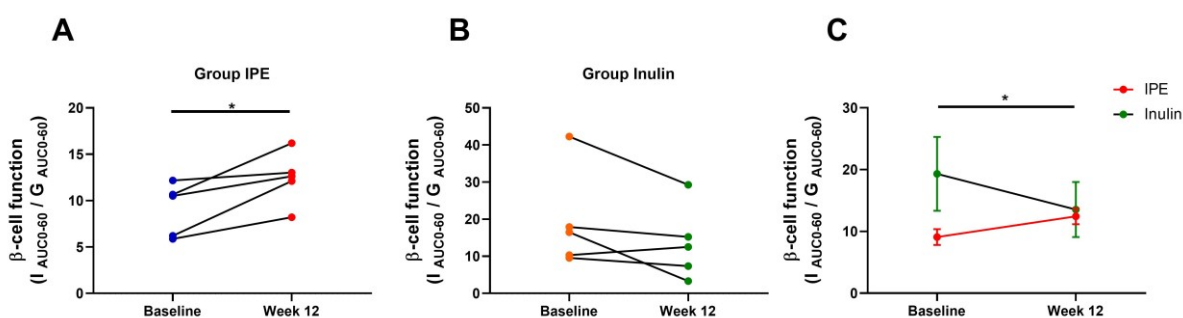


Figure 6.16: β -cell function in both of the groups following the supplementation of IPE or Inulin for 12 weeks.

Data represents (A) individuals baseline IPE (●) and post intervention IPE (●), (B) individuals baseline Inulin (●) and post intervention Inulin (●), (C) mean $\pm$ SEM of differences between IPE (●) and Inulin (●) groups. Paired t-test was used within groups and un-paired t-test was used between groups. Data at which values differed significantly were represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: tAUC, total area under the curve, IPE, Inulin-propionate ester.

6.5 Discussion

6.5.1 Main findings

- Composite appetite and hunger scores were significantly lower following IPE supplementation. However, this effect was not significant statistically when compared between the groups.
- Weight was lost successfully following IPE for 12 weeks, but it was not significant when compared between the groups.
- Energy intake did not differ within or between the groups.
- Plasma glucose did not differ within or between the groups.
- Insulin secretion was improved following IPE supplementation. These improvements were not apparent within the inulin group.
- Plasma GLP-1 and PYY concentrations did not differ within or between the groups.

6.5.2 Detailed discussion

The current study was conducted to investigate the effect of SCFAs on appetite control while following a below-daily-requirement energy diet for 12 weeks. Based on the results observed from the previous experimental chapters, the current study explores the effect of elevated colonic SCFA, one of the resultant products from complex carbohydrate fermentation, on suppressing appetite and whether or not we can extend the information gathered to recommend IPE supplementation while following a weight loss diet as a strategy for diet compliance.

6.5.2.1 Appetite measures

Although there was no difference in ad libitum meals, there was a significant reduction in appetite composite score and its elements (hunger) following IPE supplementation for 12 weeks when compared with baseline. These effects were not observed following the inulin supplementation for the same period. This finding clearly highlights the effect of propionate supplementation on decreasing appetite response while following a lower energy diet for 12

weeks. However, there was no significant difference in the time-group interactions, meaning that these differences were not statistically significant when compared between groups.

Interestingly, there were no changes in plasma GLP-1 and PYY levels within and between the two interventions, suggesting the IPE has induced lower appetite response independently of plasma gut hormone levels. This suggestion is in line with studies from the same group and others in the literature, which suggested the ability of IPE to reduce appetite following 24 weeks of supplementation as part of the participants' daily habits (i.e. they were instructed to maintain the same diet and not to attempt to lose weight) without effects on plasma GLP-1 and PYY levels (Chambers *et al.*, 2014). Another study highlighted the role of IPE on the gut–brain axis as an important regulator of appetite. With the aid of fMRI, Byrne *et al.* reported an activation of the caudate and nucleus accumbens reward brain regions following the acute ingestion of IPE in humans (Byrne *et al.*, 2016). Together, these data suggest a role of peripheral propionate that is made available for systemic circulation (Bloemen *et al.*, 2009) to reach the brain centres responsible of affecting appetite responses. Likewise, these data also do not rule out the effect of propionate on central appetite regulation through signalling the vagal nerve in the gut or portal vein via stimulation of FFAR3 (Lal *et al.*, 2001; Kimura *et al.*, 2011; De Vadder *et al.*, 2014; Kentish and Page, 2015).

6.5.2.2 Weight change

The results of the current study showed a significant reduction in the mean weight loss of the IPE group after 12 weeks of supplementation when compared with their baseline. This is an interesting finding, as it is known that weight loss following dietary intervention causes an increase in appetite in the long run, and it is considered the main reason that all of the weight lost is often regained (Sumithran *et al.*, 2011). However, in the current study, the opposite occurred while supplementing with IPE and maintaining a lower energy diet. This is a promising result from a pilot study in terms of the ability to maintain the weight loss through the diet while not struggling against the body's natural physiology to increase the appetite response. However,

there were no significant differences in the time-group interactions, meaning that these differences were not statistically significant when compared between groups.

Interestingly, this weight loss following IPE supplementation was not accompanied by a decrease in the intake from ad libitum meals or in the actual energy intakes from the participants' food diaries. The Goldberg protocol was used to identify misreported diaries, and only five out of 20 diaries were considered as under-reported in that group, while the rest were considered as acceptable reporters. Considering the weight lost and the percentage of change in energy intake in mind, this means that the participants adhered to the recommended energy allowance. This might also mean that the level of energy expenditure was as high as their baseline to induce weight loss, as the recommended energy allowance stayed the same over the 12weeks study period. Taken together, these results highlight the promising effects of IPE in terms of maintaining appetite and increasing compliance to the prescribed diet. However, further studies are needed.

6.5.2.3 Glycaemic and insulinemic responses

The results of the current study indicated no difference in the postprandial glycaemic or insulin responses following the ingestion of both of the supplements for 12 weeks. However, there was a change in the behaviour of the postprandial insulin responses. Thus, insulin resistance and secretion were studied to determine the effect of these interventions on the insulinemic responses. While no differences were found between the two interventions on HOMA-IR (as an insulin resistance measurement), there was a significant reduction of the IGI (an insulin secretion and beta-cell function measurement), suggesting improvements in both the early- and late-phase insulin responses following IPE supplementation. These findings are in line with the results of Pingitore et al., whose proposed mechanism of action was dependent on the FFAR2 discovered in the beta cells of the pancreas and involved induction of propionate in vitro to increase insulin secretion independent of changes in GLP-1 levels and prevention of the human islets from apoptosis. Interestingly these effects were significant between the groups (Pingitore *et al.*, 2017). Taken together, the results of the current study suggest a role for the proportion of propionate

made available in the peripheral circulation (Bloemen *et al.*, 2009), with a direct effect on pancreatic FFAR2 and/or indirectly via the gut–brain axis through FFAR3 stimulation of the vagus nerves or portal vein (Lal *et al.*, 2001; Kimura *et al.*, 2011; De Vadder *et al.*, 2014; Kentish and Page, 2015).

6.5.3 Strengths, weaknesses, and future recommendations

It would be ideal to supply physical activity monitors to the participants to address the exact activity level needed when estimating energy requirements, to determine whether weight loss was driven by physical activity or food intake, and to monitor changes in activities in response to supplementation. Moreover, it would be beneficial to record vital signs (e.g. heart rate and blood pressure) to provide an indication of the effects of supplementation on the sympathetic nervous system. The small number of participants is a weakness of this study. However, this was a pilot study to preliminary answer a question. Since the results are promising, a future study should be planned with sufficient power to produce significant differences between groups and make it possible to determine whether SCFA propionate can suppress appetite and increase compliance with low-energy diets to promote weight loss, considering the above mentioned recommendations. Accordingly, a larger study of 158 participants should be designed. A power calculation based on the results of the current pilot study found that propionate-ester will result in a composite appetite score mean loss of 9.8 ± 5.5 mm, while inulin supplements will cause a composite appetite score mean loss of 5.1 ± 11.9 mm. A power calculation showed that 63 volunteers would be needed per group to detect a significant difference between groups ($p=0.05$, 0.80 power). Therefore, 158 volunteers will be recruited to allow a 25% drop-out rate (79 participants per group).

6.6 Conclusion of the study

This pilot study suggests that IPE has a positive effect on appetite while following a low-energy diet. However, the appetite-suppressing effect is clearly not to be due to a change in GLP-1 or PYY gut hormones, and so it remains unclear whether SCFAs, specifically IPE, have a positive effect on central appetite regulation in humans, as proposed in previous in rodent studies.

Chapter 7

7. General Discussion

7.1 Summary of main findings

Findings of Chapters 3, 4, and 5:

Primary comparison of findings looking at the effect of starch branching enzyme mutation in peas:

- The SBE-deficient line of pea seeds and flour had lower postprandial glycaemic and insulin effects when compared with the wild counterparts.
- The lower postprandial glycaemia was independent of delayed GE; rather, it was reflected in small intestinal glucose availability, suggesting limited digestibility in the upper GI tract due to a structurally dependent mechanism.
- Tracking ^{13}C glucose analysis reveals a significantly lower breakdown of SBE-deficient pea seeds into ^{13}C glucose, suggesting their limited digestibility when compared with wild pea seeds. These results were reflected in the higher 24-hour urinary excretory levels of ^{13}C propionate and ^{13}C butyrate, suggesting that SBE-deficient pea seeds and flour were both available for microbiota fermentation.
- There were inconsistencies in and no significant effects of genetic mutation on GLP-1 and PYY gut hormones as well as on VAS.

Secondary comparison of findings looking at the effect of cellular structure in peas:

- SBE-deficient pea seeds had lower postprandial glucose and insulin responses when compared with SBE-deficient pea flour. This was independent of delayed GE, but it was reflected as a trend towards an increase in breath hydrogen excretion when compared with SBE-deficient flour, with no differences in small intestinal glucose concentration.
- Wild flour seeds had higher postprandial glucose and insulin response compared to wild pea seeds. However, this was not accompanied by differences in small intestinal glucose concentrations.
- Tracking ^{13}C glucose analysis reveals a significantly lower breakdown of SBE-deficient pea seeds into ^{13}C glucose, suggesting their limited digestibility compared with SBE-deficient

pea flour. The same results did not reach significance when comparing wild pea seeds and flour.

- There was inconsistency in and no significant effects of structural processing on GLP-1 and PYY gut hormones as well as VAS.

Chapter 6

- IPE supplementation for 12 weeks significantly reduced subjective appetite, as reflected by VAS, while maintaining a lower energy diet, when compared to baseline.

7.2 Introduction

Between 1993 and 2014, the proportion of men categorised as obese increased from 13.2% to 24.3%, and there was a similar increase in women from 16.4% to 26.8%. Moreover, obesity is further predicted to rise to 60% of men and 50% of women by 2050 (National Obesity Observatory, 2016). This occurred in parallel with an increased consumption of high-energy, low-fibre foods with no structures (due to processing), which has been implicated as a major factor in the current obesity crisis (Eaton and Konner, 1985). Meanwhile, supplementation with RS has been shown to regulate energy homeostasis, improve insulin sensitivity, alter central appetite regulation, and reduce body weight and fat mass gain in animals (Cani *et al.*, 2005; So *et al.*, 2007; Shen *et al.*, 2009) and humans (Robertson *et al.*, 2005; Cani *et al.*, 2006; Pedersen *et al.*, 2013; Wang *et al.*, 2019). Thus, altering starch structurally to increase its complex or resistant fractions is an attractive strategy for regulating appetite and energy homeostasis. Pea seeds were chosen due to their known natural mutation affecting starch biosynthesis, in addition to their associated health benefits when compared to refined cereal crops. Mutation in the *rr* locus of pea seeds results in a defect in one of the starch branching enzymes, leading to different starch characteristics and, importantly, a higher amylose-to-amylopectin ratio, lowering their digestion profile. The extent and rate of starch digestion in the upper GI tract is significantly related to the proportion of starches available for fermentation by the lower gut. As evidence suggests that RS consumption is associated with several physiological benefits, which are mediated by their fermentation products, SCFAs, the effect of delivering a high amount of one of these SCFAs,

propionate, was also examined. Our research group suggested that, based on recent human studies, propionate might be an important SCFA contributing to appetite regulation (Chambers *et al.*, 2014; Byrne *et al.*, 2016). Thus, it was the SCFA of choice in the last study of the current work. This thesis aimed to explain the effect of different starch structures and the role of the upper GI tract in digesting plant starch as well as the effects of by-products generated from their fermentation on energy homeostasis and appetite modulation.

7.3 Detailed discussion of findings

Primary comparison of findings looking at the effect of starch branching enzyme mutation in peas:

As a result of the natural mutation, SBE-deficient pea seeds have a higher ratio of amylose to amylopectin, which can lead to the acquisition of similar properties as foods with high RS. Non-viscous fibre does not tend to delay the GE rate and thus independently decrease postprandial plasma glucose in the same way as viscous fibre (Juvonen *et al.*, 2009). It has been concluded from Chapter 3 that SBE-deficient peas and flour result in lower postprandial glucose response when compared with their wild counterparts. Interestingly, this was independent of delaying the GE rate. This was an important parameter to measure, as it confirms that plasma glucose kinetics are not affected by GE rate (Marathe *et al.*, 2013) and that stomach digestion and the time needed for similar products to leave the stomach are not affected by different processing. Accordingly, it is of interest to explore the digestion process further down in the GI tract to determine the effect of genetic mutation as a primary outcome on starch digestion and whether the same effect is retained upon processing the same lines of peas.

Chapter 4 data indicated lower postprandial small intestinal glucose levels following the consumption of SBE-deficient peas when compared with the consumption of wild peas. This limited digestibility could be attributed to the high amylose-to-amylopectin ratio of SBE-deficient pea starch granules. Looking back at the collaborative in vitro microscopy (Figure 4.18 in Chapter 4), these results can be expected, considering the pleiotropic effect of the mutation. The SBE-deficient pea seed have a thicker CW, which acts as a physical barrier, protecting the entrapped

starch from digestion and limiting the space available for water absorption in response to hydrothermal processing (i.e. cooking), thus restricting gelatinisation (Edwards, Warren, *et al.*, 2015). Together, these explanations support the outcomes of Chapter 3 and suggest a need to determine the quality of a carbohydrate and its relative postprandial glucose response. Both the amylose-to-amylopectin ratio and the structural physico-chemical differences are equally important. With regard to the flours, no differences were shown in small intestinal glucose levels. Observations from the literature could explain these results. It was shown previously that the initial stages of amylase digestion target the amorphous regions of starches (Gallant *et al.*, 1992). Previous in vitro studies have shown that, depending on the botanical origin of the plant, the outer layer of legumes can be composed of an amorphous starch while the inner core is crystalline (Edwards *et al.*, 2014, 2018). Moreover, the rate of amylase digestion has been shown to be initially the same between those peas genotypes but decreased in the SBE-deficient line over time (Skrabanja *et al.*, 1999; Tahir *et al.*, 2011; Edwards *et al.*, 2018). Thus, these findings imply that the amorphous starches in both lines of peas after being processed into flour are digested at a comparable rate during the resident time in the duodenum, but the time spent in the duodenum may not be long enough to observe differences in analyses of crystalline high-amylose starch granule digestion.

The limited digestibility of food in the upper GI increases the delivery of carbohydrate to the colon and increases fermentation. Examining pea digestion further in the lower gut was done by tracking ^{13}C stable isotope-enriched peas. Concentrations of ^{13}C glucose in plasma (a marker of ^{13}C pea digestion) following the consumption of ^{13}C SBE-deficient peas were significantly reduced, which is in line with the results from Chapters 3 and 4. Meanwhile, fractional ^{13}C SCFA enrichment in 24-hour urine (a marker of ^{13}C pea fermentation) was higher in response to SBE-deficient peas when compared with wild peas. These data support the limited digestibility of SBE-deficient peas and are in line with the features illustrated previously in Figure 4.18 of Chapter 4. In contrast, while the same analysis showed no differences in plasma ^{13}C glucose levels between pea flours of both pea lines, fractional ^{13}C SCFA enrichment in 24-hour urine revealed higher ^{13}C SCFA excretion in response to SBE-deficient flour consumption when compared to wild flour

consumption. These findings suggest that, although milling increases the bioavailability of starch to digestion and absorption (as was seen in the plasma ^{13}C glucose results of pea flours), both SBE-deficient peas and flour retained a considerable amount of their RS and were available for fermentation in the lower gut, and thus they both serve as ideal candidates for microbiota fermentation. Future work should investigate whether delivering saccharolytic materials, such as in peas, to the lower gut is associated with positive metabolic effects with long-term supplementation and exposure to microbial activity (e.g. microbes that are associated with glucose homeostasis, such as bifidobacterium).

Despite the significant levels of SCFAs produced in response to mutant pea seed consumption, there were comparable and/or inconsistent results seen in VAS and gut hormones in Chapters 3, 4 and 5, which do not support a role of SCFAs in regulating appetite. However, it must be noted that this might be due to the small production level of SCFAs and the short supplementation period of peas. It may be that long-term exposure is required for the changes in gut hormones and subjective VAS to appear, or there might have been an appetite response independent of gut hormones and VAS. Future work should look at the effect of long-term pea supplementation from both lines on appetite response and examine the effect on energy intake, gut hormone secretion, and subjective appetite as well as whether these effects are related to the systematic circulation via the vagus nerves, or hedonic nervous system, thus methods as nerve denervation and visualise brain areas activation using fMRI should be considered in future studies.

Secondary comparisons of findings looking at the effect of cellular structure in peas:

The comparisons considering physical alteration were included to examine the effect of processing as a secondary outcome on starch digestibility and subsequent metabolic outcomes. It was shown that wild and SBE-deficient whole pea seeds had lower postprandial glucose and insulin responses when compared with their flour counterparts. These results were independent of delaying GE. Moreover, SBE-deficient peas showed no differences in small intestinal glucose concentrations when compared with SBE-deficient flour. In addition, ^{13}C glucose was in line with these observations, and SBE-deficient peas resulted in lower ^{13}C glucose concentrations and later

peaks than SBE-deficient flour. However, ^{13}C SCFAs excreted in 24-hour urine showed no differences between SBE-deficient peas and flour. Together, these data suggest that milling and processing can affect the starch quality and enhance its digestion in the upper GI to some extent. The data also suggest similar fermentation activity after escaping upper GI digestion. Again, there were comparable and/or inconsistent results seen for VAS and gut hormones in Chapters 3, 4 and 5, which do not support a role for SBE-deficient pea seeds and flour in regulating appetite.

Collectively, the findings from the pea studies showed that starch quality can be manipulated to affect its digestibility in the human GI. Therefore, as most of the energy delivered from peas is a result of starch digestion, the potential 'available' energy present in the mutant peas will not always be the actual metabolisable energy to be absorbed. This is an important concept to consider when establishing nutritional regulations for managing weight and appetite, and it highlights the major limitation of the current nutrition labelling system, which lacks a specification regarding food structure. Nevertheless, the results from the pea studies could also be used and replicated with other legume and/or plant crops. Peas have been used in the current studies only as a vehicle to translate the mutation and the subsequent effects, and the same mutation could be applied to other crops to see if the findings are similar.

The discovery of the co-expression of FFAR2 and FFAR3 on L cells led to the hypothesis that SCFAs might reduce appetite through the stimulation of anorexigenic gut hormones (Karaki *et al.*, 2008; Tazoe *et al.*, 2008). Despite the increased SCFA production in response to SBE-deficient peas, no clear differences in appetite response were observed. This might be due to the acute setting of trials involving peas and/or the insufficient amount of SCFAs produced to exert profound effects on appetite. Meanwhile, propionate has previously been shown to acutely stimulate the secretion of GLP-1 and PYY in both human (Chambers *et al.*, 2014) and murine colonic crypt cultures (Psichas *et al.*, 2015). Since L cells are most abundant in the colon, it is likely that IPE would stimulate a greater anorexigenic gut hormone response as a result of selectively delivering propionate to the colon. Previous work from our lab has demonstrated that enriching the colon with propionate reduced appetite in humans. This has been translated into a significant reduction in both body weight gain and the development of abdominal adipose tissue following habitual

supplementation of IPE for 24 weeks (Chambers *et al.*, 2014). The findings from the pilot study in Chapter 6 suggest that IPE was able to significantly decrease subjective appetite response, represented as VAS, while maintaining a lower energy diet when compared to baseline. However, there were no differences in GLP-1 or PYY responses within or between groups. This finding is in line with the results reported by Chambers *et al.*, who found no differences in gut hormones over a 24-week supplementation period (Chambers *et al.*, 2014). This might be explained by the desensitisation of FFAR responses to SCFAs over time, and the long-term beneficial effects of IPE supplementation may not be mediated by GLP-1 and PYY. One proposed mechanism of action is triggering of the hedonic appetite system, which is related to eating behaviour. Work from the Frost group demonstrated that acute IPE supplementation reduced the activation of brain reward circuits (i.e. caudate and nucleus accumbens) as assessed by fMRI. Interestingly, this was accompanied by a reduction in ad libitum energy intake in the same study (Byrne *et al.*, 2016), but it was independent to changes in PYY or GLP-1 gut hormone levels. It is also plausible to speculate that elevated colonic propionate levels might alter hepatic metabolism and thus induce appetite changes systematically via the induction of vagal signalling in the gut or portal vein through stimulation of FFAR3, which could elevate energy expenditure (Lal *et al.*, 2001; Kimura *et al.*, 2013; De Vadder *et al.*, 2014; Kentish and Page, 2015). However, the exact underlying mechanism remains to be elucidated.

Conventional weight loss plans are effective, but maintaining the weight loss is challenging (Hall and Kahan, 2018). Weight regain is a common occurrence, and it is attributed to the decreased gut hormone levels throughout the weight loss period, which result in an increased appetite as part of the physiological adaptation to lower energy intake (Sumithran *et al.*, 2011; Sumithran and Proietto, 2013). In Chapter 6, weight loss was significant in the IPE group when compared to baseline. This is a surprising result to see after 12 weeks of following a lower energy diet, particularly as it coincided with a lower subjective appetite response and no changes in gut hormones. Being able to lose weight while not only maintaining appetite hormones but also decreasing subjective appetite is a promising result for follow-up. However, the differences in weight loss between the groups in the current study were not significant. This might be due to

the low number of participants. Based on the results of the current pilot study, propionate-ester caused a composite appetite score mean loss of 9.8 ± 5.5 mm, while inulin supplements caused a composite appetite score mean loss of 5.1 ± 11.9 mm. A power calculation shows that 63 volunteers will be needed per group to detect a significant difference between groups ($p=0.05$, 0.80 power). Thus, 158 volunteers should be recruited in future work to allow a 25% drop-out rate (i.e. 79 participants per group).

7.4 Current limitations and Future recommendations

The research limitations and future considerations are listed below:

- With regard to the pea studies, appetite responses were not measured properly (e.g. no energy intake quantification was done). It would be interesting to investigate the effect of pea supplementation on appetite in future work. In such work, peas should be incorporated into the habitual diet long enough to examine the changes, if any, in gut microbial activity, appetite response represented by VAS, gut hormones, energy intake quantification (i.e. ad libitum meal), and weight.
- In the context of the current studies, it would have been beneficial if a proper control had been added as an extra intervention to the peas studies, to address the effect of available starch on digestion independent of both pea lines. This control group could have received the same content of the test meal except for the pea intervention.
- With regard to the secondary comparisons of peas and flour in Chapters 3 and 5, they were cooked in different settings (whole pea seeds required around an hour of boiling while flour only required 2 minutes of microwaving). Although each structure was found to be well cooked with these durations, it is well known that different cooking methods can greatly affect starch characteristics and thus digestibility. Future work should consider a standardised cooking protocol to confirm the changes observed.
- With regard to Chapter 6, future work is needed to verify these promising preliminary effects in a properly powered study. Moreover, as the exact underlying mechanism through which propionate suppresses appetite remains to be elucidated, further work is needed to understand how propionate suppresses appetite, particularly to gain insights on gut–liver–brain signalling. It would be ideal to collect samples from the portal vein as well as venous samples following the consumption of IPE to gain insight into the metabolic and hormonal signals being produced. However, portal sampling is highly invasive and thus cannot be carried out in human research studies. Additionally, the role of hepatic metabolism and vagal signalling in mediating SCFA appetite-suppressing effects should be investigated further. The recruitment of patients who have undergone vagotomies would

enable investigation of the role of vagal signalling for the first time in humans in this context. In addition, it would be interesting to investigate whether increased colonic propionate enrichment could reduce long-term energy intake and maintain body weight or the weight lost while following weight loss diet. It is recommended that IPE supplementation be started after the weight loss has taken place to confirm its ability to help in keeping the weight off while maintaining and/or suppressing appetite.

7.5 Conclusion

In summary, the data presented in this thesis support the hypothesis that manipulating a food's structure physically or chemically can significantly affect starch digestibility, energy homeostasis, and microbiota fermentation. SBE-deficient peas and flour both had positive effects on glucose response independent of GE. These beneficial effects on glycaemia were further observed as lower breakdown in the small intestine, an important part of the GI tract in nutrient digestion. The lower digestion rate implies less susceptibility to hydrolysis compared to the vulnerable wild pea seeds, which was later identified as a greater release of SCFAs in response to resistant structure fermentation. Further work is required to understand whether long-term supplementation of SBE-deficient peas can suppress appetite through the fermentation process. In addition, the findings of this thesis support the hypothesis that the SCFA propionate plays a role in appetite regulation in humans. However, the lack of mechanistic insight into the factors positively driving the effects of SCFAs on appetite reduction prevents solid conclusions.

8. References

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9. Appendices

Appendix 1: Information Sheet for Research Participants

Dietary resistant starch from peas for healthy glucose homeostasis

STUDY 1 - Evaluating the Impact of Resistant Starch from Peas on Gastric Emptying

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

You are being invited to take part in a research study investigating the effect of dietary fibre on the diet. Before you decide if you would like to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation.

Thank you for reading this

What is the purpose of this study?

Diabetes mellitus type 2 is a disease that is caused by insulin resistance, whereby the body fails to respond or produce insulin, a hormone that regulates carbohydrate metabolism. Without regulated carbohydrate metabolism glucose remains within the bloodstream; having disadvantageous effects. Insulin is secreted by β -cells that are found within a specific region of the pancreas, known as the Islet of Langerhans. These β -cells can deteriorate and fail to release insulin due to lifestyle factors such as age and obesity, as well as genetic factors.

Resistant starches are found within certain food products, particularly fruits, vegetables and whole grains, and are believed to be beneficial to β -cells. This is because the resistant starch is not digested within the gut and is instead used by bacteria within the gut. The bacteria ferment the resistant starch to produce short chain fatty acids (SCFAs), which are believed to improve β -cell function.

We are investigating the effects of different food products containing resistant starches found naturally in peas. The aim of this study is to see if resistant starch improves glucose metabolism by slowing how quickly ingested food moves through the stomach and gastrointestinal (GI) tract.

This study was reviewed and received favourable opinion by South East-Coast Surrey Research Ethics Committee.

Who is suitable to participate?

- Male and female healthy volunteers (aged 18 to 65 years)
- Normal to overweight individuals (body mass index (BMI) 20-35 kg/m²)
 - BMI is equal to body weight (kg) divided by height squared (m²)

You are NOT suitable to participate if you have:

- Gained or lost ≥ 3 kg weight in the past two months
- Current smokers
- History of substance abuse
- Excessive alcohol intake
- Pregnancy
- Diabetes
- Cardiovascular disease
- Cancer
- Any gastrointestinal disease e.g. irritable bowel syndrome or inflammatory bowel disease
- Kidney disease
- Liver disease
- Pancreatitis
- Use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance, including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones.

Do I have to take part?

It is entirely up to you whether or not to take part. If you do we will ask you to sign a consent form. You are free to withdraw at any time and you do not have to give a reason. A decision either not to take part or to withdraw from the study will not affect the standard of care you receive.

Will I get paid for participating?

You will be reimbursed £200 for completing the study.

What will I have to do?

Visit 1 – Health Screening:

If you do decide to participate, you will initially be invited to attend a health screening to confirm your eligibility for the study. You will be asked to attend the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital where you will be interviewed and examined by one of the researchers. You will have a blood test (to check your liver, kidneys, cholesterol and ensure that you are not anaemic or diabetic) and height, weight and blood pressure measurements will also be taken. You will also have an electrocardiogram (ECG). This is a non-invasive test to look at the health of your heart. All women of child bearing age will have a pregnancy test.

The health screening should last about 1 hour.

Study Days 1, 2, 3 and 4:

On the day before the study we will ask you to complete a 24 hour food diary in which you write down all the food and drink that you have consumed during those 24 hours. You will also be asked to refrain from strenuous exercise, caffeine and alcohol. You will then be requested to fast overnight (you are allowed to drink water).

On the study day, you will be asked to arrive at the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital at approximately 9:00am. A small plastic tube (cannula) will be inserted into your arm. This will be in place for the duration of the study day and will be used to take blood samples without causing you any further discomfort.

At approximately 10:00am you will consume a standard breakfast that will contain different resistant starches on each study visit. The breakfast will also contain a very small amount of ¹³Carbon. The ¹³Carbon breath test is a commonly used research tool for measuring how quickly ingested food moves through the stomach. By measuring the level of ¹³Carbon that appears in your breath we can work out how quickly your stomach empties food after eating. For 5 hours after eating the breakfast you will be asked every 15 minutes to blow through a straw into a glass tube to collect breath samples. You will also be asked to breathe into a breath analyser every hour after consuming the breakfast so that we can measure your breath hydrogen levels. This will provide us with another measurement of how quickly the different resistant starches eaten at breakfast have moved through the stomach.

During the study you will have blood samples taken at certain time points. A total of 100ml of blood will be taken (about 20 teaspoons of blood). These will be taken through the plastic cannula.

During the study you will be asked to fill in questionnaires (visual analogue scales) to measure your feelings of hunger and nausea.

We will also ask you to collect urine each time you go to the toilet during the study visit. You will be provided with an appropriate measurement container to collect urine and will be asked to note the total volume following each collection.

Measurements will be taken for 5 hours after you eat the breakfast. At the end of the study, at approximately 3:00pm, we will remove the cannula and you will be able to go home. During the course of the study day you will be able to read or watch DVDs if you wish.

We request that you do not start any other new diets or intensive exercise regimes in-between the four study visits as this may give us conflicting results. There would also need to be a period of at least 7 days between study visits.

What are the possible disadvantages and risks of taking part?

In the event that we discover something about your health that you were unaware of, for example if your kidney tests are abnormal or if you have diabetes, we would immediately inform you of this and with your consent inform your GP so that you can be referred to an appropriate specialist. If you require more urgent assessment we would arrange this for you immediately within the hospital.

Some of the procedures in this study, such as the recording of your weight, height and blood pressure present no risk to you. Other procedures, such as taking blood samples, can cause mild discomfort. The risks of taking a blood sample include: slight discomfort when the needle is inserted and possible bruising and a localised infection. These procedures will only be carried out by experienced doctors under aseptic conditions to minimise all these risks.

There are no major side effects associated with eating foods containing resistant starch; however, some people may experience mild abdominal bloating.

What happens when the research study stops?

Once the study has finished, the results of the study can be made available to you and/or your GP if you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided.

What if new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time and you do not need to give a reason. Any stored blood or urine samples that can still be identified as yours will be destroyed.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Professor Gary Frost; g.frost@imperial.ac.uk; 020 8383 3242). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

What can I do if I have any complaints or concerns?

If you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Principal Investigator, Professor Gary Frost, through his secretary on 020 8383 3242 or by email at g.frost@imperial.ac.uk

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

All electronic data about you will be stored on Imperial College departmental database. This is a confidential computer system which requires a specific password for access and can only be viewed by authorised persons. It is a requirement that your GP is informed of your participation in this study.

What will happen to the results of the research study?

The results are likely to be published six months following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP.

Contact for Further Information

The researchers and doctors involved in the study, Professor Gary Frost and Dr Ed Chambers, will be available by telephone

During working hours through Professor Gary Frost's secretary	020 8383 3242
At all other times through Hammersmith Hospital switchboard	020 8383 1000

Appendix 2: Dietary resistant starch from peas for healthy glucose homeostasis

STUDY 1: Evaluating the impact of resistant starch from peas on gastric emptying

Please initial the box if you agree with each statement.

1. I have been given the opportunity to ask questions and discuss the study ☐
2. I have received satisfactory answers to all my questions ☐
3. I have received enough information about the study ☐
4. I confirm that I have read and understand the information sheet :
*"Evaluating the impact of resistant starch from peas on gastric emptying–
Version ____Date_____ for the above study.* ☐
5. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
6. I understand that sections of any of my research notes may be looked at by responsible individuals from Imperial College, Imperial College NHS Healthcare Trust, and regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
7. I agree that that my Identifiable information can be stored on Imperial College London computer systems ☐
8. I agree to have my GP informed about participating in this study. ☐
9. I agree to have my blood taken. ☐
10. I agree to have my collected tissue samples stored and used in future ethically approved studies. ☐
11. I agree to have my collected samples analysed at the laboratories of the Department of Investigative Medicine, Hammersmith Hospital and the Institute of Food Research (IFR), Colney, Norwich. ☐
12. I agree to take part in this study. ☐

Name of Patient

Date

Signature

Name of Researcher

Date

Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 3: Information Sheet for Research Participants
Dietary resistant starch from peas for healthy glucose homeostasis
STUDY 2 - Evaluating the Impact of Resistant Starch from Peas on
Small Intestinal Digestion

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

You are being invited to take part in a research study investigating the effect of dietary fibre on the diet. Before you decide if you would like to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation.

Thank you for reading this

What is the purpose of this study?

Diabetes mellitus type 2 is a disease that is caused by insulin resistance, whereby the body fails to respond or produce insulin, a hormone that regulates carbohydrate metabolism. Without regulated carbohydrate metabolism glucose remains within the bloodstream; having disadvantageous effects. Insulin is secreted by β -cells that are found within a specific region of the pancreas, known as the Islet of Langerhans. These β -cells can deteriorate and fail to release insulin due to lifestyle factors such as age and obesity, as well as genetic factors.

Resistant starches are found within certain food products, particularly fruits, vegetables and whole grains, and are believed to be beneficial to β -cells. This is because the resistant starch is not digested within the gut and is instead used by bacteria within the gut. The bacteria ferment the resistant starch to produce short chain fatty acids (SCFAs), which are believed to improve β -cell function.

We are investigating the effects of different food products containing resistant starches found naturally in peas. The aim of this study is to see how resistant starches influence the digestion of food in the stomach and gastrointestinal (GI) tract.

This study was reviewed and received favourable opinion by South East-Coast Surrey Research Ethics Committee.

Who is suitable to participate?

- Male and female healthy volunteers (aged 18 to 65 years)
- Normal to overweight individuals (body mass index (BMI) 20-35 kg/m²)
 - BMI is equal to body weight (kg) divided by height squared (m²)

You are NOT suitable to participate if you have:

- Gained or lost ≥ 3 kg weight in the past two months
- Current smokers
- History of substance abuse
- Excessive alcohol intake
- Pregnancy
- Diabetes
- Cardiovascular disease
- Cancer
- Any gastrointestinal disease e.g. irritable bowel syndrome or inflammatory bowel disease
- Kidney disease
- Liver disease
- Pancreatitis
- Use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance, including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones.

Do I have to take part?

It is entirely up to you whether or not to take part. If you do we will ask you to sign a consent form. You are free to withdraw at any time and you do not have to give a reason. A decision either not to take part or to withdraw from the study will not affect the standard of care you receive.

Will I get paid for participating?

You will be reimbursed £400 for completing the study.

What will I have to do?

Visit 1 – Health Screening:

If you do decide to participate, you will initially be invited to attend a health screening to confirm your eligibility for the study. You will be asked to attend the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital where you will be interviewed and examined by one of the researchers. You will have a blood test (to check your liver, kidneys, cholesterol and ensure that you are not anaemic or diabetic) and height, weight and blood pressure measurements will also be taken. You will also have an electrocardiogram (ECG). This is a non-invasive test to look at the health of your heart. All women of child bearing age will have a pregnancy test.

The health screening should last about 1 hour.

Visit 2 – Study Visit

On the day before the study visit we will ask you to refrain from strenuous exercise, caffeine and alcohol. You will then be requested to fast overnight from 10 pm (you are allowed to drink water).

On the study day, you will be asked to arrive at the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital at approximately 8:00am. You will stay at the Clinical Research Facility for a 4 day visit (three nights).

A small plastic tube (cannula) will be inserted into your arm. This will be in place for the duration of the study visit and will be used to take blood samples without causing you any further discomfort.

Two thin, bendy plastic tubes will be placed through your nose and into your stomach and small intestine. The tubes will be placed by a Gastroenterologist. They will put some lubricant on the end of each tube to make it slippery and place them through your nose and down into your stomach and small intestine. You will be sat upright as the tubes are placed and asked to drink water through a straw as this will help the tubes to go down. It will feel a bit uncomfortable whilst the tubes are being put in, however, any discomfort should subside once the tubes have been positioned. These tubes will allow us to take samples from your stomach and small intestines after you have eaten to see how different foods are digested. These tubes will remain in place for the duration of the 4 day visit.

On each of the 4 study mornings you will consume a standard breakfast that will contain different resistant starches each morning. Blood samples will be taken at certain time points for 3 hours after you eat the breakfast. A total of 80ml of blood will be taken each morning (about 16 teaspoons of blood). These will be taken through the plastic cannula. Following each test breakfast, samples from your stomach and small intestines will be taken every 15 minutes for 3 hours.

Following the 3 hour measurement period, you will be able to read or watch DVDs at the Clinical Research Facility. We will provide all of the food you require for the duration of the 4 day study visit. You will be requested to fast every evening of the visit from 10 pm (you are allowed to drink water).

Following the final samples on the fourth study day, the tubes in your stomach and small intestines and the plastic cannula will be removed and you will be able to go home.

What are the possible disadvantages and risks of taking part?

In the event that we discover something about your health that you were unaware of, for example if your kidney tests are abnormal or if you have diabetes, we would immediately inform you of this and with your consent inform your GP so that you can be referred to an appropriate specialist. If you require more urgent assessment we would arrange this for you immediately within the hospital.

Some of the procedures in this study, such as the recording of your weight, height and blood pressure present no risk to you. Other procedures, such as taking blood samples, can cause mild discomfort. The risks of taking a blood sample include: slight discomfort when the needle is inserted and possible bruising and a localised infection. These procedures will only be carried out by experienced doctors under aseptic conditions to minimise all these risks.

The potential risks of the stomach and small intestines tubes include blockage of a tube, a misplaced tube or infection. This procedure will only be undertaken by a Gastroenterologist and care will be taken to minimise any discomfort from the procedure. The tubes will be placed using a system that provides 'real-time' information about the position of the tube during placement to minimise discomfort and all of these risks.

There are no major side effects associated with eating foods containing resistant starch; however, some people may experience mild abdominal bloating.

What happens when the research study stops?

Once the study has finished, the results of the study can be made available to you and/or your GP if you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided.

What if new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?

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If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Professor Gary Frost; g.frost@imperial.ac.uk; 020 8383 3242). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

What can I do if I have any complaints or concerns?

If you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Principal Investigator, Professor Gary Frost, through his secretary on 020 8383 3242 or by email at g.frost@imperial.ac.uk

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

All electronic data about you will be stored on Imperial College departmental database. This is a confidential computer system which requires a specific password for access and can only be viewed by authorised persons. It is a requirement that your GP is informed of your participation in this study.

What will happen to the results of the research study?

The results are likely to be published six months following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP.

Contact for Further Information

The researchers and doctors involved in the study, Professor Gary Frost and Dr Ed Chambers, will be available by telephone

During working hours through Professor Gary Frost's secretary	020 8383 3242
At all other times through Hammersmith Hospital switchboard	020 8383 1000

Appendix 4: Dietary resistant starch from peas for healthy glucose homeostasis
STUDY 2: Evaluating the impact of resistant starch from peas on small intestinal digestion

Please initial the box if you agree with each statement.

- | | | |
|-----|---|--------------------------|
| 7. | I have been given the opportunity to ask questions and discuss the study | ☐ |
| 8. | I have received satisfactory answers to all my questions | ☐ |
| 9. | I have received enough information about the study | ☐ |
| 10. | I confirm that I have read and understand the information sheet:
<i>"Evaluating the impact of resistant starch from peas on small intestinal digestion– Version 2 – 250215"</i> for the above study. | ☐ |
| 11. | I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. | ☐ |
| 12. | I understand that sections of any of my research notes may be looked at by responsible individuals from Imperial College, Imperial College NHS Healthcare Trust, and regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. | ☐ |
| 7. | I agree that that my Identifiable information can be stored on Imperial College London computer systems | ☐ |
| 13. | I agree to have my GP informed about participating in this study. | ☐ |
| 14. | I agree to have my blood taken. | ☐ |
| 15. | I agree to have my collected tissue samples stored and used in future ethically approved studies. | ☐ |
| 16. | I agree to take part in this study. | ☐ |

Name of Patient

Date

Signature

Name of Researcher

Date

Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 5: Information Sheet for Research Participants
Dietary resistant starch from peas for healthy glucose homeostasis
STUDY 3 - Evaluating the Impact of Resistant Starch from Peas on
Starch Delivery to the Colon

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

You are being invited to take part in a research study investigating the effect of dietary fibre on the diet. Before you decide if you would like to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation.

Thank you for reading this

What is the purpose of this study?

Diabetes mellitus type 2 is a disease that is caused by insulin resistance, whereby the body fails to respond or produce insulin, a hormone that regulates carbohydrate metabolism. Without regulated carbohydrate metabolism glucose remains within the bloodstream; having disadvantageous effects. Insulin is secreted by β -cells that are found within a specific region of the pancreas, known as the Islet of Langerhans. These β -cells can deteriorate and fail to release insulin due to lifestyle factors such as age and obesity, as well as genetic factors.

Resistant starches are found within certain food products, particularly fruits, vegetables and whole grains, and are believed to be beneficial to β -cells. This is because the resistant starch is not digested within the gut and is instead used by bacteria within the gut. The bacteria ferment the resistant starch to produce short chain fatty acids (SCFAs), which are believed to improve β -cell function.

We are investigating the effects of different food products containing resistant starches found naturally in peas. The aim of this study is to see how resistant starch fermentation on the colon influences metabolic pathways involved in the production of energy for the body.

This study was reviewed and received favourable opinion by South East-Coast Surrey Research Ethics Committee.

Who is suitable to participate?

- Male and female healthy volunteers (aged 18 to 65 years)
- Normal to overweight individuals (body mass index (BMI) 20-35 kg/m²)
 - BMI is equal to body weight (kg) divided by height squared (m²)

You are NOT suitable to participate if you have:

- Gained or lost ≥ 3 kg weight in the past two months
- Current smokers
- History of substance abuse
- Excessive alcohol intake
- Pregnancy
- Diabetes
- Cardiovascular disease
- Cancer
- Any gastrointestinal disease e.g. irritable bowel syndrome or inflammatory bowel disease
- Kidney disease
- Liver disease
- Pancreatitis
- Use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance, including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones.

Do I have to take part?

It is entirely up to you whether or not to take part. If you do we will ask you to sign a consent form. You are free to withdraw at any time and you do not have to give a reason. A decision either not to take part or to withdraw from the study will not affect the standard of care you receive.

Will I get paid for participating?

You will be reimbursed £200 for completing the study.

What will I have to do?

Visit 1 – Health Screening:

If you do decide to participate, you will initially be invited to attend a health screening to confirm your eligibility for the study. You will be asked to attend the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital where you will be interviewed and examined by one of the researchers. You will have a blood test (to check your liver, kidneys, cholesterol and ensure that you are not anaemic or diabetic) and height, weight and blood pressure measurements will also be taken. You will also have an electrocardiogram (ECG). This is a non-invasive test to look at the health of your heart. All women of child bearing age will have a pregnancy test.

The health screening should last about 1 hour.

Study Days 1, 2, 3 and 4:

On the day before the study we will ask you to complete a 24 hour food diary in which you write down all the food and drink that you have consumed during those 24 hours. You will also be asked to refrain from strenuous exercise, caffeine and alcohol. You will then be requested to fast overnight (you are allowed to drink water).

On the study day, you will be asked to arrive at the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital at approximately 9:00am. A small plastic tube (cannula) will be inserted into your arm. This will be in place for the duration of the study day and will be used to take blood samples without causing you any further discomfort.

At approximately 10:00am you will consume a standard breakfast that will contain different resistant starches on each study visit. The resistant starch will contain a small amount of ^{13}C Carbon. The ^{13}C Carbon breath test is a commonly used research tool for measuring how quickly ingested food moves through the stomach. By measuring the level of ^{13}C Carbon that appears in your breath we can work out how quickly your stomach empties food after eating. In order to track the digestive process at 40 min and 180 min you will have to consume two capsules which will contain the non-radioactive tracer molecules $^2\text{H}_2$ -Glucose and $^2\text{H}_3$ Acetate. For 6 hours after eating the breakfast you will be asked every 15 minutes to blow through a straw into a glass tube to collect breath samples.

You will also be asked to breathe into a breath analyser every hour after consuming the breakfast so that we can measure your breath hydrogen levels. This will provide us with another measurement of how quickly the different resistant starches eaten at breakfast have moved through the stomach.

During the study you will have blood samples taken at certain time points. A total of 110ml of blood will be taken (about 22 teaspoons of blood). These will be taken through the plastic cannula. You will also be asked to breathe into a breath analyser every hour so that we can measure your breath hydrogen levels.

Measurements will be taken for 6 hours after you eat the breakfast. At the end of the study, at approximately 4:00pm, we will remove the cannula and you will be able to go home. During the course of the study day you will be able to read or watch DVDs if you wish.

You will be given a rack of labelled test tubes to take home with you to continue to collect breath samples every hour until you go to bed. You will also be asked to collect breath samples when you wake the following morning. You will be asked to store these samples at home and bring them with you the next morning.

On the study day, you will also be asked to collect a 24 hour urine sample and a stool sample. We will explain to you in detail how to do this and you will be given an appropriate container.

We request that you do not start any other new diets or intensive exercise regimes in-between the four study visits as this may give us conflicting results. There would also need to be a period of at least 7 days between study visits.

What are the possible disadvantages and risks of taking part?

In the event that we discover something about your health that you were unaware of, for example if your kidney tests are abnormal or if you have diabetes, we would immediately inform you of this and with your consent inform your GP so that you can be referred to an appropriate specialist. If you require more urgent assessment we would arrange this for you immediately within the hospital.

Some of the procedures in this study, such as the recording of your weight, height and blood pressure present no risk to you. Other procedures, such as taking blood samples, can cause mild discomfort. The risks of taking a blood sample include: slight discomfort when the needle is inserted and possible bruising and a localised infection. These procedures will only be carried out by experienced doctors under aseptic conditions to minimise all these risks.

There are no major side effects associated with eating foods containing resistant starch; however, some people may experience mild abdominal bloating.

What happens when the research study stops?

Once the study has finished, the results of the study can be made available to you and/or your GP if you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided.

What if new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time and you do not need to give a reason. Any stored blood or urine samples that can still be identified as yours will be destroyed.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Professor Gary Frost; g.frost@imperial.ac.uk; 020 8383 3242). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

What can I do if I have any complaints or concerns?

If you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Principal Investigator, Professor Gary Frost, through his secretary on 020 8383 3242 or by email at g.frost@imperial.ac.uk

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

All electronic data about you will be stored on Imperial College departmental database. This is a confidential computer system which requires a specific password for access and can only be viewed by authorised persons. It is a requirement that your GP is informed of your participation in this study.

What will happen to the results of the research study?

The results are likely to be published six months following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP.

Contact for Further Information

The researchers and doctors involved in the study, Professor Gary Frost and Dr Ed Chambers, will be available by telephone

During working hours through Professor Gary Frost's secretary	020 8383 3242
At all other times through Hammersmith Hospital switchboard	020 8383 1000

Appendix 6: Dietary resistant starch from peas for healthy glucose homeostasis
STUDY 3: Evaluating the impact of resistant starch from peas on starch delivery to the colon

Please initial the box if you agree with each statement.

- | | | |
|-----|---|--------------------------|
| 13. | I have been given the opportunity to ask questions and discuss the study | ☐ |
| 14. | I have received satisfactory answers to all my questions | ☐ |
| 15. | I have received enough information about the study | ☐ |
| 16. | I confirm that I have read and understand the information sheet :
<i>"Evaluating the impact of resistant starch from peas on starch delivery to the colon– Version 3 – 290915"</i> for the above study. | ☐ |
| 17. | I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. | ☐ |
| 18. | I understand that sections of any of my research notes may be looked at by responsible individuals from Imperial College, Imperial College NHS Healthcare Trust, and regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. | ☐ |
| 7. | I agree that that my Identifiable information can be stored on Imperial College London computer systems | ☐ |
| 17. | I agree to have my GP informed about participating in this study. | ☐ |
| 18. | I agree to have my blood taken. | ☐ |
| 19. | I agree to have my collected tissue samples stored and used in future ethically approved studies. | ☐ |
| 20. | I agree to take part in this study. | ☐ |

Name of Patient

Date

Signature

Name of Researcher

Date

Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 7: Information Sheet for Research Participants

Colonic Propionate, Appetite and Weight Loss

Effect of increased colonic propionate on weight loss during a hypo-caloric diet.

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

You are being invited to take part in a research study investigating the role of a novel dietary fibre in the diet. Before you decide if you would like to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP if you wish. Part 1 tells you the purpose of the study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. You are free to withdraw at any time without explanation.

Thank you for reading this

Part 1

What is the purpose of this study?

High fibre diets are known to have health benefits and have been widely advertised as a method of helping people to lose weight. We are investigating one of the breakdown products of fibre, short chain fatty acids (SCFA), which are believed to be responsible for the beneficial effects of fibre. One of these SCFA, propionate, may have a role in reducing appetite leading to weight loss and a reduced risk of diabetes and cardiovascular disease.

It is not possible to eat propionate alone as it is broken down rapidly in the body. We are investigating a novel dietary supplement, propionate ester. This is propionate chemically bound to the natural dietary fibre inulin. Inulin is a fibre normally found in garlic, Jerusalem artichoke, chicory and onion. Inulin is a natural dietary fibre that has been used in many dietary studies without any significant side effects. The propionate ester has previously been tested in humans and presents no health risks.

This is a randomised controlled study to investigate the effects of propionate ester versus inulin supplemented with a low caloric diet on appetite and weight loss.

Who is suitable to participate?

- Any overweight male or female adults aged between 18 to 65 years old, whose their BMI is in the range of 25-35 kg/m² (BMI is equal to body weight (kg) divided by height squared (m²))

You are NOT suitable to participate if you have:

- Weight change of $\geq 3\text{kg}$ in the preceding 2 months
- Undergone any weight loss surgery or gastric procedures to promote weight loss
- Current smokers
- History of substance abuse
- Excess alcohol intake
- Pregnancy
- Diabetes
- Cardiovascular disease
- Cancer
- Gastrointestinal disease
- Kidney disease
- Liver disease
- Pancreatitis
- Use of medicines likely to interfere with energy metabolism, appetite regulation and hormonal balance, including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones.

It is entirely up to you whether or not to take part. If you do we will ask you to sign a consent form. You are free to withdraw at any time and you do not have to give a reason. A decision either not to take part or to withdraw from the study will not affect the standard of care you receive.

What will I have to do?

If you do decide to participate, the study will consist of 5 separate study visits. We request that you do not start any new diets or intensive exercise regimes in-between the study visits as this may give us conflicting results.

Visit 1- Health Screening

You will be asked to attend the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital where you will be interviewed and examined by one of the research doctors. You will have a blood test (to check your liver, kidneys, cholesterol and ensure that you are not anaemic or diabetic) and height and weight measurements and blood pressure will be taken. You will also have an electrocardiogram (ECG). This is a non-invasive test to look at the health of your heart. All women of child bearing age will have a pregnancy test. The health screening should last about 1 hour.

Visits 2: Acclimatisation Study Day (familiarization visit)

The main objective of this visit is to fully acclimatize you to the study before the baseline visit. This visit will take approximately 5 hours.

On the day before the study we will ask you to refrain from strenuous exercise, caffeine and alcohol. You will then be requested to fast overnight (you are allowed to drink water).

On the study day, you will be asked to arrive at the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital at approximately 8:00am. A small plastic tube (cannula) will be inserted into your arm. This will be in place for the duration of the study day and will be used to flush saline without causing you any further discomfort.

We will then measure your energy expenditure. This will involve a large, clear Perspex calorimeter canopy being placed over your head and upper body to measure your oxygen consumption for 30 min. We will demonstrate this to you during the initial health screening visit. The resulted number will be used to design a low calorie healthy diet to follow during the study.

You will be asked to complete a 7-day food intake diary in which you write down all the food and drink that you have consumed during the last 7 days. A dietitian will develop an individualised low caloric diet plan. The dietitian also will give you dietary instruction and will answer all your relevant questions.

At approximately 9:00am you will consume a standard breakfast. During the study you will have saline flushes at certain time points. These will be done through the plastic cannula.

During the study you will be asked to fill in questionnaires (visual analogue scales) to measure your feelings of hunger and nausea. You will also be asked to breathe into a breath analyser every hour so that we can measure your breath hydrogen levels. We will also ask you to collect urine each time you go to the toilet during the study visit. You will be provided with an appropriate measurement container to collect urine and will be asked to note the total volume following each collection.

At approximately 1:00pm you will be served a free buffet meal and asked to eat this until you are comfortably full. Once you have finished eating you will be able to go home. We will provide all of the food for the study day. During the course of the study day you will be able to read or watch DVDs if you wish.

Visit 3 – Study Day 1

Approximately 7 days after Visit 2 you will repeat the study day. This is to ensure that our measures of appetite are accurate and are not affected by the initial anxiety some participants experience when first starting a study. The only change will be that we take a total of 90ml (18 teaspoons of blood) instead of saline flushing during Visit 2. You will also be asked to complete a 24 hour food diary in which you write down all the food and drink that you have consumed during those 24 hours. This visit will take approximately 5 hours.

You will also be assigned to one of the randomisation dietary supplement groups (Propionate ester group or Inulin group) to ingest 10 g of either once a day for 84 days (12 weeks). Supplements will be given to you in ready-to-use sachets and we will ask you to mix the contents into your low calorie individual diet.

You will be asked to collect your urine throughout the study time. You will be provided with an appropriate measurement container to collect urine and will be asked to note the total volume following each collection.

You will be asked to collect a stool sample. You will collect a sample of the first stool you pass after 10:00 pm on the study day. You will be provided with detailed instructions of how to do this and will be given appropriate containers.

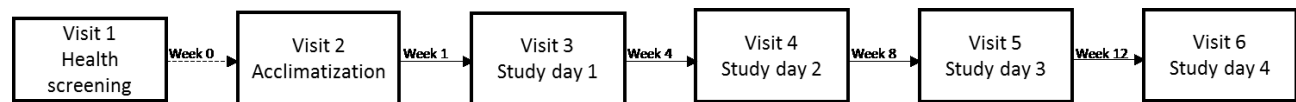
Visits 4 and 5 – Study Days 2 and 3

Short visits would take place on week 4 and week 8 to assess weight and dietary compliance to the diet. Your weight and height will be measured and a 7-day food intake diary will be collected.

Visit 6 – Study Day 4

At the end of 84 day supplementation period, you will attend the NIHR/Wellcome Trust Imperial Clinical Research Facility at Hammersmith Hospital and all measurements collected during Study Day 1 will be repeated.

The timings of the 6 study visits are summarised below:



Will I get paid for participating?

You will be reimbursed for any inconvenience caused due to the study. You will receive £125 for completing the entire study. You will receive £25 following each of the study days (Visits 2-6). Additionally, the cost of local public transport to and from the CRF will be reimbursed up to a maximum of £25 per day (proof of purchase is required) for study days only

What are the possible advantages and disadvantages and risks of taking part?

Throughout the intervention period you will be provided with a personalised dietary plan designed to lose 5% body weight over the 12 weeks. We cannot guarantee that you will lose weight by participating in the study, as this will depend on your compliance to the personalised dietary plan we will provide.

In the event that we discover something about your health that you were unaware of, for example if your kidney tests are abnormal or if you have diabetes, we would immediately inform you of this and inform your GP so that you can be referred to an appropriate specialist. If you require more urgent assessment we would arrange this for you immediately within the hospital.

Some of the procedures in this study, such as the recording of your weight, height and blood pressure present no risk to you. Other procedures, such as taking blood samples, can cause mild discomfort. The risks of taking a blood sample include: slight discomfort when the needle is inserted and possible bruising and a localised infection. These procedures will only be carried out by experienced doctors under aseptic conditions to minimise all these risks.

There are no major side effects associated with taking the propionate ester or inulin food products. Propionate ester and inulin have previously been tested in human volunteers. Some participants may find the taste unpleasant or may experience mild nausea or abdominal bloating initially, however, this should settle down after a few days.

What happens when the research study stops?

Once the study has finished, the results of the study can be made available to you and/or your GP. Your personal data will be retained for 10 years following completion of the study. There will be no continued provision for the dietary intervention following completion of the study, however you will be provided with an information pack explaining simple lifestyle changes that can promote health benefits. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided in Part 2.

What if there is a problem

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

This completes part 1.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

Part 2

What if new information becomes available?

Sometimes during the course of a research study, new information becomes available about the treatment that is being studied. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time and you do not need to give a reason. Any stored blood or urine samples that can still be identified as yours will be destroyed.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigator (Professor Gary Frost; g.frost@imperial.ac.uk; 020 8383 3242). The normal National Health Service complaint mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office.

What can I do if I have any complaints or concerns?

If you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Principal Investigator, Professor Gary Frost, through his secretary on 020 8383 3242 or by email at g.frost@imperial.ac.uk

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential. Any information about you that leaves the hospital will have your name and address removed so that you cannot be recognised from it.

Your collected samples during the participation time will be kept strictly confidential and used for future analysis and research.

All electronic data about you will be stored on Imperial College departmental database. This is a confidential computer system which requires a specific password for access and can only be viewed by authorised persons. It is a requirement that your GP is informed of your participation in this study.

What will happen to the results of the research study?

The results are likely to be published six months following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP.

Contact for Further Information

The researchers and doctors involved in the study, Professor Gary Frost and Dr. Ed Chambers, will be available by telephone

During working hours through Professor Gary Frost's secretary	020 8383 3242
At all other times through Hammersmith Hospital switchboard	020 8383 1000

Appendix 8: Colonic Propionate, Appetite, and Weight Loss

Effect of increased colonic propionate on weight loss during a hypo-caloric diet.

19. I have been given the opportunity to ask questions and discuss the study ☐
20. I have received satisfactory answers to all my questions ☐
21. I have received enough information about the study ☐
22. I confirm that I have read and understand the information sheet
 “Study: Effect of increased colonic propionate on weight loss during a hypo-caloric diet
 – Version 3– ” for the above study. ☐
23. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. Upon withdrawing, my data will be disposed of. ☐
24. I understand that sections of any of my research notes may be looked at by responsible individuals from Imperial College, Imperial College NHS Healthcare Trust, and regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
7. I agree that that my Identifiable information can be stored on Imperial College London computer systems ☐
21. I agree to have my GP informed about participating in this study. ☐
22. I agree to have my GP to be notified of test results. ☐
23. I agree to have my collected tissue samples stored and used in future ethically approved studies. ☐
24. I agree to take part in this study. ☐

Name of Patient

Date

Signature

Name of Researcher

Date

Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes