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Development of Functional Bread with Beta Glucan and Black Tea and Effects on Appetite Regulation, Glucose and Insulin Responses in Healthy Volunteers

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Author's declaration

I declare that the original work presented in this thesis is the work of the author Abbe Maleyki Mhd Jalil. I have been responsible for the organisation, recruitment, sample collection, laboratory work, statistical analysis and data processing of the whole research, unless otherwise stated.

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Dedication

I would like to dedicate my thesis to my parents (Hasnah, Norehan, Mhd Jalil and Mohd Nasser), my wife (Nadia Nazeerah Nasser) and my three little boys (Anas, Aqeel and Ammar). I was very lucky that they were able to be close to me and encourage my every step. I will be eternally grateful for their support, love and prayers.

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Abbreviations

AMG	Amyloglucosidase
BT	Black tea bread
βG	β-Glucan bread
βGBT	β-Glucan plus black tea bread
BMI	Body mass index
CCK-A	Cholecystokinin receptors A
CCK-B	Cholecystokinin receptors B
CHO	Carbohydrate
CG	Catechin gallate
CI	Confidence interval (95%)
CO_2	Carbon dioxide
CV	Coefficient of variation
EGCG	Epigallocatechin gallate
ECG	Epicatechin gallate
EC	Epicatechin
EE	Energy expenditure
ECG	Epicatechin gallate
EGCG	Epigallocatechin gallate
EFSA	European Food Safety Authority
Fe^{3+}	Ferric ion
Fe^{2+}	Ferrous ion
$FeCl_3$	Ferric chloride
$FeSO_4.7H_2O$	Ferric (II) sulfate heptahydrate
FV	Fruit and vegetable
GAE	Gallic acid equivalents
GPR41	G protein-coupled receptor 41
GPR43	G protein-couple receptor 43
GC-FID	Gas chromatography-fluorescence ionisation detector
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GTE	Green tea extract
GTP	Green tea (poly)phenols
GOPOD	Glucose oxidase/peroxidase reagent
GTE	Green tea extract
GT	Green tea
H_2	Hydrogen
HACS	High amylose corn starch
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HCO_3^-	Bicarbonate ion
HR	Hazard ratio
kcal	kilocalorie
kJ	kilojoule

KI	Potassium iodate
KOH	Potassium hydroxide
NaOH	Sodium hydroxide
Na_2CO_3	Sodium carbonate
NCD	Non-communicable disease
NPY	Neuropeptide Y
NSP	Non-starch polysaccharides
OFN	Oxygen-free nitrogen
OGTT	Oral glucose tolerance test
PAs	Proanthocyanidins
PYY	Peptide tyrosine tyrosine
PREDIMED	Prevention with Mediterranean diet study
RS	Resistant starch
RDS	Rapidly digestible starch
RR	Relative risk
SCFA	Short chain fatty acid
SGLT1	Sodium-dependent glucose transport
TAUC	Total area under the curve
TF	Theaflavins
TPL	Tea (poly)phenols
TPTZ	2, 4, 6- Tris(2-pyridyl)-s-triazine
TR	Thearubigins
T2DM	Type 2 diabetes mellitus
WB	White bread
WHO	World Health Organization
Y2R	Y2 receptor

Abstract

In the UK, dietary fibre intake is below the recommended level of 30 g/day. The manipulation of behavioural change is challenging, hence finding alternative ways to improve diet is important. The development of functional foods such as bread with added functional ingredients such as β-glucan and black tea may be more feasible and acceptable than changing to a new eating pattern. β-Glucan and black tea are often eaten separately, however there may be a food-matrix interaction between starch, protein (gluten), tea (poly)phenols and β-glucan when added together in a bread. We hypothesise that β-glucan and black tea will be digested slowly and display a blunted postprandial glycaemia. Some undigested residues will reach the colon, where it will be metabolised to short chain fatty acids (SCFA). SCFA, particularly propionate, have the potential to increase satiety by stimulating G protein receptors, however the effects on food intake need to be tested.

This project described: i) development of a functional bread containing black tea, BT; β-glucan, βG; β-Glucan and black tea, βGBT) and compare it to normal white bread (WB) (**study 1**); ii) determination of bread palatability, perceived satiety and subsequent energy intake following ingestion (**study 2**); iii) determination of postprandial glucose and insulin responses, and appetite hormones (CCK, PYY and GLP-1) among healthy volunteers (**study 3** – *in vivo* study).

In **study 1**, the breads were developed and tested for starch functionality, antioxidant potential and *in vitro* fermentability mimicking human colonic fermentation. βG and βGBT breads reduced early (10-min) *in vitro* starch hydrolysis and this could be due to action of β-glucan that 'protected' some of the starch granules (microscopic study) against amylolysis. Digestion with α-amylase increased antioxidant potential and total (poly)phenols content of BT and βGBT breads compared with WB. *In vitro* propionate concentration did not increase significantly when fermented with β-glucan. High inter-individual variation was observed for individual SCFA production. The addition of black tea had no apparent effect on SCFA production.

Study 2 is a randomised, crossover study design conducted in healthy volunteers. Breads

were given as breakfast and perceived satiety (perceived fullness, hunger, satiety, desire to eat and prospective food intake) was measured postprandially for 3 h. *Ad libitum* lunch was given after 3 h and energy intake estimated. BT bread was the most acceptable among all breads. βG and βGBT breads showed adverse taste, texture and palatability but showed similar overall acceptability as WB and BT breads. Female subjects showed lower preference for taste, texture and palatability of βG and βGBT compared with WB. βG and βGBT had positive effects on perceived satiety as follows: 1) decreased hunger; 2) increased fullness; and 3) decreased desire to eat. However, eating βG and βGBT at breakfast did not reduce energy intake at lunch compared with WB.

Study 3 was similar to study 2. Only βG bread showed significantly lower glucose $TAUC_{0\text{-}180\ min}$ compared with BT and βGBT but has no apparent effect on insulin response. No significant changes were observed for CCK and GLP-1 responses for all breads. However, βG and βGBT showed lower PYY $TAUC_{0\text{-}180\ min}$ compared with BT. *In vitro* starch hydrolysis did not correlate with *in vivo* postprandial glycaemic responses.

In conclusion, these studies suggest that breads with β-glucan and/or black tea have positive effects on perceived satiety *in vivo* and show good overall acceptability. However, there is no clear evidence that they affect appetite regulation. Breads containing 7 g β-glucan per 50 g of available carbohydrate reduced *in vivo* glucose response without altering insulin responses. There was no additional effect of adding black tea together with β-glucan to bread on the *in vivo* postprandial glycaemic response. It is too early to generalise the results from *in vitro* batch fermentation and starch hydrolysis and this needs to be considered when planning future dietary interventions looking at both *in vitro* and *in vivo* studies. Overall this study concluded that adding soluble dietary fibre to bread is feasible in controlling glycaemic responses and may help increase daily dietary fibre intake.

Chapter 1: Introduction

Once considered a 'disease' of the affluent society, overweight and obesity are now on the rise in low and middle-income countries, particularly in urban settings. In developing countries with emerging economies, the rate of increase in childhood overweight and obesity has been more than 30% higher than that of developed countries (WHO, 2015a). Obesity is a global problem, with more than 1.9 billion adults over the age of 18 years old being overweight and 600 million of these obese in 2014 (WHO, 2015b). Obesity is a major risk factor for developing chronic diseases such as cardiovascular diseases (heart disease and stroke), diabetes, musculoskeletal disorders (eg. osteoarthritis) and certain cancers (endometrial, breast and colon) (WHO, 2015c).

Obesity is preventable. Obesity prevention is largely dependent on a supportive environment and communities to shape individual choice, promoting healthier food choice and regular physical activity by making these easily accessible, available and affordable (WHO, 2016a). Behavioural changes are the single most important strategy for obesity prevention, i.e limiting high energy dense foods, increasing fruit and vegetable intake, including legumes, wholegrains and nuts in the diet and engaging in regular physical activity. In addition, policy makers and food industries could play a significant role in combating obesity through promoting healthy diets by ensuring that healthy and nutritious choices are available and affordable to all consumers.

A recent recommendation by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) delineated a minimum of 400 - 500 g of fruits and vegetables per day for the prevention of non-communicable diseases including heart disease, cancer, diabetes and obesity (Rimm et al., 1996; Liu et al., 2000; Joshipura et al., 1999; WHO, 2016b). Fruits and vegetables are rich in vitamins, minerals, dietary fibres and also contain signficant amounts of (poly)phenols. Recent *in vivo* and *in vitro* investigations have shown that (poly)phenols improved blood pressure (Rodrigo et al., 2012), improved cardiovascular risk factors (Nicholson et al., 2008, Perez-Jimenez and Saura-Calixto, 2008, Andriantsitohaina et al., 2012) and reduced diabetes risk (Williamson

et al., 2012). However, the presence of (poly)phenols in fruits and vegetables is mostly associated with the plant cell walls and may be bound within the food matrix limiting their bioavailability (Goñi et al., 2009). Hence, it is difficult to interpret the possible protective effects of fruits and vegetables from epidemiological studies. Studying the interactions of functional ingredients from dietary fibre isolates (e.g. β-glucan concentrate) and (poly)phenol-rich beverage (e.g. black tea) in a food matrix and their actions on human health could partly answer this question. Tea (*Camellia sinensis*) is one of the most popular drinks and a major source of (poly)phenols in the UK diet (Yahya et al., 2015). Black tea contains significant amounts of glycosylated flavonoids, typically 30-40% of the dry mass of black tea infusion (Kuhnert, 2010). Tea (poly)phenols are present freely in tea infusion and have been used in a variety of bakery products including bread, biscuit and cake (Pasrija et al., 2015; Lu et al., 2010; Sharma and Zhou, 2011). The combination of these two ingredients is the basis of the studies described in this thesis.

In the UK, a minimum of 30 g of dietary fibre a day is recommended for adults (SACN, 2015). Dietary fibre intake among Western countries is generally less than adequate for the prevention of major non-communicable diseases (Marlett et al., 2002). One feasible way to increase dietary fibre intake is by incorporating fibre into foods and ready meals. Dietary fibres can be used in a variety of food matrices such as bakery products, beverages, pasta and noodles, breakfast cereal and beverages. Hence, the development of palatable foods containing functional ingredients (eg. β-glucan concentrate) is one of the options to increase dietary fibre intake in the general population.

The European Food Safety Authority (ESFA) approved a health claim in which 4 g of β-glucan per 30 g available carbohydrate from either oats or barley is recommended to reduce the glycaemic response without disproportionally increasing postprandial insulinaemia (Agostoni et al., 2011). β-Glucan is a soluble viscous dietary fibre and has mixed β-(1 to 3) and β-(1 to 4) linkages. *In vitro* studies showed how bread with added functional viscous fibres (guar gum) reduced starch hydrolysis by forming a 'physical barrier' to starch-alpha-amylase interactions (Brennan et al., 1996). Ekstrom et al. showed a correlation between *in vitro* starch hydrolysis and glycaemic and insulinaemic responses

after guar gum supplementation in an acute human study (Ekstrom et al., 2013). The addition of β-glucan to muesli, cookies and breads reduced post-prandial glycaemic and insulinaemic responses in humans (Granfeldt et al., 2008; Casiraghi et al., 2006; Juntunen et al., 2002). Hence, β-glucan can be used as an active ingredient in formulating products aimed at reducing postprandial blood glucose.

Bread is the most popular starchy food in Europe (Bakers, 2014). In addition, breads have been used as a model to study food matrix interactions (Juntunen et al., 2002; Juntunen et al., 2003). Food constituents can interact in several ways when cooked together in a product such as bread and these interactions may become more complex and influence other components during food processing. Gluten and starch in bread are directly influenced by different stages of bread making (mixing ingredients, proofing and baking) (Rosell, 2011). The preparation of bread with onion skin (polyphenols-rich) dose-dependently (0.1 to 0.5%) increased *in vitro* antioxidant activity compared with control bread without onion skin (Gawlik-Dziki et al., 2013). Preparation of breads with whole-kernel and endosperm rye bread showed reduced starch hydrolysis compared with white bread (Juntunen et al., 2002; Juntunen et al., 2003).

The addition of dietary fibre and (poly)phenols may cause conformational changes in the gluten network in bread (Sivam et al., 2013). Gluten possessed hydrophilic characteristics when added with fruit (poly)phenols and dietary fibre (apple pectin) by forming hydrogen bonds with water, (poly)phenols and starch. Hence, the addition of apple pectin and (poly)phenol extracts in dough development and bread baking, directly influenced the cross-linking of gluten polymers, which could lead to greater water holding and softer bread (Sivam et al., 2011). These changes will affect the texture, visual appeal and palatability of the final products (Yuan et al., 2014). It is a challenge to develop a good product with the addition of functional ingredients, as there must be a balance between product acceptability and the amount required for any health benefits (Hall et al., 2010). Jenkins et al. measured the palatability of high fibre diets (β-glucan) in order to identify whether their dietary approaches were feasible for the general population (Jenkins et al., 2002). Palatability of high fibre β-glucan diets was comparable to that of white bread,

measured on a 6-point scale (-3 being dislike extremely, 0 being neutral and +3 being like extremely). In bread, Ellis et al. demonstrated that the addition of guar gum at 5.0 g/100 g is the upper limit before the products become unacceptable (Ellis et al., 2001). Hence, it is important to investigate the product's pleasantness to ensure any health benefits after consuming the products, as if they are not acceptable they will not be eaten.

1.1 Food matrix interactions of dietary fibre, (poly)phenols and macronutrients

β-Glucan and (poly)phenols have shown multiple health benefits in *in vitro* and *in vivo* studies. However, there is a dearth of studies available on the effects of having these two functional ingredients together. There may be additive or synergistic effects on health benefits when these two ingredients are combined in functional foods such as bread.

1.1.1 (Poly)phenols-dietary fibre interaction

(Poly)phenols may be closely associated with dietary fibre within the same food matrix like in fruits, vegetables and cereals. (Poly)phenol generally accumlate in with in the intra-cellular matrix (central vacuoles of guard cells and epidermal cells) within the plant cell. In beverages (coffee, cocoa, tea, beer and wine), (poly)phenols accounted for 3 to 63% of soluble dietary fibre and 1 to 51% in insoluble dietary fibre (Goñi et al., 2009). However, it must be noted that these beverages contain very little fibre when compared with fruits and vegetables. In wine, almost 30-60% of (poly)phenols are located within dietary fibre (Diaz-Rubio and Saura-Calixto, 2011). These researchers were the first to coin the term 'the wine (poly)phenols gap' because a significant amount of (poly)phenols-linked to dietary fibre (soluble) may pass undigested in the small intestine and reach the colon. Dietary fibre-(poly)phenols complex may have bigger impact on health benefits than having (poly)phenols or dietary fibre alone. In *chapati* (an Indian flat bread), treatment with amylase significantly increased the free phenolics, namely genistic, caffeic and syringic acids and also soluble dietary fibre suggesting the presence of phenolics bound to dietary fibre (Hemalatha et al., 2012).

Specific (poly)phenol adsorption to β-glucan has been studied using *in vitro* systems (Simonsen et al., 2009). This study aimed to investigate the possible interactions between

vanillin-related (poly)phenols (as flavor) and β-glucan. This combination may have implications for flavour retention and release when added in a food system. Moreover, this matrix may also be metabolised differently in the gastrointestinal (GI) tract when consumed. Simonsen et al. showed β-glucan purified from two sources, namely Glucagel (barley) and PromOat (oat) had similar ability to form complexes with (poly)phenols (Simosen et al., 2009). In this system, an ultrafiltration technique was applied to investigate the adsorption capacities of (poly)phenols into β-glucan defined as moles of (poly)phenols adsorbed by 1 mol of β-glucan. After ultrafiltration, unbound (poly)phenols passed through the membrane as filtrate, whilst β-glucan-(poly)phenol complex was retained on the membrane (Wang et al., 2013). Epigallocatechin gallate (EGCG) showed higher adsorption capacities into β-glucan and the adsorption of epicatechin gallate (ECG) was higher than aglycone epicatechin (EC). This suggests that galloylation of catechins increased adsorption capacities of flavan-3-ols (Wang et al., 2013). The presence of strong hydrogen bonding governed the interactions between EGCG and β-glucan (Wu et al., 2011). Although the method employed in this system mimics physiological condition (37°C, pH 7), it does not take into consideration the dynamics of pH and enzymic changes in the GI tract (from stomach to colon). The extent to how this adsorption affects human health is unknown and warrants further *in vitro* and *in vivo* study.

The presence of three hydroxyl groups on the galloyl moiety is important for hydrogen bonding, and the presence of aromatic rings is important for hydrophobic interactions (Tang et al., 2003). By employing thin layer chromatography, Tang et al. suggested that these interactions are particularly important in gallotannin-cellulose interactions. Cellulose is built up with β-1,4 glucosidic bonds similar to the β-glucan bond configuration. The galloyl groups serve as a functional group and the strength of interaction is dependent on molecular size, the galloyl groups and hydrophobicity of (poly)phenols. Simonsen et al. demonstrated (poly)phenol aglycones (4′-hydroxy-3′-methoxyacetophenone, 3,5-dimethoxy-4-hydroxybenzaldehyde, 3,5-dimethoxy-4-hydroxybenzoic acid, and ethyl 4-hydroxy-3-methoxybenzoate) were adsorbed more to the β-glucan molecule than their glucoside molecules (Simonsen et al., 2009). These aglycones have a smaller molecular weight, and therefore showed higher water solubility, which would increase interactions

with β-glucan. Further enzymic treatment of (poly)phenol-β-glucan complex with β-glucanase released some of the intact fibres which moved freely in solution. Another study showed in combination of β-glucan (300 mg) and 100 mg tea (poly)phenols interactions were governed by strong hydrogen bonds (Wu et al., 2011). The studies discussed above were based on an *in vitro* system and the conditions did not mimic the human GI tract. Further study is required to determine the impact of these interactions *in vivo*.

1.1.2 Interaction of (poly)phenols, dietary fibre and macronutrients

To date, there are few existing studies investigating the food matrix interactions of added (poly)phenols and dietary fibres in prepared foods such as bread and in food development processes (dough or pasting) (Barros et al., 2012; Sivam et al., 2013; Whistler et al., 1998). Studying these interactions requires sophisticated techniques such as fluorescence emission, UV-vis adsorption, circular dichroism, Fourier transform infrared and mass spectrometry, nuclear magnetic resonance, X-ray diffraction and light scattering techniques (Ulrih, 2015). Moreover, the health benefits of food matrix interactions are unknown and this is an area of interest. In wine, 1.5 – 2.3 g/L of dietary fibre is associated with total (poly)phenols (35 – 60%) after strong acid hydrolysis (Saura-Calixto and Diaz-Rubio, 2007). This might indicate that (poly)phenols associated with dietary fibre are not bioavailable in the small intestine and may reach the colon for bacterial fermentation. Saura-Calixto et al. demonstrated that grape antioxidant dietary fibre (a functional product) was 50% fermentable (when compared with 100% fermentability of lactulose) in an *in vitro* batch fermentation (Saura-Calixto et al., 2010). The main phenolic acids produced during fermentation were hydroxyphenylacetic acid, hydroxyphenylvaleric acid and 3- or 4- hydroxyphenylpropionic acid. The same metabolites were detected in human plasma after supplementation of grape antioxidant dietary fibre.

Dietary fibre may also be associated with other macromolecules such as protein and starch (Saura-Calixto and Diaz-Rubio, 2007). Proteins are complex molecules because of their secondary and tertiary structures. There is an interaction between gluten (protein) and starch during food processing and this plays an important role when functional ingredients such as soluble dietary fibre are added (Jekle et al., 2016). Zhou et al. demonstrated that

the addition of konjac glucomannan (a polysaccharides from konjac tuber) in wheat flour caused conformation changes in gluten structure (Zhou and Zhao, 2014). Native gluten is in α-helix form and β-sheets become a secondary structure when mixed with konjac glucomannan. The presence of hydroxyl (OH) from konjac glucomannan formed strong intermolecular hydrogen bonding which led to flexible gluten conformation and therefore more elastic dough.

Amino acids are the building blocks of protein conformed of a carboxylic acid group, hydrogen, functional side group (R) and an amino group. These unique chemical characteristics allow them to interact with (poly)phenols in the following ways: 1) hydrophobic bond: bond between ring structure of (poly)phenols and hydrophobic sites of proteins (eg. proline). Higher proline content in a protein favours (poly)phenols binding thorough hydrophobic interactions. The presence of intermolecular hydrogen bonds between H of one protein and OH of another protein strengthens the interactions between the (poly)phenols and protein complex 2) Hydrogen bonds: interaction between H-acceptor sites of protein and hydroxyl (OH) group of a (poly)phenols 3) Ionic bonds: interaction between positively charged protein and negatively charged OH of (poly)phenols 4) London bonds: a weak interaction between non-polar polarisable aromatic rings of flavonoids and non-polar polarisable protein side chains (Bordenave et al., 2014).

1.1.2.1 (Poly)phenols and protein interactions in a food system

The presence of both dietary fibre and (poly)phenols in breads could cause conformational changes in the protein network during breadmaking. Sivam et al. proposed the enhanced 'Loop and Train' model to illustrate the conformational changes in dough with added fibre and (poly)phenols (Sivam et al., 2013). This model proposes that at low hydration levels, protein-protein interactions formed with H from glutamine residues of *β*-spiral structures (loops). The 'Train' region is related to *β*-sheets of protein moiety while the 'Loop' with extended hydrated β-turns. As hydration proceeds, β-turns in adjacent β-spirals form interchain β-sheets and get plasticised. Further hydration leads to the formation of loop regions by breaking some of the H bonds between glutamine and water (Shewry et al., 2002). At this point, the addition of (poly)phenols and fibre reduces the mobility of

hydrated segments because of the competition among protein, (poly)phenols and fibre for water (Sivam et al., 2013).

In a food system such as bread, the addition of (poly)phenols from apple and blackcurrant with pectin increased intermolecular contact and binding sites for H bonding which resulted in the formation of extended chains (Sivam et al., 2013). The addition of apple pectin and (poly)phenols extracts from kiwi, blackcurrant or apple in dough development and bread baking, directly influenced the cross-linking of gluten polymers which could lead to more water holding and softer bread (Sivam et al., 2011). The type of (poly)phenols used may have different mechanisms in forming complexes with bread protein (gluten). Highly polar phenolic acids (*i.e*. the caffeic acid present in kiwi) are more mobile in bread than low polarity (poly)phenols (i.e. anthocyanins and proanthocyanins in blackcurrant and apple). Caffeic acid is attracted to charged components in protein and/or directly incorporates into the protein meshwork with less steric hindrance (Sivam et al., 2011; Sun-Waterhouse et al., 2009).

1.1.2.2 (Poly)phenols and starch interactions in a food system

(Poly)phenol interactions with starch components have been studied during pasting and dough formation. Barros studied the addition of different sorghum phenolic extracts (high molecular proanthocyanidins (PAs) vs (poly)phenol monomers) on pasting properties and resistant starch (RS) (Barros et al., 2012). At the level of 10% (w/w) of starch basis, PAs increased RS level of normal starch two times compared with monomeric (poly)phenols. Mechanistically, smaller monomers are adsorbed into corn starch (from maize) granules due to large surface pores (approximately 1 μm diameter) while large PAs will be trapped within the pores and thus become resistant to enzymic attack compared with smaller monomers (Whistler et al., 1998). Debranching of the amylopectin regions further increases RS in normal starch and this suggests the involvement of linear starch helix in starch-PAs interactions (Chai et al., 2013). The addition of PAs in high amylose starch increased RS by 52% compared with corn starch (from maize). During heat treatment, amylose and PAs possessed strong hydrophobic regions, which are readily exposed and this could explain (poly)phenol-starch interactions. PAs form complexes with single

helical regions of amylose that is stabilised by hydrophobic and hydrogen bonding, hence resistant to enzymic action.

Chai et al. demonstrated the formation of complexes between tea (poly)phenols and amylose (Chai et al., 2013). The presence of tea (poly)phenols in the complexes interrupted the normal process of amylose recrystallisation which leading to a low order of crystalline structure (more rigid structure and hence resistant to enzymic action) (**Figure 1-1**). Wu et al. showed the addition of 16% tea (poly)phenols (w/w basis) prevented starch retrogradation at the molecular level (Wu et al., 2011). Both the outer surface of amylose helices and tea (poly)phenols are rich in hydroxyl groups and hence, the hydrogen bonding might governed the interaction between starch and tea (poly)phenols during gelatinisation (Wu et al., 2011).

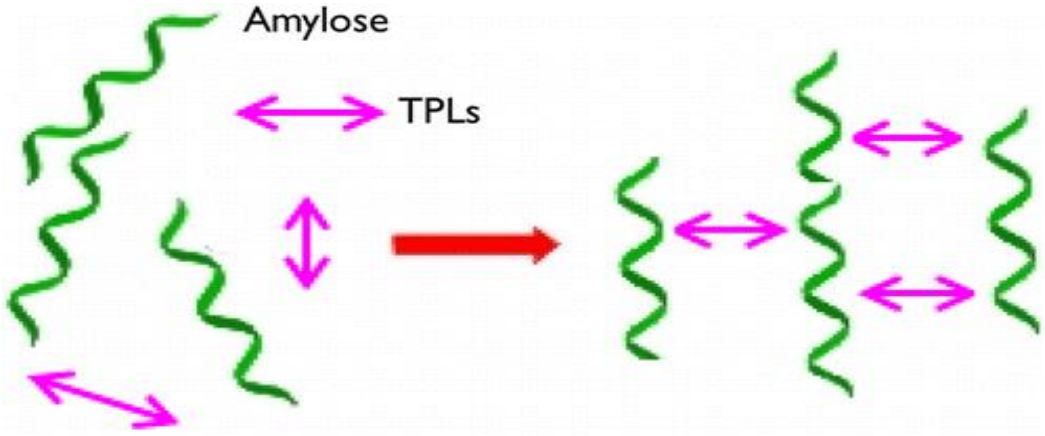


Figure 1-1. Interaction between tea (poly)phenols (TPL) and amylose through hydrogen bonding (Chai et al., 2013).

1.2 Functional foods

The food-matrix effects of (poly)phenols with dietary fibre and other micronutrients were discussed in the previous section of this study. These interactions allow the development of specific foods that confer enhanced health benefits. In 1980, the concept of functional food was introduced in Japan (EUFIC, 2016a). Increasing life expectancy and expanding numbers of the elderly means that health costs need to be controlled, and hence promotes the development of specific food to improve health or reduce disease risks. In 1991, the

concept of Foods for Specified Health Use (FOSHU) was introduced in Japan. FOSHU approval is granted by the Ministry of Health, Labour and Welfare after reviewing comprehensive scientific data to support the food claims when they are taken as part of ordinary diet (EUFIC, 2016a; Japan Ministry of Health, 2016).

In Europe, functional food is stated as "A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease" (Bellisle, 1998). Some functional foods are developed around particular functional ingredients such as dietary fibres, phytochemicals, antioxidants, prebiotics, probiotics, or plant stanols and sterols (EUFIC, 2016a).

The global functional food market was estimated to be £30 billion between 2004 and 2005 (Euromonitor, 2006). Emerging and ongoing research focusing on the importance of fruits, vegetables and wholegrain cereals and recent research on dietary antioxidants and/or combinations of other ingredients have both provided the impetus for the development of the functional food market in Europe. According to EUFIC, socio-economic and demographic changes govern to the need of food with additional health benefits. There is concerted action between governments, researchers, health professionals and the food industry have to see how changes in life expectancy, improved quality of life and an increasing cost of health care can be managed more efficiently (EUFIC, 2016b).

Bread is one of the most widely consumed food products in Europe with an average intake of 50 kg bread per person per year (Bakers, 2014). Bread is also a good target for the further development of functional food products and food-matrix interaction between its constituents during different stages of processing (Juntunen et al., 2002). The addition of dietary fibre to foods such as bread may be considered as a functional food when this imparts a function beyond the normal expected function, such as reducing cholesterol concentrations, reducing glucose and insulin responses (Prosky, 2000).

In the UK, there is a traditional British malt loaf available on the market (Soreen, 2016).

This is called sticky malt loaf and is prepared by adding 150 mL of hot black tea and 175 g malt extract from barley per loaf (**Figure 1-2**) (BBC, 2016). However, the ingredients and method of preparation of this loaf is different from the preparation of normal white bread. This loaf appears as dark brown and is chewier when eaten than normal bread. This bread received an average of 5 stars from 50 online readers (BBC, 2016). Hence, this kind of bread offers a huge opportunity for the development of functional food with added health benefits. However, this loaf has a high sugar content of 22 g per portion. This would reduce their ability to be considered as a functional food due to current fears about sugar (SACN, 2015).

Figure 1-2. Traditional British sticky malt loaf from BBC Good Food (BBC, 2016).

1.3 β-Glucan in functional foods

The definition of dietary fibre varies between country and/or organisation. Current definitions of dietary from around the world are tabulated in **Table 1-1** (Jones, 2014). In the UK, non-starch polysaccharides determined by the Englyst method was adopted for the dietary recommendations (Lunn and Buttriss, 2007). Non-starch polysaccharides (NSP) can be divided into those that beneficially regulate blood glucose (but not all NSP) and lipid absorption from the small intestine and the second are those becoming a substrate for an incomplete fermentation in the colon and have an effects in the bowel habit and referred

as insoluble NSP (Cummings and Stephen, 2007). Examples of soluble NSP are β-glucan, guar gum, gum Arabic, pectin, gum karaya and gum tragacanth while insoluble NSP as cellulose and chitin (**Table 1-2**).

β-Glucan is a soluble dietary fibre with mixed linkage of (1→3), (1→4)-β-D-glucan (Nielsen et al., 2008). Oats and barley β-glucan have a dominant (1→3), (1→4)-β-D-glucan while yeast β-glucan has a (1→3), (1→6)-β-D-glucan linkage (**Table 1-3**). β-Glucan content varies between cultivar, demography and method of extraction. Isolated β-glucan can be produced from different extraction procedures, i.e. water extraction and alcohol-based enzymic extraction and different sources (barley, oats and yeast) (Cleary et al., 2007; Panahi et al., 2007). The quality of β-glucan is low when produced using water because of the high shear rate involved during processing. During this process, endogenous enzymes (i.e. cellulase and β-glucanase) hydrolyse β-glucan resulting in changes in the molecular weight and lower viscosity. In contrast, alcohol extraction does not solubilise β-glucan, which remains intact within the cell wall. This technique preserves the molecular weight of β-glucan and has higher viscosity.

The bread baking process decreased molecular weight and solubilised β-lucan while cooking oats as porridge increased solubilised β-glucan (Johansson et al., 2007; Åman et al., 2004). Brennan and Cleary demonstrated increased resistance to extension in bread with added 5% β-glucan concentrate when compared with control (Brennan and Cleary, 2007). This bread showed reduced *in vitro* sugar release but no changes were observed in bread containing 2.5% β-glucan. β-Glucan has high water holding capacity and limits the water available for starch hydration due to competition of β-glucan for water and the formation of viscous β-glucan that inhibits enzyme accessibility to partially gelatinised starch granules.

Table 1-1. Current dietary fibre definitions from around the world. Adapted from (Jones, 2014)

Organisation	Definition
CODEX Alimentarius Commission 2009 (Sets International guidance standards for food and food imports)	•Dietary fibre means carbohydrate (CHO) polymers with ten or more monomeric units[1], which are not hydrolysed by the endogenous enzymes in the small intestine (SI) of humans and belong to the following categories: •Edible CHO polymers naturally occurring in the food as consumed •CHO polymers, obtained from food raw material by physical, enzymic, or chemical means[2] •Synthetic CHO polymers[2] [1]The footnote allows international authorities to decide whether those compounds with DP of 3–9 would be allowed. [2] For the isolated or synthetic fibres in category '2' or '3', they must show a proven physiological benefit to health as demonstrated by generally accepted scientific evidence to competent authorities Includes resistant oligosaccharides, resistant starch and resistant maltodextrins when footnote 2 is included.

Health Canada (HC) 2010 (A department within the Canadian government responsible for national public health)	•Dietary fibre consists of naturally occurring edible carbohydrates (DP > 2) of plant origin that are not digested and absorbed by the small intestine and includes accepted novel dietary fibres. •Novel dietary fibre is an ingredient manufactured to be a source of dietary fibre. It consists of carbohydrates (DP > 2) extracted from natural sources or synthetically produced that are not digested by the small intestine. It has demonstrated beneficial physiological effects in humans and it belongs to the following categories: •Has not traditionally been used for human consumption to any significant extent, or •Has been processed so as to modify the properties of the fibre, or has been highly concentrated from a plant source Includes resistant oligosaccharides, resistant starch and resistant maltodextrins.
European Food Safety Authority (EFSA) 2009 **(The Panel on Dietetic Products, Nutrition and Allergies develops scientific opinions on reference values for the European Union)**	•Non-digestible carbohydrates plus lignin, including all carbohydrate components occurring in foods that are non-digestible in the human small intestine and pass into the large intestine Includes non-starch polysaccharides, resistant starch and resistant oligosaccharides.
Food Standards Australia and New Zealand (FSANZ) 2001 (Responsible for development and administration of the food standards code listing requirements for additives, safety, labeling, and genetically-modified foods)	•Dietary fibre means that fraction of the edible part of plants or their extracts, or synthetic analogues that: •Are resistant to digestion and absorption in the, usually with complete or partial fermentation in the large intestine; and •Promotes one or more of the following beneficial physiological effects: i) laxation ii) reduction in blood cholesterol iii) modulation of blood glucose Includes resistant polysaccharides, oligosaccharides (DP >2) and lignins and resistant starches.
American Association of Cereal Chemists (AACC) 2001	•The edible parts of plants or analogous CHOs' that are resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the

(Gathers scientific and technical data for global use by grain-industry professionals; currently known as AACCI)	large intestine •Dietary fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. •Dietary fibres promote beneficial physiological effects including laxation, and or blood cholesterol attenuation, and/or blood glucose attenuation. Includes resistant oligosaccharides, resistant starch and resistant maltodextrins.
Institute of Medicine (IOM) 2001 (U.S. and Canadian advisory organization of the National Academy of Sciences; provides science- based research and evidence-based analysis to improve national health)	•Dietary fibre consists of non-digestible CHOs' and lignin that are intrinsic and intact in plants. •Functional fibre consists of isolated, non-digestible CHOs' with beneficial physiological effects in humans. •Total fibre is the sum of dietary fibre and functional fibre. Includes resistant oligosaccharides, resistant starch and resistant maltodextrins.
NSP Non-Starch Polysaccharides	•The skeletal remains of plant cells that are resistant to digestion by enzymes of man measured as non α-glucan polymers measured by the Englyst (Type 2 Method). •It includes NSP, which is comprised of cellulose, hemicelluloses, pectin, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages and hydrocolloids, all of which are principally found in the plant cell wall. Does not include oligosaccharides, resistant starch and resistant maltodextrins.

Table 1-2. Different types and characteristics of soluble dietary fibres

Type	Origin	Monomer unit	Type of bond	Characteristics	Food application	Reference
Guar gum (Indian cluster bean)	Leguminous Cyamopsis tetragonoloba plant	Galactomannan	Linear chain (1→4)-β-D-mannopyranosyl backbone with (1→6)-α-D-galactopyranosyl branch at O-6	Viscous	Thickener and emulsion stabiliser	(Goldstein et al., 1973)
Pectin	Orange, lemon, grapefruit, lime	Homogalacturonnan	(1→4)-α-D-galacturonic acid	Viscous	Gelling, thickening, emulsifier	(Kaya et al., 2014)
Gum Arabic	Exudate of *Acacia* senegal and Acacia seyal trees	Galactose, arabinose, rhamnose, glucuronic acid	(1→3)-β-D-galactopyranosyl backbone with 2 to 5 (1→3)-β-D-galactopyranosyl join to the main backbone by (1→6) linkage	Non-viscous gum	Stabiliser, emulsifier, thickening	(Ali et al., 2009)
Gum karaya	Sterculia species	Galacturonic acid, rhamnose	(1→4)-β-D-galactose backbone with (1→2)-β-D-galactose branch or by (1→3)-β-D-glucuronic acid to the galacturonic acid of the main chain	Viscous solution	Stabilising low pH emulsion (sauces and dressings)	(Verbeken et al., 2003)
Gum tragacanth	Astragalus species	Galacturonic acid, galactose, arabinose, xylose, fucose,	(1→4)-α-D-galactose backbone with (1→3)-β-D-xylose sidechain with	Viscous solution even at low concentration,	Sauces and dressings, shiny and clear appearance on fruit-	(Verbeken et al., 2003)

		rhamnose	attached chains of (1→2)-α-L-fucose, (1→2)-β-D-galactose	acid resistant	based bakery products	
Pullulan	Fermentation product of yeast *Aerobasidium pullulans*	3 glucose units	(1→4)-α-D-glucose backbone and branch at (1→6)-α-D-glucose at terminal glucose	Viscous, film forming	Gelatine-free capsule, tablets for dietary supplements, film for breath freshers	(Anton et al., 2014)

Table 1-3. Different types and characteristics of β-glucan

Type	Origin	MW (x 10^6) (Da)	Purity	Bond	Reference
Barley β-glucan	Hull-less barley grain (*Hordeum vulgare*)	0.191	80%	β-(1,3)- and β-(1,4)-glucosidic	(DKSH, 2016)
Oat β-glucan	Oat bran (*Avena sativa*)	1.12	33%	β-(1,3)- and β-(1,4)-glucosidic	(Tate and Lyle, 2016)
Yeast β-glucan	Baker's yeast (*Saccharomyces cerevisiae*)	0.1 to 0.2	50-65%	β-(1,3)- and β-(1,6)-glucosidic	(Borchani et al., 2016)
Laminarin	Brown microalgae (*Laminaria digitata*)	< 0.01 – 0.06	10 – 98%	β-(1,3)- and β-(1,6)-glucosidic	(Custodio et al., 2016)

1.4 Physiological effects of soluble viscous fibres in the upper gut

Epidemiology studies have shown a protective effect of dietary fibre against obesity, diabetes, cancers and cardiovascular diseases (CVDs) (**Table 1-4**). In a large multicentre prospective study from eight European countries (Denmark, France, Germany, Italy, the Netherlands, Spain, Sweden and the UK), total dietary fibre intake (>26 g/day) was associated with lower T2DM risk than the lowest quartile of total dietary fibre intake (< 19 g/day) (Kuijsten et al., 2015). In a large UK Women's Cohort Study, greater NSP intake had no additional benefits on CVD mortality but may lower fatal risks associated with stroke among overweight women (Threapleton et al., 2013a). An intake of 7 g/d of total dietary fibre was associated with an overall decrease of 7% in stroke risk (Threapleton et al., 2013b).

In the National Institute of Health (NIH)-AARP Diet and Health Study, whole grain and not total dietary fibre was associated with renal cell carcinoma and colorectal cancers (Daniel et al., 2013; Schatzskin et al., 2007). A systematic review showed 10 g of total dietary fibre reduced the risk for colorectal cancer in 16 studies (RR = 0.90, 95% CI 0.86 to 0.94) (Aune et al., 2011). Another study found total dietary fibre reduced the risk of head and neck cancer but only in women (Lam et al., 2011). However, not all studies showed a favorable relationship between dietary fibre intake and cancer. A recent systemic review found a null relationship between whole grain and cancers (e.g. lymphoma and prostate) in 14 out of 20 studies (Makarem et al., 2016). Six studies showed negative associations between whole grain intake and colorectal cancer. The null association between whole grain and cancers might be attributed to factors such as differences in the whole grain definitions, variations in the method of dietary fibre intake measurement (24-hour diet recall *vs* food frequency questionnaire) and lack of adjustment of cancer risks (e.g. body mass index, family history of cancer, physical activity and intake of red and processed meat).

There have been many *in vitro* and *in vivo* studies exploring the mechanisms by which dietary fibres act physiologically. Insoluble fibre has different effects to soluble fibre. An *in-vivo* study (in pigs) demonstrated lower digestibility of insoluble dietary fibre (e.g. oat

bran) in the intestine and increased faecal bulk compared with oat flour (Knudsen et al., 1993). In rats, supplementation with soluble fibre (70 g guar gum and xanthan gum) increased stomach and small intestinal content viscosity when compared with wheat bran (Cameron-Smith et al., 1994). These soluble fibres showed blunted postprandial glycaemic responses at 15 and 30 min when compared with wheat bran. Soluble fibres such as β-glucan are highly fermented by colonic microbiota and lead to the production of short chain fatty acids (SCFA) such as acetate, propionate and butyrate, which may be another route for their effects on postprandial glucose, liver metabolism satiety and colonic health.

As discussed in **Section 1.4**, there are different types of soluble dietary fibre, each of them possesses unique characteristics and some may also share similar characteristics (eg. viscous, soluble in water). There are several mechanisms by which soluble fibres may affect the rate of digestion and absorption of dietary carbohydrate (Englyst and Englyst, 2005) (**Figure 1-3**). These include the chemical characteristics (e.g. viscous and non-viscous), food-matrix (e.g. liquid, semi-solid and solid), and macronutrient composition (e.g. isocaloric or isovolumetric). In this context, viscosity plays an important role in delivering the health benefits of soluble β-glucan. The glucose and cholesterol-lowering properties of β-glucan are highly dependent on their amount and molecular weight being solubilised in the gastrointestinal tract (Wang and Ellis, 2014).

Table 1-4. Association between total dietary fibre (or whole grain) intakes and chronic diseases (T2DM, cancers and CVDs).

Study	Participants (n)	Follow-up (mean years)	Outcome	Reference
EPIC-InterAct	26, 088 (men and women)	10.8	↓ diabetes risk (HR 0.82; 95% CI 0.69, 0.97)	(Kuijsten et al., 2015)
National Institute of Health (NIH)-AARP Diet and Health Study	1,867 (men and women)	11	↓ head and neck cancer (in women only) (HR 0.77; 95% 0.64-0.93)	(Lam et al., 2011)
National Institute of Health (NIH)-AARP Diet and Health Study	491, 841 (men and women)	9	↓ renal cell carcinoma (whole grain) (HR 0.84; 95% CI 0.73, 0.98)	(Daniel et al., 2013)
National Institute of Health (NIH)-AARP Diet and Health Study	197, 623 (men and women)	5	↓ colorectal cancer (whole grain) (RR 0.79; 95% CI 0.70, 0.89)	(Schatzkin et al., 2007)
Swedish Mammography Cohort and the Cohort of Swedish Men	69,677 (men and women)	10.3	↓ total stroke risk (RR 0.90; 95% CI 0.81, 0.99)	(Larsson et al., 2014)
UK Women's Cohort Study	27, 373 women	14.4	↓ total stroke risk (HR 0.89; 95% CI 0.81, 0.99)	(Threapleton et al., 2013a)

HR, Hazard ratio; RR, relative risk; CI, Confidence interval; T2DM, type 2 diabetes mellitus; AARP, American Association of Retired Person.

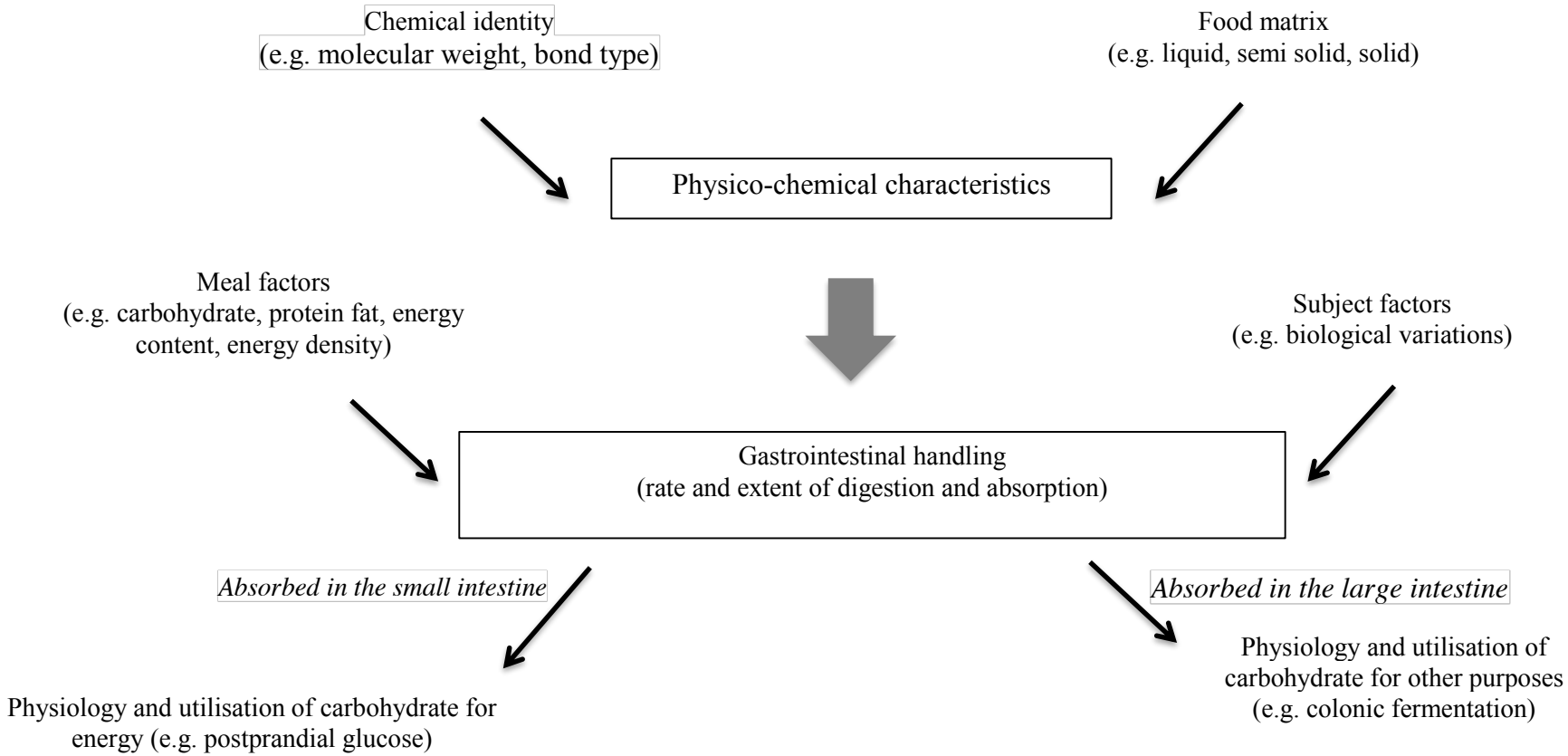


Figure 1-3. Food and non-food related factors influencing digestibility of soluble β-glucan in the gastrointestinal tract (Englyst and Englyst, 2005).

1.4.1 Effects of viscous fibres on gastric emptying

The digestion of food begins in the mouth and the process continues in the stomach and small intestine. In the mouth, digestion begins with the breakdown of starch to maltose, maltotriose and α-limit dextrins by salivary α-amylase. Oro-sensory stimulation of food plays an important role in regulating energy intake. Bypassing oral stimulation (by nasogastric feeding) decreased satiety and increased appetite hormones (Spetter et al., 2014). Physiologically, higher amounts and different food texture in the mouth leads to increased chewing and higher oral processing time, as more time is needed for enough saliva to be added to form a cohesive bolus for swallowing (Zijlstra et al., 2010). Increased oral processing time from 1 to 8 min reduced *ad libitum* energy intake at lunch (Wijlens et al., 2012). Thus, oral exposure time is as important as gastric filling (intragastrically infused) (8 min/100 mL and 8 min/800 mL) in reducing energy intake. Another study found that participants consumed more test product (chocolate custard) when the eating rate (g/min) was increased from 15 to 45 g/min (Zijlstra et al., 2009). This study concluded that manipulating oral exposure time by reducing eating rate is an important factor in food intake regulation. Food form (solid *vs*. liquid) might also have an impact on oral processing time. One study showed that biscuits containing 4 g of β-glucan with orange juice increased eating time when compared with control biscuits (without β-glucan) (Pentikäinen et al., 2014).

Oral processing time may control the rate of food (bolus) delivery to the stomach. Gastric volumetric signals and intestinal nutritive signals from the gastrointestinal tract work in concert in limiting food intake (Powley and Phillips, 2004). These two negative feedbacks synergise in the control of feeding and both were actioned through vagal afferents. Satiety signals are generated from stomach by two signals: distention at high volume and nutrient content via mechanoreceptors and chemoreceptors in the stomach linked to the brain via vagal afferents to stop food intake (Deutsch, 1985).

Theoretically, most soluble dietary fibres will increase stomach viscosity, but this does not necessarily mean that it will reduce stomach emptying. Different methods used to measure both stomach viscosity and gastric emptying makes the data interpretation difficult.

Measurement of stomach viscosity in human is difficult and time consuming. Alternatively, pigs are commonly used as a model to study stomach viscosity and gastric emptying with a simple gastric cannulation technique (Low et al., 1985; Rainbird, 1986; Rainbird and Low, 1986). Rainbird showed that guar gum supplementation significantly reduced stomach emptying rate at 1, 2 and 4 h, when added to a high energy meal and when compared to a low energy meal (Rainbird, 1986). Supplementation of high molecular weight guar gum significantly increased stomach viscosity in pigs and dose-dependently reduced glucose absorption and apparent insulin secretion (Ellis et al., 1995). However, guar gum supplementation as semisolid or solid meal had little effect on gastric emptying (Rainbird and Low, 1986; Van Nieuwenhoven et al., 2001). In a liquid meal, maximum viscosity was more favourable than a solid meal because of earlier and more complete mixing in the stomach. In addition, small intestine motor activity differs greatly between solid and liquid meals (V Schonfeld et al., 2001). The small intestine contracted more frequently after solid than liquid meals. Solid meals induced slower gastric emptying as opposed to liquid meals and hence had prolonged postprandial pattern.

Studying the physico-chemical properties of dietary fibre in relation to viscosity, i.e. hydration properties of dietary fibre, might partly answer this question. The action of dietary fibre on gastric emptying is related to its ability to hydrate in the stomach and the differences in rates of viscosity development are important to mimic its physiological effects (Holt et al., 1979). Guar gum had different hydration properties at different temperatures (32 and 22 °C) and pH (1 and 4) (O'connor et al, 1981). This study showed that powdered guar gum was the most viscous at the pH and temperature similar to stomach conditions. Feeding oat bran flour increased stomach viscosity at 1 h compared to wheat flour (Johansen et al., 1996). There was a strong and positive correlation between viscosity and β-glucan concentration in the liquid phase of digesta ($r = 0.45$). An increased in stomach viscosity might be due to the ability of soluble β-glucan to retain more water within the stomach. Pentikainen et al. demonstrated that β-glucan enriched biscuits and juice significantly increased viscosity when measured under simulated stomach digestion (Pentikäinen et al., 2014).

In humans, Edwards et al. showed that a combination of xanthan and locust bean gum (1% w/v, total volume of 250 mL) reduced gastric emptying when compared with control (drink without xanthan and locust bean gum) (Edwards et al., 1987). This fibre mixture showed higher *in vitro* viscosity when measured at different concentrations compared with control. However, this mixture did not show any favourable effects on blood glucose. A further test was performed to mimic gastric conditions (acidification with HCl and saline) and duodenal secretions (reneutralisation with sodium carbonate and saline). Acidification and reneutralisation reduced the viscosity of the mixture (dilutional effects) and this study concluded that gastric emptying is not necessarily related to their effects on plasma glucose and insulin levels.

Non-invasive techniques such as gamma scintigraphy and stable radioisotope could provide useful information on the effects of dietary fibre on gastric emptying. This method is considered to be the most accurate method for the estimation of gastric emptying (Scarpellini et al., 2013). By using sodium acetate labelled with 1-^{13}C, Clegg et al. demonstrated that 4 g of agar significantly delayed gastric emptying but lacked any physiological effects, particularly on blood glucose (Clegg et al., 2014). Meal viscosity or gastric viscosity alone does not necessarily decrease gastric emptying. Gastric viscosity and not meal viscosity changed intragastric distribution (move backward, retropulsion) which in turn reduced gastric emptying rate (Guerin et al., 2001). Meals enriched with fibre moved from distal to proximal stomach and were consistent with the slower stomach emptying time. This study concluded that delayed gastric emptying was related to changes in intragastric distribution in the stomach. Another study showed that guar gum (9 g in solution) significantly reduced peak glucose response and was due to a delay in small intestine transit time (Blackburn et al., 1984). No effect was observed on gastric emptying time.

1.4.2 Effects of viscous soluble fibres on digestion and absorption

After digestion, nutrients are passively or actively absorbed by the enterocytes after crossing two diffusion barriers: the unstirred water layer external to the cell and the protein lipid membrane of the microvilli (Cerda et al., 1987). Nutrients are made accessible to the

epithelium by two mechanisms as follows: 1) intestinal contractions creates turbulence and convection, followed by mixing of luminal content and finally bring the nutrients from the centre of the lumen close to the epithelium; and 2) diffusion of nutrients across unstirred layer of fluid adjacent to the epithelium (Eastwood, 2003). It has been suggested that viscous dietary fibres inhibit the access of nutrients to the epithelium. An *in vitro* model showed that the incorporation of guar gum blunted glucose uptake produced by increasing the rate of contraction (using dialysis tubing) (Edwards et al., 1987). This study suggests that viscous guar gum inhibited convection of the luminal content in relation to mixing contractions and hence reduced the absorption of glucose.

Related to a reduction in the mixing in the small intestinal contents with soluble fibre, there have been several studies suggesting an increase in the hypothetical unstirred water layer. It is the structured water layer close to the mucosal surface in the small intestine. This is the rate-limiting step for the solutes to diffuse across the enterocytes from the luminal bulk. Hence, this action retards the rate of diffusion towards and away from the surface. The reduce peak plasma glucose observed after guar gum ingestion was not explained by changes in the distribution of radio-labelled glucose drink in the small intestine, suggesting that the effect was not related to a reduced contact area in the small intestine (Blackburn et al., 1984). In another experiment using intestinal perfusion, the intestinal absorption of glucose was impaired during acute pectin supplementation (Flourie et al., 1984). *In situ* glucose measurement in the human jejunum showed pectin did not enhance glucose dependent sodium transport but significantly increased the unstirred layer thickness. The pectin concentration was highly correlated with unstirred layer thickness. Additionally, an *in vitro* study using perfused rat jejunal sacs showed that guar gum (0.25 and 0.50 %, w/v) increased the thickness of the unstirred layer of about 48% when compared with sacs incubated without guar gum (Johnson and Gee, 1981). Using the same dose, Cerda et al. showed that 0.25% and 0.5% (w/v) guar gum increased thickness of unstirred water layer in rabbits' jejunal sacs (Cerda et al., 1987). In another jejunal study in rats, inclusion of β-glucan increased the unstirred water layer compared with control (Lund et al., 1989).

Thus, the effects of viscous fibres may be related to their ability to decrease gastric

emptying and delay absorption of nutrients from the small intestine's lumen (Jenkins et al., 1978). This may also be related to slowed transit in the small intestine. Supplementation of guar gum or pectin (14.5 g in water with 50 g glucose) increased mouth-to-cecum transit time by 0 to 2 h and this effect was significantly correlated with the viscosity of the tested fibres (Jenkins et al., 1978). When hydrolysed guar gum was used no effect was observed on mouth-to-caecum transit time. Lembcke et al. showed that the flattening effects of guar gum on blood glucose were due to a delay gastric emptying (Lembcke et al., 1984).

1.4.3 Postprandial glucose and insulin

The food-matrix and chemical composition of soluble fibres such as β-glucan play an important role in the gastrointestinal handling of glucose and insulin responses (Englyst and Englyst, 2005). Viscosity of β-glucan is directly related to molecular weight and also depends on food sources (Cleary et al., 2007; Panahi et al., 2007). There were negative correlations between *in vitro* viscosity of soluble guar gum and plasma glucose responses (Jenkins et al., 1978; Edwards et al., 1987). Under *in vitro* digestion conditions, Pentikainen et al. showed that β-glucan dose-dependently increased gastric and small intestinal viscosity regardless of food matrix (solid or liquid) (Pentikäinen et al., 2014). This was supported by Jenkins et al., who showed that less viscous guar gum (hydrolysed) was ineffective in reducing postprandial glucose (Jenkins et al., 1978). As discussed in **Section 1.4.1**, the effects of viscous fibres on postprandial glucose is more likely through the inhibition of convection (slowing intestinal mixing) rather than inhibits glucose transport across the epithelium.

Drinks prepared with different viscosities of β-glucan have different effects on glucose and insulin responses (**Table 1-5**). High viscosity β-glucan decreased glucose and insulin responses compared to low viscosity (Juvonen et al., 2009; Panahi et al., 2007). However, Wood et al. demonstrated that 79-96% of changes in plasma glucose and insulin were due to the viscosity of the products (Wood et al., 1994). β-Glucan is in a form of a random-coil polymer and viscosity arises from coil entanglement above a critical concentration and molecular weight. The effective responses are obtained with higher doses of low molecular weight or lower doses of high molecular weight β-glucan.

Panahi et al. demonstrated that snack bars prepared with different levels of β-glucan (1.5, 3 and 6 g) reduced individual glucose responses, but had no effects on total AUC. The solubility of β-glucan was inversely related to β-glucan content in a solid snack bar (Panahi et al., 2014). The author hypothesise that in solid food, solubility was lower with increasing β-glucan content (6 g of β-glucan) due to 'intra' (between β-glucan molecules) and 'inter' (with other molecules present in bar matrix) interactions in the solid bar matrix. Regand et al. showed high molecular weight β-glucan in a bar (6.0 g β-glucan per 60 g carbohydrate) had no effect on individual glucose responses but reduced total AUC when compared with low and medium molecular weight β-glucan bar (Regand et al, 2001). Ellis et al. demonstrated that molecular weights (low and high) and particle size of guar gum when prepared in breads had no effect on glucose responses but were equally effective in reducing total insulin responses (AUC) (Ellis et al., 1991).

β-Glucan (3.0 g) in bread containing 55 g carbohydrate reduced total insulin AUC by 9% compared with control bread (Vitaglione et al., 2009). The preparation of β-glucan in biscuits (3.5 g β-glucan in 40 g carbohydrate) reduced total AUC glucose and insulin responses by 52% and 23%, respectively (Casiraghi et al., 2006). However, glucose and insulin responses were unchanged when the same amount of β-glucan was added to crackers. Different processing techniques used in making biscuits and crackers led to differences in moisture content between the two. Cookies had lower dough moisture of 5-10% as opposed to crackers with 10-20% and this resulted in lower starch gelatinisation in cookies compared with crackers. Juntunen et al. suggested that the structural and compositional properties of cereal grains rather than total dietary fibre alone play an important role in the regulation of insulin response (Juntunen et al., 2003). This can be partly explained by the differences in starch structure and the continuous matrix between the starch granules. This study showed that in rye bread, the starch granules were closely packed and formed a continuous matrix, while in wheat bread the starch granules were dispersed.

Table 1-5. Effect of β-glucan containing foods on postprandial glycaemia and insulin levels in healthy subjects.

Study design	Subjects, n (M/F)	Study duration (h)	Dose	Main outcomes	Reference
Liquid food-matrix					
Randomised	N = 20 (4/16)	3 h	Low viscosity β-glucan (10 g) (<250 mPas) High viscosity β-glucan (10 g) (>3000 mPas) (shear rate: 50 s^{-1}) *Food-matrix*: Beverage, 300 mL (isocaloric)	• Glucose not significant • Insulin $AUC_{0\text{-}180min}$ significantly lower (27%) in high *vs* low viscosity ($p = 0.007$)	(Juvonen et al., 2009)
Randomised, double	N = 11 (5/6)	2 h	Oat A: 6 g β-glucan (high viscosity) (*enzymic extraction*) Oat B: 6 g β-glucan (low viscosity) (*aqueous extraction*) *Food matrix*: 300 mL with 75 g glucose (isocaloric)	• ↓ glucose response (AUC) in Oat A (20% vs Oat B, 17% vs control) • Insulin not reported	(Panahi et al., 2007)
Non-randomised	N = 9 (4/5) N = 11 (6/5)	3 h	Study 1 (dose-response) (1.8, 3.6 and 7.2 g β-glucan) Study 2 ('hydrolysed' experiment) (7.2 g β-glucan hydrolysed for 15 and 60 min) *Food matrix*: 500 mL with 50 g glucose (isocaloric)	• Study 1: dose-dependently reduced glucose but no effect on AUC. Insulin not changed • Study 2: no significant changes in glucose and insulin responses	(Wood et al., 1994)

Semi-solid food-matrix					
Non-randomised, non blind	N =10 (4/6)	3 h	14.5 g oat gum (78% purity, β-glucan) 14.5 g guar gum (82% purity, galactomannan) *Food-matrix*: Gel-like pudding (500 mL containing 50 g glucose (isocaloric)	• ↓ glucose response *vs* control ($p < 0.05$) at 20 – 60 min in both groups • ↓ insulin response *vs* control ($p < 0.05$) at 20 – 60 min in both groups	(Braaten et al., 1991)
Randomised	N = 20 (15/5)	3 h	Oat bran (10.3 g) Oat bran and wheat bran (10.1 g) *Food-matrix*: Pudding (300 g) with water (isolcaloric)	• Glucose not significantly changed • Insulin significantly lower in oat bran *vs* combination at 30, 45 and 60 min	(Juvonen et al., 2011)
Solid food-matrix					
Randomised, crossover	N = 12 (not specified)	2 h	Oat or barley β-glucan at 1.5, 3 and 6 g *Food –matrix*: Snack bar (90 g) (isocaloric)	• Oat: all doses reduced glucose ($p < 0.05$) but no change in AUC • Barley: 1.5 g reduced glucose ($p < 0.05$) but no change in AUC	(Panahi et al., 2014)
Randomised, crossover	N = 14 (7/7)	3 h	β-Glucan (from barley) (3 g) *Food-matrix*: 100 g bread (isocaloric)	• ↓ glucose response at 30 min *vs* control bread ($p < 0.05$) and ↓ AUC by 10% • Insulin not significant	(Vitaglione et al., 2009)
Non randomised,	N = 10 (5/5)	2 h	Crackers: 3.6 g β-glucan	• ↓ glucose response in cookies (15 and 30 min, p	(Casiraghi et al., 2006)

crossover			Cookies: 3.5 g β-glucan *Food-matrix*: cookies (85 g) and crackers (95 g)	< 0.05), ↓ AUC 52% • ↓ insulin response in cookies (30 and 45 min, p < 0.05), ↓ AUC 23%	
Non-randomised, single blind	N = 20 (10/10)	2 h	5.4 g β-glucan *Food-matrix*: bread (169 g) (energy content slightly differ)	• Glucose unchanged • ↓ insulin response by 22% *vs* white bread	(Juntunen et al., 2002)
Randomised	N = 12 (6/6)	2 h	Low, medium and high molecular weight β-glucan (6.2 g) *Food-matrix*: granola bar (60 g) (isocaloric)	• ↓ glucose $AUC_{0\text{-}120min}$ by 19% in high *vs* low, medium and control group • Insulin not reported	(Regand et al., 2011)
Randomised, crossover	N = 12 (12/0)	6 h	Naturally high β-glucan (5.2 g) containing pasta (564 g) β-glucan (5.0 g) enriched pasta (563 g) *Food-matrix*: pasta as a mixed meal with chicken gravy, turkey ham and butter (isocaloric)	• Glucose unchanged • ↓ total $AUC_{0\text{-}60min}$ insulin for enriched pasta	(Bourdon et al., 1999)

AUC, area under the curve.

Bourdon et al. prepared two different types of pasta from naturally high β-glucan content and flour enriched with β-glucan on glucose and insulin responses (Bourdon et al., 1999). The pastas were consumed as a mixed meal with chicken gravy, turkey ham and butter. There were no effects of having these pastas on glucose response, but there was a decreased total insulin response ($AUC_{0\text{-}60min}$) for enriched pasta. This study suggests that the addition of β-glucan in a mixed meal obscured the glycaemic response due to the stimulation of hormone cholecystokinin (CCK). Rushakoff et al. demonstrated that CCK infusion did not increased glucose response after a meal, suggesting the importance of CCK in regulating postprandial glycaemia (Rushakoff et al., 1993). Mixed meals evoked higher insulin secretion when compared with a single meal (Bornet et al., 1987).

In semi-solid food, Braaten et al. was the first to investigate the effects of purified β-glucan on postprandial blood glucose and insulin responses (**Table 1-5**). In this study, 50 g of oat β-glucan was consumed together with 50 g of glucose in 500 mL of water and compared to control (50 g glucose in 500 mL water) (Braaten et al., 1991). The supplementation reduced peak (30 min) plasma glucose response when compared with control. The other mechanism whereby β-glucan could reduce postprandial glucose is through the production of propionic acid by the colonic bacteria, which has been shown to play a role in hepatic glucose metabolism in healthy volunteers (Venter et al., 1990). Berggren et al. showed that the supplementation of a physiologically relevant dose of 0.15 g sodium propionate per day reduced fasting glucose in rats (Berggren et al., 1996). Nilsson found an inverse correlation between GLP-1 and glucose response after intake of barley kernel bread in healthy volunteers (Nilsson et al., 2008). Colonic fermentation produced short chain fatty acids that in turn promote the secretion of GLP-1 (Reimer et al., 1996; Cani et al., 2005). A study showed how chronic supplementation of propionate decreased fasting glycaemia, but had no effect on hepatic glucose production nor whole body glucose utilisation in rats (Boillot et al., 1995). However, the effects of propionate on glucose metabolism in rats are not consistent. Battilana et al. suggested that the lowering effects of β-glucan was due to delayed and reduced glucose absorption from the gut and was not related to the fermentability of dietary β-glucan in the colon (Battilana et al., 2001). The outcome measures of this study were observed for up to 9 h and thus allowed at least some of the β-

glucan to reach the colon for colonic fermentation.

1.5 Physiological roles of β-glucan in lower gastrointestinal tract

1.5.1 Colonic fermentation

The gut microbiota is diverse, dynamic and affected by factors such as diet, ethnicity, ageing, drugs and disease (Nardone and Compare, 2015). The human intestine contains two hundred trillions of microbial cells and more than 1000 bacterial species (Herrera and Guarner, 2014). Bacteria in the human colon are dominated by bacteriodes, clostridia, fusobacteria, eubacteria, ruminococci, peptococci, peptostreptococci and bifidobacteria (Manson et al., 2008). These microbes play a pivotal role in normal physiology and susceptibility to disease through various metabolic activities and host interactions. For instance, the interaction between the pre-existing gastric microbiota and *Helicobacter pylori* infection might influence an individual's risk of gastric disease, including gastric cancer.

Physiologically, the proximal colon is a *saccharolytic* environment and is the main site for fermentation (**Figure 1-4**). There is low substrate availability in the distal colon and proteolysis is the main activity. Starch and non-starch polysaccharides may have different effects on the gut microbiota (Leitch et al., 2007). Breads prepared with oat bran and barley fractions were given to ileostomist subjects for 2 consecutive days and the soluble β-glucan was detected in an ileostomy effluents collected after 24 h of last food intake (Sundberg et al., 1996). This indicated that β-glucan escaped undigested in the stomach and small intestine and might reach the large intestine for bacteria fermentation. Similar results were observed when β-glucan was added in bread (Lia et al., 1996). The dry weight of effluents showed the presence of residue and nutrient complexes that could be a substrate for colonic fermentation.

Supplementation of bran with human faecal inocula increased *Clostridium hathewayi*, *Eubacterium rectale* and *Roseburia* species. Fermentation with starch increased the bacteria *Ruminococcus bromii*, *Bifidobacterium adolescentis*, *Bifidobacterium breve* and *Eubacteria rectale* while fermentation with mucin increased the bacterial species of

Bifidobacterium bifidum and *Ruminococcus lactaris*. These bacteria have the capability to ferment dietary fibre at different pH. Butyrate was produced four-fold higher at pH of 5.5 (proximal colon) compared with pH 6.5 (distal colon) (Scott, 2008).

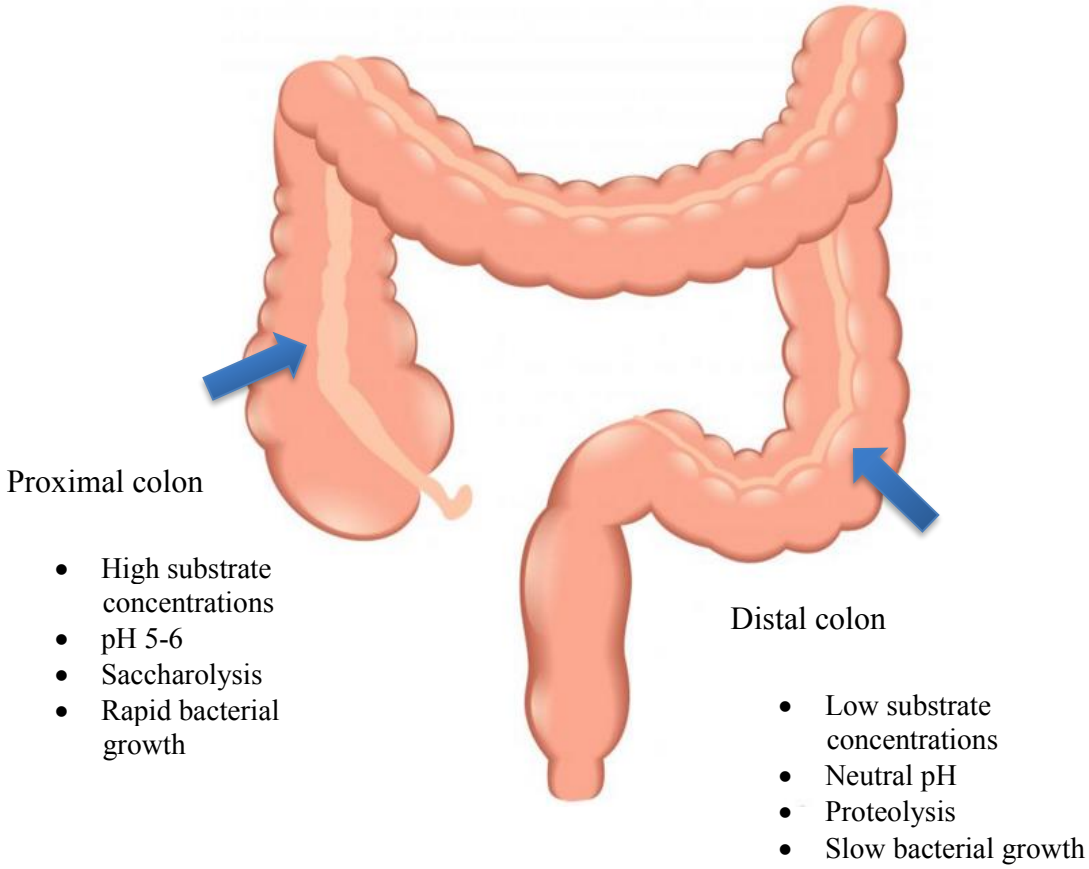


Figure 1-4. Human proximal and distal colon. Adapted from Guarner (2014).

In vitro digestion of both rye kernel bread (RKB) and boiled rye kernel (RK) significantly increased total SCFA production when compared with inoculum (Ibrugger et al., 2014). However, RK kernel showed a significantly higher total SCFA when compared with RKB, suggesting the importance of the food matrix for bacterial fermentation. RK might preserve its intact structure and contain a higher resistant starch, while RKB might have been milled and has lower resistant starch. The structural differences between RKB and RK may also affect on how the bacteria reach the resistant starch for the production of SCFA. The increment in total SCFA relatively increased the quantity of bifidobacteria and was

associated with reduced bacteriodes when compared with inoculum (control). The study also showed that the population of *C. coccoides* group was substantially reduced after RKB. *In situ* hybridisation techniques showed that chronic consumption of maize-derived whole grain cereal for 21 days increased the level of faecal bifidobacteria, when compared with control cereal in humans (Carvalho-Wells et al., 2010).

1.5.2 Colonic fementation products

Any carbohydrate which reaches the colon is subject to bacterial fermentation. This process begins with the metabolism of monomeric sugar into pyruvate, through the Embden-Meyerhoff pathway, and are immediately converted to end-products such as SCFA and gases. Colonic fermentation by saccharolytic bacteria produces major SCFA, namely acetate, butyrate and propionate along with trace amounts of other SCFA (Herrera and Guarner, 2014). The gases produced after fermentation are hydrogen (H_2), methane (CH_4) and carbon dioxide (CO_2). In humans, an average of 500-600 mmol of SCFA are found in the colon after dietary fibre intake or because of malabsorbed carbohydrates (McBurney and Thompson, 1989). The presence of SCFA reduces the luminal pH and this suppresses harmful pathogens and helps the absorption of Ca, Mg and Fe in the caecum.

SCFA are absorbed by Na/H exchange with the Na hydrogen exchanger-3 (NHE_3) transporter in the colon (Roy et al., 2006). SCFA absorption is governed by active (apical carrier-mediated) and passive (non-ionic diffusion) transport. The pH in the colon ranges from 5.5 to 7.5 and the pKa of SCFA is 4.8 and hence most SCFA are present in the anionic form. However, this anion will be converted to undissociated (protonated) form before being transported through passive transport (non-ionic diffusion). Hence, non-ionic diffusion is more apparent than active transport (Charney et al., 1998).

Generally, acetate is the main anion but the molar ratios of all the SCFA change substantially from colon to portal blood and hepatic vein indicating uptake of butyrate by the colonic epithelium and propionate by the liver (Cummings et al., 1987). Acetate has been shown to play a central role in appetite regulation, while propionate is a precursor for hepatic glucose production and also stimulation of hepatic vagal afferents to reduce food

intake (Chambers et al., 2014). Acetogenic bacteria produce acetate from CO_2 and H_2. Rectal acetate infusion significantly increased serum cholesterol and decreased serum free fatty acids suggesting that acetate is involved in cholesterol synthesis (Wolever et al., 1991). In contrast, propionate infusion decreased cholesterol synthesis when added together with acetate, suggesting that propionate inhibits the utilisation of acetate for cholesterol synthesis. However, the physiological levels of these SCFA produces from the colonic fermentation might not be relevant for the observed effects. Butyrate is the main source of nutrient for epithelial cells lining the colon colonocytes (Roediger, 1982). Colonocytes have the capability to utilize ketone bodies (acetoacetate) and at the same time produced acetoacetate and β-hydroxybutyrate from SCFA. SCFA are rapidly absorbed in the colon and further metabolised as follows:

1) Uptake of butyrate by the colonic mucosa and to lesser extent acetate and propionate for energy production.

2) Hepatocytes (in the liver) metabolise propionate for gluconeogenesis (glucose production) and acetate for lipogenesis.

3) Production of energy from the oxidation of residual acetate in muscle cells and other tissues (Cummings et al., 1987).

Major SCFA produced from the fermentation of soluble dietary fibres from *in vitro*, animal and human studies are tabulated in **Table 1-6**. *In vitro* fermentation of different types of dietary fibre with human faecal produced different patterns of SCFA. More butyrate is produced from the fermentation of rapidly available starch, resistant starch and insoluble wheat bran, while soluble fibres such as guar gum and β-glucan produced large proportions of propionate (James et al., 1997; Weaver et al., 1992; Cummings and Macfarlane, 1991; Adiotomre et al., 1990). Production of acetate, propionate and butyrate from *in vitro* fermentation of β-glucan-rich fractions from barley and/or oats were in the range of 11–48 mM, 5.5–18 mM and 7–14 mM, respectively. The molar proportions of acetate:propionate:butyrate production differ from one study to another, in the range of 39-66, 13–45 and 11–35 mM, respectively.

These *in vitro* fermentation studies were carried out using samples from 2-6 donors (**Table 1-6**). In a multicentre fermentation study, the greatest intra-laboratory variation was observed with those utilizing samples from only 4 donors (Edwards et al., 1996). In a recent study, et al. demonstrated 2-fold differences in acetate, propionate and butyrate productions from fermentation of partially hydrolysed guar gum between 6 donors (Carlson et al., 2016). This study showed 1 out of 6 donors showed unexceptionally high SCFA when compared to other subjects. Two studies measured SCFA in faecal samples, and this could underestimate the amount of SCFA being produced from fermentation, as only 5% of SCFA are recovered in faecal samples (Kien et al., 2006; Topping and Clifton, 2001).

β-Glucan is fermented by intestinal microbiota and significantly increases propionate resulting in an acetate:propionate:butyrate production ratio of 51:32:17 which was considered as propionate-rich (Hughes et al., 2008). In mice, supplementation of oat bran with different molecular weight β-glucans (2348, 1311, 241, 56, 21 or < 10 kDa) increased the production ratio of propionic acid and butyric acid/acetic acid with increasing molecular weight (Immerstrand et al., 2010). There were no significant differences between individuals and total SCFA production from the fermentation of β-glucan either from barley and oats (Hughes et al., 2008; Jozefiak et al., 2006). β-Glucan from barley has higher purity but lower in molecular weight compared with oats β-glucan and hence, this could explain why there was no significant difference in SCFA production.

Table 1-6. Production of major SCFA (acetate, propionate and butyrate) after the fermentation of soluble dietary fibres in *in vitro*, animal and human study.

Type of soluble fibre	**Amount of fibre (mg)**	**Fermentation medium (Number of faecal donors)**	**Study duration (h)**	**A, P and B‡ (mM) and/or molar proportion of A:P:B (at end point)**	**Total, mM or total (%)**	**Comments**	**Reference**
In vitro study							
High and low molecular weight β-glucan (0.46 – 3.48 x 10^5 g/mol)	100 mg	Brain-heart infusions medium (2 donors)	24 h	13.5 - 17 mM 5.5 - 6 mM 10 - 12 mM Molar proportions (range): 47 – 49: 17 – 19: 34 - 35	29-35 mM	Low molecular weight had higher total SCFA	(Kim and White, 2011)
Purified oat β-glucan	100 mg	Brain-heart infusions medium (2 donors)	24 h	39:27:34	81-89 %	Exact SCFA not reported. Most β-glucan has been fermented at 8 h	(Sayar et al., 2007)
High and low molecular weight β-glucan (from oats and	10 mg	Peptone, yeast extract, vitamins and minerals	48 h	11 - 25 mM 10 - 17 mM 7 - 10 mM Molar	28 - 52 mM	No prebiotic effects (no effect on bifidobacteria and lactobacilli) but modulated microbial	(Hughes et al., 2008)

barley) 150 and 230 kDa		(3 donors)		proportion (range): 39 – 48: 33- 36: 19 - 25		community (*C. histolyticum*). No difference between oat and barley in term of APB production. Considered as propionate rich	
Partially hydrolysed guar gum	500 mg	Peptone fermentation media (6 donors)	24 h	13 - 26 mM 20 - 45 mM 9 - 23 mM Molar proportions (range): 28 - 30: 48: 21 - 24	42 - 94 mM	Reported high interindividual variation between donors. 2-fold difference among individual	(Carlson et al., 2016)
Oat concentrate	200 mg	(6 donors)	24 h	48 mM 18 mM 14 mM Molar proportions: 60:22:18	80 mM	Samples were pre-digested prior to fermentation	Nordlund et al., 2012
Animal study							
β-Glucan concentrate	9% in pig's feed but food intake was not reported	Digesta from pig's stomach, caecum and colon (n = 32)	Dissected after 14 days	69 - 72* 24 - 29 12 Molar proportions (range):	105 - 113	Enhanced bacterial growth in the stomach and colon	(Metzler-Zebeli et al., 2011)

				64 - 66: 23 – 27: 11			
Barley and oat	49 g β-glucan in barley-based and 66 g β-glucan in oats-based chicken's feed	Digesta from broiler chicken (n = 96) ileum and caeca	35 days	63 – 88 * 13 – 43 21- 25 Molar proportions: 57 – 63: 13 – 28: 16 – 24	98 – 156 *	No difference between barley and oats in term of SCFA production	(Jozefiak et al., 2006)
Different molecular weight β-glucan from oats (< 10, 21, 56, 241 and 1311 kDa)	7.2 – 8.4 g β-glucan from oats in mice's feed	Digesta from caecum	28 days	6 – 8 § 2.6 - 3 0.9 – 3 Molar proportions (range): 57 – 63 21 -27 9 – 21	9.5 -14 §	Propionate was higher in all groups *vs* control. Increased production ratio of propionate and butyrate/ acetate in increasing molecular weight	(Immerstrand et al., 2010)
Human study							
Barley kernel and barley porridge	^{13}C-labelled barley. Barley kernel: 86 g in 450 mL Barley	^{13}C-breath content (n = 5)	12 h	Not reported	Not reported	Only 3 volunteers produced propionate (porridge). Kernel group never produce propionate. High intervariations between individuals	(Verbeke et al., 2010)

	porridge: 86 in 320 mL						
PolyGlycoPlex ®(glucomannan, sodium alginate and xanthan gum)	5 g at week 1 followed by 10 g during week 2 and 3	Direct measurement of SCFA from faecal sample (n = 27)	Week 1, week 2 and week 3	40 ** 12 12 Molar proportions: 62:19:19	64**	Direct measurement from faecal sample. Samples were stored in refrigerator for 10 h then frozen.	(Reimer et al., 2012)

‡, A, acetate, P, propionate, B, butyrate; *micromol/g digesta; ** mmol/g faeces; § µmol/caecal content.

1.6 Stimulation of gastrointestinal hormones

Cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) are considered gut hormones or neurotransmitters in a satiety signal because they increased food intake in knock-out animals or in response to receptor antagonism (Naslund and Hellstrom, 2007). These hormones also showed a temporal profile (time dependant) with satiety and meal termination. The release of these gut hormones depends on the quantity and quality of foods (macronutrients and/or dietary fibres) being ingested. The effects of these hormones are dependent of the interaction with specific receptors in the gut and the brain (gut-brain axis) (Mendieta Zeron et al., 2013). The site of hormone secretion, receptors involved and their major action is shown in **Table 1-7**.

The primary function of the GI tract is for digestion and absorption. Within this system, there is a GI neuroendocrine system that forms an important relationship between the brain and gut that optimises digestion and absorption (Chaudhri et al., 2006). In the brain, the arcuate nucleus (ARC) is the centre for molecular signals due to its location near to the median eminence. Circulating factors such as gut hormones CCK, PYY and GLP-1 then modify the activity of two populations of neuron within the ARC (Chaudhri et al., 2006). The first population of neuron co-express cocaine- and amphetamine transcript and propiomelanocortin and inhibits food intake. The second population of neuron co-express neuropeptide Y (NPY) and agouti-related protein which increases food intake (Cone et al., 2011). Both populations are directed to the paraventricular nucleus and other areas important in the regulation of food intake (Schwartz et al., 2000). These gut hormones also play an important role in glucose metabolisms as follow: altering food intake and body weight, thereby insulin sensitivity; affecting stomach emptying and motility, thereby fluctuation in glucose levels after a meal; affecting insulin secretion, thereby plasma glucose levels (Heijboer et al., 2006).

Regulation of gut hormones is inter-related and dependent on the site of action. CCK appears in the bloodstream postprandially as soon as the nutrients enter the duodenum. The release of GLP-1 and PYY is delayed until the nutrients reach further down the distal small intestine and colon (Pilichiewicz et al., 2007a). Both I- and L-cells (lower intestine and the

colon) are stimulated when they come into contact with their luminal content along the intestinal axis (Gribble et al., 2012). Kokkinos et al. demonstrated that eating moderately slow (30 min) significantly increased PYY and GLP-1 compared to eating very fast (5 min) (Kokkinos et al., 2010). A similar study by Li et al. found that chewing food by 40 chews associated with lower energy intake and higher GLP-1 and PYY responses (Li et al., 2011). The presence of dietary lipids, proteins and carbohydrates (eg. sugars) triggered the secretion of CCK, GLP-1 and PYY (Pilichiewicz et al., 2007a; Pilichiewicz et al., 2007b). Studies have demonstrated that soluble dietary fibres (eg. β-glucan) increase the release of intestinal peptides such as ghrelin, CCK, PYY and GLP-1 (Juvonen et al., 2009; Vitaglione et al., 2009). These peptides reduce the rate of gastric emptying, increase gastric distension and hence act as a pivotal role in controlling the appetite (Kissileff et al. 1981; Blundell et al., 2010).

Table 1-7. Source, specific receptors and mode of action of hormones related to food intake.

Hormones	**Source**	**Major receptor**	**Mode of action**
Cholecystokinin (CCK), CCK-55-CCK-33, CCK-22, CCK-8	Intestinal I cells, neurons	CCK2	Slows gastric emptying Release of pancreatic enzyme Causes gall bladder contraction Increases gut motility Increases satiety
Glucagon	Pancreatic α cells	Glucagon	Gluconeogenesis Glycogenolysis
Glucagon-like peptide-1 (GLP-1)	Gastrointestinal L cells (lower intestine and colon)	GPR41 and GPR43	Slows gastric emptying Releases insulin (glucose dependent) Vagal and central nervous system effects Increases satiety

Peptide tyrosine-tyrosine (PYY), PYY_{1-36}, PYY_{3-36}	Gastrointestinal L cells (lower intestine and colon)	Neuropeptide Y (NPY) Y2 receptor (Y2R)	• Suppression of gastric acid secretion • Slows gastric emptying • Inhibition of intestinal motility • Inhibition of gallbladder contraction • Vagal & central nervous effects • Increases satiety
Glucose-dependent insulinotropic polypeptide (GIP)	K cells in the duodenum, jejunum (upper small intestine)	GIP-R	Stimulates insulin synthesis and secretion

1.6.1 CCK

Cholecystokinin (CCK) is present in different molecular forms, CCK-55, -33, -22 and -8 (Rehfeld et al., 2001). CCK-33 is the predominant fasting plasma and postprandial CCK derived from gut I-cells with 51 and 57%, respectively. CCK-22 is second most abundance CCK found in plasma with approximately 34 and 32%, respectively, during fasting and postprandial state (Rehfeld et al., 2001). CCK-8 is a predominant form in the neuron (Rehfeld et al., 2003). CCK is stimulated by the presence of protein, amino acids and fat but glucose causes a smaller elevation in plasma CCK (Liddle et al., 1985). The interaction of dietary fat in the small intestine resulted in feedback inhibition of gastric emptying, which acts through prolonged gastric distention (Heddle et al., 1989, Read et al., 1994). Reynolds et al. demonstrated that carbohydrate quality (different dietary fibre types, but matched for macronutrients and fibre content) elicited different CCK responses. High 'quality' (low glycaemic index) fibre type meal evoked higher CCK responses compared with low 'quality' (high glycaemic index) fibre meal (Reynolds et al., 2008). This study suggests the diet induced slower gastric emptying and hence reduced the access of carbohydrate and other macronutrients within the proximal small intestine. This slower

transit time allows prolong stimulation of I-cells, which in turn further decrease gastric emptying and maintains higher CCK levels.

CCK stimulates secretion of pancreatic enzymes, causes contraction of gallbladder, slows gastric emptying and increases motility in the large intestine (Beyer et al., 2008). CCK has a profound effect when administered through jejunal feeding rather than gastric feeding, which lead to greater insulin sensitivity and delayed gastric emptying (Luttikhold et al., 2016). There are two distinct types of G protein-coupled receptor CCK, namely CCK-1 and CCK-2. CCK-1 receptors are primarily found in the pancreas, on vagal efferent and enteric neurons. CCK-2 receptors are found in the afferent vagus nerve and also within the stomach (pyloric area) (Dockray, 1987). CCK receptors are both present in the brain and the stomach and therefore play significant roles in the regulation of food intake (Rehfeld et al., 2003).

In animal models, Moran suggested that low doses of CCK inhibit food intake by direct activation of vagal afferent CCK-1 receptors. High doses of CCK are secondary to the activation of pyloric CCK receptors and dependent on the ability of CCK to inhibit gastric emptying (Moran et al., 1988; Moran et al., 1997; Moran et al., 2000). In an early study by Gibbs et al., exogenous CCK dose-dependently reduced food intake in rats (Gibbs et al., 1973). Another study showed that an intravenous infusion of CCK reduced meal size and duration in human (Kissileff et al., 1981). However, the effect of CCK is short-lived and repeated administration reduced food intake but increased meal frequency, and hence overall intake is unchanged (Gibbs et al., 1973; West et al., 1987a; West et al., 1987b).

In healthy subjects, supplementation of isocaloric and isovolumetric low viscosity β-glucan (low molecular weight) beverage increased CCK level by 80% compared with high viscosity β-glucan (high molecular weight) (Juvonen et al., 2009) (**Table 1-8**). This study suggests the importance of molecular weight and viscosity in the release of CCK. Low viscosity beverage leads to lower chyme viscosity compared with high viscosity and hence increase the interaction between nutrients and gastrointestinal mucosa required for efficient stimulation of CCK. Acute supplementation of viscous insoluble cellulose (highly viscous) (extracted from corn stalks and husks) in beverage reduced CCK compared with control

beverage among hypercholesterolaemic men and women (Geleva et al., 2003). However, long-term supplementation (6 weeks) did not change the CCK levels. This study suggests higher interindividual variability may be primarily responsible for the inconsistent results. This was supported by another study that showed how sodium alginate supplementation with beverage for 10 days had no effect on CCK (Odunsi et al., 2010).

1.6.2 Peptide YY (PYY)

Peptide tyrosine-tyrosine (PYY) was first characterised in porcine upper intestinal tissue as long linear chain of 36 amino acids sequence (Tatemoto et al., 1982). PYY_{3-36} is an active form of PYY and is derived from the cleavage of PYY_{1-36} by enzyme dipeptidyl peptidase-V enzyme (Unniappan et al., 2006). PYY has been expressed in both neurons and endocrine cells (Ekblad and Sundler, 2002). PYY involves in the secretion of gastric acid and pancreatic secretions and increased gastrointestinal motility (Naruse et al., 2002; Yang et al., 2002; Imamura et al., 2002).

Batterham and co-workers were the first to identify the role of PYY in energy homeostasis in humans (Batterham et al., 2002). This study showed an infusion of physiological dose of PYY_{3-36} significantly reduced food intake by 33% over 24 h. A PYY_{3-36} level was lower in the fasting state and increased proportionately in response to caloric content (Chan et al., 2006; le Roux et al., 2006). le Roux demonstrated how the physiological infusion of PYY_{3-36} (0.2 to 0.8 pmol/kg/min) dose-dependently reduced energy intake in men (le Roux et al., 2006). Essah et al. showed equicaloric low-carbohydrate, high-fat diet associated with higher PYY levels when compared with low-fat, high-carbohydrate diet (Essah et al., 2007). The abdominal vagotomy diminished the anorectic effect of PYY and suggesting the PYY_{3-36} act through the arcuate nucleus neuropeptide Y (NPY) Y2 receptor (Y2R) to inhibit food intake in a gut-hypothalamus pathway (Abbott et al., 2005; Batterham et al., 2002). NPY Y2 is a presynaptic receptor that is highly expressed on NPY neurons in the arcuate nucleus and is highly accessible by peripheral hormones (Broberger et al., 1997). NPY Y2R is also present in the vagal afferent fibres located on the vagal nerve. This forms an interconnection between gastrointestinal tract and the brain, which is important for gut hormones to sensitise the brain to inhibit food intake (Koda et al., 2005).

β-glucan, when prepared in bread, reduced PYY by 16% when compared with normal bread (**Table 1-8**) (Vitaglione et al., 2009). PYY increased by 418% when prepared in low viscosity β-glucan in beverage compared with high viscosity beverage (Juvonen et al., 2009). In semi-solid food (pudding) the same level of β-glucan had no effect on PYY (Juvonen et al., 2011). Different products may have undergone different processing techniques and might have different effects on their solubility. Johansson et al. demonstrated that processing methods had significant impact on β-glucan content (Johansson et al., 2007). This study showed preparation of oat porridge by boiling in hot water for 10 min significantly increased soluble β-glucan, while preparation in bread using normal baking technique significantly reduced solubilised β-glucan. Ronda et al. showed degradation of β-glucan during bread making which may be due to the activity of β-glucanase (Felicidad Ronda et al., 2015). Some studies determined *in vitro* viscosity of products using digestion model mimicking human gastrointestinal digestion (Juvonen et al., 2009; Panahi et al., 2007; Wood et al., 1994). However, it must be noted that the results derived from *in vitro* viscosity are not directly translated to human, due to interaction with other food components, dilutional effects of digestive fluids and enzymic degradation of β-glucan (Wang and Ellis, 2014). In dietary fibre studies, it is common to observe the dietary fibre intake as a composite meal (Reimer et al., 2010; Kristensen and Jensen, 2011). Reimer et al. showed longer study duration of 2 weeks using composite meal (eaten with cereal) reduced PYY by 30% when compared with control (Reimer et al., 2010). Kristensen et al. showed no effects of adding flax mucilage in beverage and eaten with bun (Kristensen et al., 2013).

Table 1-8. Effects of viscous soluble fibres on postprandial CCK, PYY and GLP-1.

Study design	Subjects, N (M/F)	Study duration (h)	Dose	Main outcomes	Reference
Liquid food-matrix					
Randomised	N = 20 (4/16)	3 h	Low viscosity β-glucan (10 g) (<250 mPa.s) High viscosity β-glucan (10 g) (>3000 mPa.s) (shear rate: 50 s^{-1}) *Food-matrix*: Beverage, 300 mL (isocaloric)	• ↑ CCK by 79.6% • ↑ PYY by 417.8% • ↑ GLP-1 by 155.4% in low viscosity beverage *vs* high viscosity beverage ($AUC_{0\text{-}180\ min}$)	(Juvonen et al., 2009)
Solid food-matrix					
Randomised, crossover	N = 14 (7/7)	3 h	β-Glucan (from barley) (3 g) *Food-matrix*: 100 g bread (isocaloric)	↑ AUC PYY (16%)	(Vitaglione et al., 2009)
Semi-solid food-matrix					
Randomised	N = 20 (15/5)	3 h	Oat bran (10.3 g) Oat bran and wheat bran (10.1 g) *Food-matrix*: Pudding (300 g) with water (isolcaloric)	Plasma PYY not affected	(Juvonen et al., 2011)
Composite meal					

Randomised	N = 54 (25/29)	1 week – 5 g/day 2 weeks – 10 g/day	PolyGlycoPlex (glucomannan, sodium alginate and xanthan gum). Visit 1 = 2.5 g, twice a day for 1 week visit 2 = and 5 g, twice a day for 2 weeks Visit 3 = endpoint *Food-matrix*: with 10 g cereal with 135 mL plain yoghurt	↓ PYY at visit 3 *vs* control by 33.31% (after adjusted for BMI, i.e. just BMI < 23 was included)	(Reimer et al., 2010)
Randomised, double blind	N = 18 (18/0)	7 h	Low-mucilage flaxseed (12 g) High-mucilage (17 g) Food-matrix: Two buns with cheese, butter, ham and 400 mL water	No significant effects in GLP-1, PYY	(Kristensen et al., 2013)

mPa.s; milli pascal per second

1.6.3 GLP-1

Glucagon-like peptide-1 is a 31-amino acid gastrointestinal peptide released from L cells of the lower intestine and colon (**Table 1-8**). GLP-1_{7-36} is an active form and the presence of proteolytic enzyme dipeptidyl peptidase-V cleaves it into inactive GLP-1_{9-36} (Kieffer et al., 1995). GLP-1 slows gastric emptying, reduces gastric acid secretion and pancreatic enzymes and reduces food intake (Baggio and Drucker, 2007). The combination of these activities leads to increase insulin sensitivity and lower postprandial glycaemia (Schirra et al., 2005; Baggio et al., 2007). GLP-1 increases postprandially after a meal accompanied by a fall in glucose and glucagon levels (Kreymann et al., 1987). GLP-1 works through entero-insular axis and hence regulates postprandial glycaemia. GLP-1 also acts through blood-brain barrier that protects the brain from fluctuation in intracerebral glucose during postprandial period (Lerche et al., 2008).

Carbohydrate (sugars) and protein stimulate the release of GLPI-1 (Layer et al., 1995). The peak plasma GPL-1 was obtained at 20 min after an oral glucose load (Schirra et al., 1996). Duodenal delivery at a rate exceeding more 1.4 kcal/min was required for sustained release of GLP-1. However, meals containing different amounts of energy from fat and carbohydrate may have different effects on GLP-1. Rijkelijkhuizen et al. found that carbohydrate-rich (energy bar and drink) (109 g, 66 % from total energy) and not fat-rich (butter, cheese, high-fat milk) (49 g fat, 52 % from total energy intake) meals increased GLP-1 in healthy subjects (Rijkelijkhuizen et al., 2010). Another study found that the equicaloric content of meals from fat (42 g, 45% from total energy) and glucose (100 g, 42 % from total energy) increased GLP-1 secretion at 150 and 30 min, respectively. No significant result was observed for protein (352 g, 13 % from total energy) on GLP-1 secretion (Elliott et al., 1993.) This suggests that carbohydrate meals stimulated the release of GLP-1 at low and high energy content.

In humans, monoinfusion of PYY_{3-36} or GLP-1 alone did not reduce food intake, but the combination of both significantly reduced it (Schmidt et al., 2014). However, Flint et al. showed GLP-1 infusion alone beneficially reduced energy intake and enhanced perceived

satiety measures (Flint et al., 1998). Studies of GLP-1 have received much attention because of their multiple actions on gastrointestinal motility, reducing postprandial glucose responses and reduced energy intake. Apart from carbohydrate and fat, numerous studies have been reported on the effects of soluble dietary fibres on GLP-1 responses (**Table 1-8**).

Juvonen et al. showed how low viscosity β-glucan in a beverage reduced the GLP-1 response when compared to high viscosity (Juvonen et al., 2009). The trend was similar to those observed for PYY concentration. Non-viscous oligofructose (10 and 55 g) in a beverage had no effect on postprandial GLP-1 responses up to 8 h (Verhoef et al., 2011; Pedersen et al., 2013). GLP-1 did not change significantly after intake of soluble low viscosity mucilage prepared in buns. The study meal was eaten as a composite meal with cheese, butter and ham (composite meal) and might have different effects (Kristensen et al., 2013). Johansson demonstrated a longer observation for up to 16 h after consuming boiled barley kernel (97 g) at dinner (Johansson et al., 2007). Barley kernel was boiled for 25 min in 125 mL of water and this study indicates that all water was absorbed into the barley kernel. Hence, it is speculative that this meal is highly viscous. This procedure is similar to the preparation of porridge and the hot-water extraction of β-glucan (Johansson et al., 2007; Panahi et al., 2007). GLP-1 significantly increased when compared with white bread, when measured the following day (10 – 16 h) after standardised breakfast and lunch. Breath hydrogen (H_2) significantly increased when measured at 10.5 to 16 h. This study suggests the role of colonic fermentation as a key factor leading to the observed increased in GLP-1.

GLP-1 is activated by short chain fatty acids (SCFA) through G protein-coupled receptor 41 (GPR41, found in adipose tissue) and GPR43 (found in immune cells) (Brown et al., 2003; Kimura et al., 2004). GPR41 and GPR43 were both activated by SCFA but with different specificity for carbon length. For GPR43, the relative affinity for SCFA are as follows: acetate (C_2) = propionate (C_3) = butyrate (C_4) > pentanoate (C_5) > hexanoate (C_6) = formate (C_1) (Brown et al., 2003). This study also showed that GPR41 was activated by the same SCFA but with different rank of potency being propionate = pentanoate = butyrate > acetate > formate. Le Paul et al. demonstrated that propionate is a potent agonist

for GPR41 and GPR43 while acetate was a more potent stimulator for GPR43 (Le Poul, et al., 2003). Butyrate and isobutyrate were more selective for GPR41. In humans, GPR43 is expressed on enteroendocrine L-cells containing PYY in the large intestine (Karaki et al., 2008).

In the previous section, we already discussed dietary fibres and their physiological effects in humans. β-Glucan is one of the potential candidates for the development of a functional bread. β-Glucan produces beneficial effects on plasma glucose and insulin, and is fully fermented in the colon for the production of SCFA. SCFA particularly propionate may have positive effects in increasing satiety. However, the development of bread with a viscous fibre such as β-glucan showed a lower palatability when compared with white bread. It is important to find an alternative way to improve palatability without losing functionality. (Poly)phenols occur naturally in foods (fruits and vegetables) and beverages such as wine, coffee, tea and chocolate are potential candidates for improving the palatability of this bread.

1.7 Dietary (poly)phenols

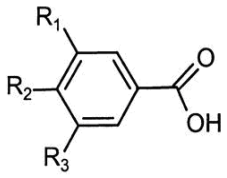
(Poly)phenols are a diverse group of bioactive compounds found in the plant kingdom. They are present abundantly in foodstuffs including whole grains (e.g. sorghum, barley in the range of 2.6 to 39.7 mg/100 g fresh weight), fruits (e.g. apples, blackberries, cranberries, grapes, peaches, pears, plums, raspberries, and strawberries in the range of 8.6 to 496.0 mg/ 100 g fresh weight), vegetables (e.g. cabbage, celery, red onion, and parsley in the range of 0.1 to 139.5 mg/ 100 g fresh weight) and beverages (e.g. black tea, coffee, cocoa and red wine in the range of 0.89 to 477.5 mg/100 g fresh weight) (Bravo et al., 1998; Chung et al., 1998; Duthie and Crozier, 2000; Neveu et al., 2010). Several lines of evidence have suggested that (poly)phenols exert protective effects against chronic diseases. Recent evidence showed that coffee (poly)phenols (chlorogenic acid) improved post-ischaemic flow-mediated dilation in healthy individuals (Ward et al., 2016). The Prevention with Mediterranean diet (PREDIMED) study demonstrated a negative correlation between total urinary (poly)phenols excretion with blood pressure and a

positive correlation with HDL-cholesterol suggesting the protective effect of (poly)phenols-rich diet against cardiovascular risk factors (Medina-Remon et al., 2016). In another PREDIMED sub-group study, the highest tertile of (poly)phenols intake was associated with a 28% reduction in new Type 2 diabetes compared with the lowest tertile of (poly)phenol intake (Tresserra-Rimbau et al., 2014). However, it must be noted that this health benefits may also be due to the presence of dietary fibre associated with the (poly)phenols.

1.7.1 Basic (poly)phenol structures

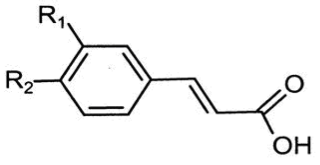
To date, there are at least 8,000 types of (poly)phenol reported in the literature. (Poly)phenolic compounds are derived from cinnamic acid and include the xanthones and flavonoids. (Poly)phenols are products of secondary metabolism of plants; they provide protection against pathogens and parasites, as well as giving the unique colour of plants (Liu, 2004). The basic generic structure consists of two aromatic rings (ring A and B) interconnected by 3 carbons in an oxygenated heterocycle C-ring (**Figure 1-5**). The structure of (poly)phenols varies from simple molecules, such as the phenolic acids, to highly polymerized compounds, such as condensed tannins (e.g. flavan-3-ol as base unit) (Harborne, 1980). The chemical structure of (poly)phenols influences their biological properties such as bioavailability, antioxidant activity, and specific interaction with cell receptors and enzymes (Scalbert and Williamson, 2000). In particular, the antioxidant activity of these compounds depends on the individual structure and number of hydroxyl groups. **Figure 1-5** shows the basic structure of five common (poly)phenols in the plant kingdom and their derivatives. Major subclasses of flavonoids are shown in **Figure 1-6**.

1) Hydroxybenzoic acids



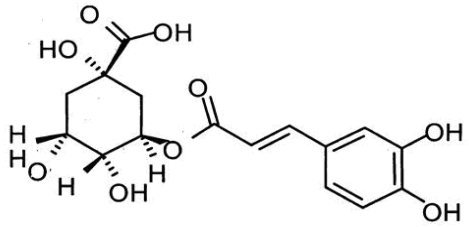
$R_1 = R_2 = OH, R_3 = H$: Protocatechuic acid
$R_1 = R_2 = R_3 = OH$: Gallic acid

2. (a) Hydroxycinnamic acids



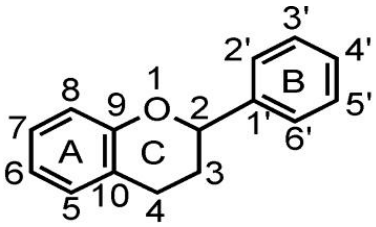
$R_1 = OH$: Coumaric acid
$R_1 = R_2 = OH$: Caffeic acid
$R_1 = OCH_3, R_2 = OH$: Ferulic acid

2. (b) Hydroxycinnamic acids derivative

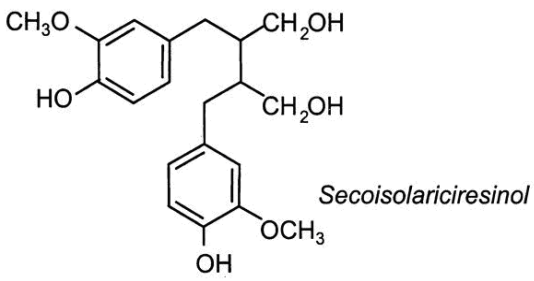


Chlorogenic acid

3) Flavonoids (basic structure)

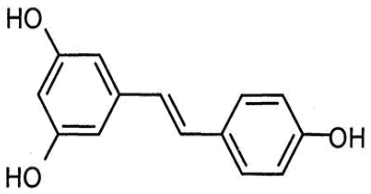


4) Lignans



Secoisolariciresinol

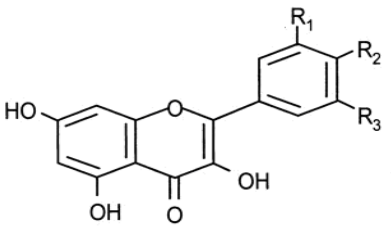
5) Stilbenes



Resveratrol

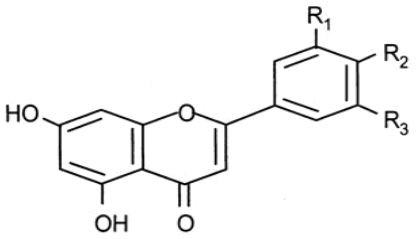
Figure 1-5. Five major groups of (poly)phenols (Manach et al., 2004).

1) Flavonols



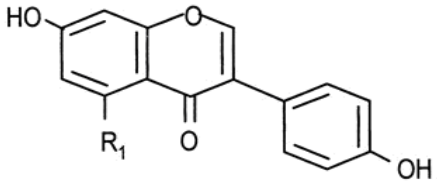
$R_2 = OH; R_1 = R_3 = H$: *Kaempferol*
$R_1 = R_2 = OH; R_3 = H$: *Quercetin*
$R_1 = R_2 = R_3 = OH$: *Myricetin*

2) Flavones



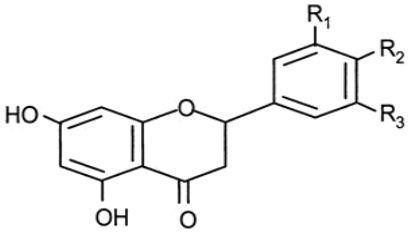
$R_1 = H; R_2 = OH$: *Apigenin*
$R_1 = R_2 = OH$: *Luteolin*

3) Isoflavones



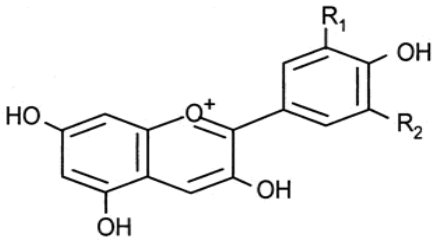
$R_1 = H$: *Daidzein*
$R_1 = OH$: *Genistein*

4) Flavanones



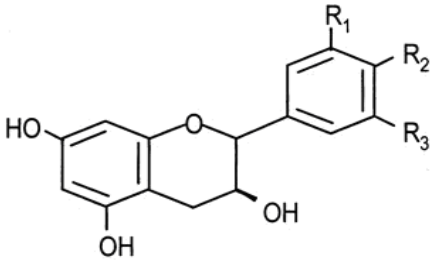
$R_1 = H; R_2 = OH$: *Naringenin*
$R_1 = R_2 = OH$: *Eriodictyol*
$R_1 = OH; R_2 = OCH_3$: *Hesperetin*

5) Anthocyanidins



$R_1 = R_2 = H$: *Pelargonidin*
$R_1 = OH; R_2 = H$: *Cyanidin*
$R_1 = R_2 = OH$: *Delphinidin*
$R_1 = OCH_3; R_2 = OH$: *Petunidin*
$R_1 = R_2 = OCH_3$: *Malvidin*

6) Flavanols



$R_1 = R_2 = OH; R_3 = H$: *Catechins*
$R_1 = R_2 = R_3 = OH$: *Gallocatechin*

Figure 1-6. Subclass of flavonoids (flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols) (Manach et al., 2004).

1.7.2 Tea (poly)phenols

Tea (*Camellia sinensis*) is one of the major sources of (poly)phenols consumed in Europe along with coffee and fruits (Zamora-Ros et al., 2015). According to the different production procedures, they are further classified as black, green and oolong tea. Black tea is the most commonly consumed in European countries, with the highest intake category being 855 mL/day (Bami et al., 2016). It is produced from the young green shoots of the tea plant (UK Tea and Infusions Association, 2016). The harvested leaf is withered before being crushed to achieve efficient disruption of cellular compartmentation. This process is also known as fermentation and takes around 3-4 h to allow the exposure of (poly)phenols compounds to polyphenol oxidase until the leaves turn brown and oxidation is complete (UK Tea and Infusions Association, 2016). Tea Processing and Blending (Kuhnert, 2010). The leaf is then dried or 'fired' quickly (moisture content below 3%) to stop the oxidation process. Black tea produces a strong aroma and a brownish colour when brewed. Green tea is produced by steaming and rolling withered tea leaves before drying or firing. This process inhibits the release of polyphenol oxidase and thus prevents any oxidation of the leaf. The whole leaf is used for making tea and the colour is very pale when brewed. Both black and green teas are processed differently and hence have different (poly)phenol composition (Balentine et al., 1997). Oolong tea is a semi-green and semi-black tea, which follows a similar production process as black tea, but the oxidation time is half that of black tea production (1-2 h) before being fired or dried.

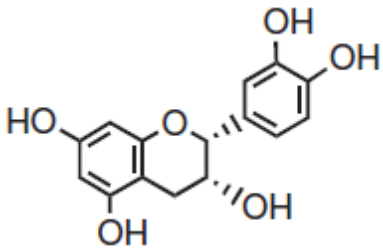
Flavan-3-ols are the main flavonoids found in teas and they constitute about 30 % of the dry weight of green tea and 9% of the dry weight of black tea (Harbowy and Balentine, 1997). The basic flavan-3-ol structure in tea is shown in **Figure 1-7**. Green tea contains more flavan-3-ols monomers compared to black tea. Lee et al. have shown that an infusion of green tea (1 L) contained 1 g catechins (Lee et al., 1995). High-performance liquid chromatography (HPLC) is widely used for the determination of flavan-3-ols, which is much easier than the determination of thearubigins (TR) and theaflavins (TF) that requires more sophisticated analysis, e.g. LC-MS-MS (Kuhnert, 2010).

Black tea contains a significant amount of glycosylated flavonoids, typically 30-40% of the

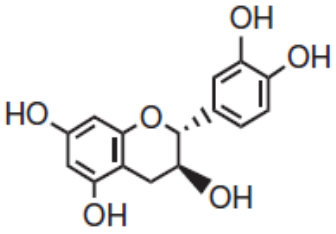
dry mass of black tea infusion and 60-70% of uncharacterised (poly)phenols fermentation products (Kuhnert, 2010). Black tea contains TF and TR, which are complex structures formed by enzyme-catalyzed oxidation (**Figure 1-8**). TF and TR are made up from two units of flavan-3-ol. TF and TR are two major groups of pigments in black tea, which are derived by enzymic oxidation of green tea fermentation (Davies et al., 1999). During black tea production, the fermentation process by polyphenol oxidase utilises flavan-3-ols monomers as substrate and subsequently decreases their content while increasing TF and TR levels (Del Rio et al., 2004). The sum of theaflavins in black tea (theaflavin, theaflavin-3-gallate, theaflavin-3´-gallate and theaflavin-3,3´-digallate) is 224 mg/L (Del Rio et al., 2004). In addition, black tea contains substantial concentrations of the flavanols quercetin (10-25 mg/L), kaempferol (7 - 17 mg/L), and myricetin (2-5 mg/L) (Hertog et al., 1993). The antioxidant activity of tea flavan-3-ols catechin, catechin gallate (CG), epicatechin (EC) and epicatechingallate (ECG) depends on the presence of an ortho-3´, 4´-dihydroxy moiety in the B ring that is involved in electron delocalisation and stabilises the radical form (Sharma and Zhou, 2011). Gallocatechin has a third hydroxy group in the B ring instead of two. Gallate compounds (ECG and EGCG) possess a galloyl moiety attached to C ring and this adds three more hydroxy groups to the compounds. Chen and Chan demonstrated that EGC possessed a higher antioxidant activity when compared with EC due to the presence of OH group at position 5´ on ring B (Chen and Chan, 1996).

Epimerisation of catechins happens by conversion to their respective isomers. Epimers of catechins, namely EGCG, EGC, ECG and EC can be converted to their non-epimers GCG, GC, CG and C (Wang et al., 2008; Chen and Chan, 1996). The levels of catechins could be easily reduced by means of epimerisation and degradation during processing (Wang et al., 2008). In this study, two specific temperature points were identified as 44°C and 98°C in the reaction kinetics. Degradation was significant below temperature 44°C. Epimerization from GCG to EGCG occurred at temperature above 44°C and the epimerization from GCG to EGCG become more prominent at 98°C. During fermentation of black tea, the degradation of catechins was faster at 35°C when compared with 20°C (Samanta et al., 2015). A lower temperature (20°C) increased the production ratio of TFs/TRs, which is desirable for the production of quality black tea. Black tea fermentation with endogenous polyphenol oxidase produced TFs from catechins and generated H_2O_2 under normal

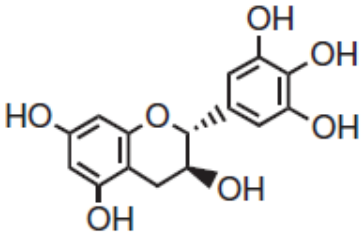
manufacturing conditions (pH 5.5).



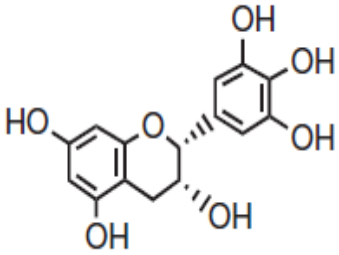
(–)-epicatechin



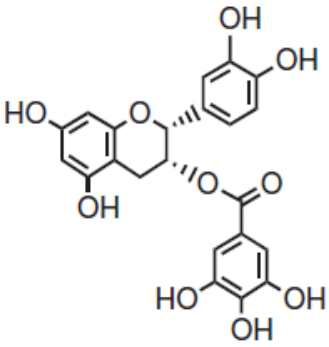
(+)-catechin



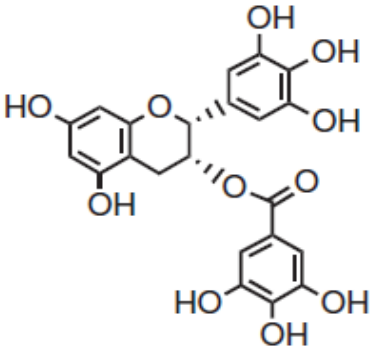
(+)-gallocatechin



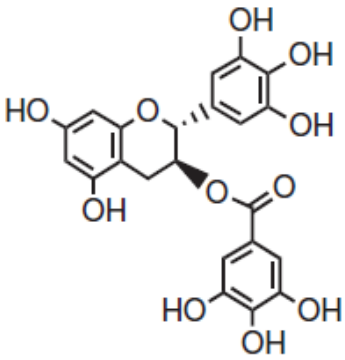
(–)-epigallocatechin



(–)-epicatechin gallate

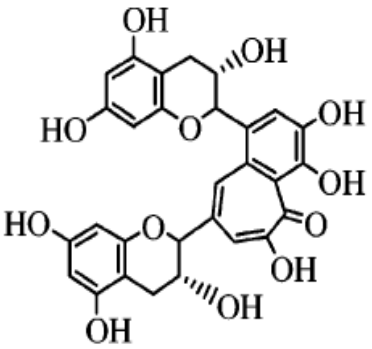


(–)-epigallocatechin gallate

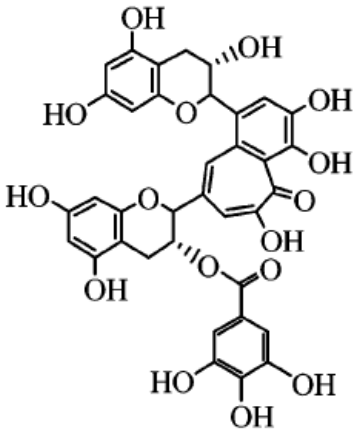


(+)-gallocatechin gallate

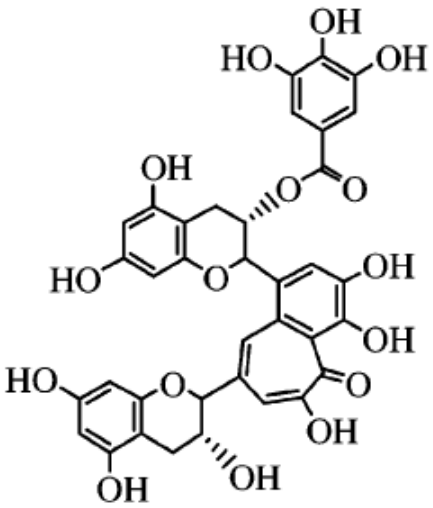
Figure 1-7. Chemical structure of the most common flavan-3-ols in tea (Roowi et al., 2010)



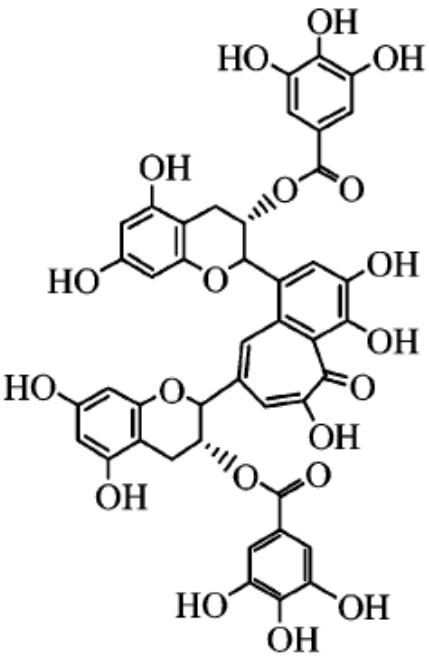
Theaflavin



Theaflavin-3-gallate



Theaflavin-3´-gallate



Theaflavin-3,3´-digallate

Figure 1-8. Theaflavins found in black tea (Del Rio et al., 2004)

1.7.3 Tea in food products

Tea is rich in (poly)phenols, which are stable at a high temperature (Del Rio et al., 2004; Lun Su et al., 2003). Hence, tea is suitable for the development of various food products such as cakes, biscuits and bread (**Table 1-10**). Tea has been added to bread as green tea extract (GTE) at the level of 150 mg/ 100 g flour with one slice of bread (53 g) containing 28 mg of tea flavan-3-ols. This is equivalent to 35% of those found in a 2 g of tea bag (250 mL of tea) (Wang and Zhou, 2004). Tea flavan-3-ols were stable and 84% of total green tea catechins were retained during bread baking (Wang and Zhou, 2004).

Sponge cake using green tea (poly)phenols (GTP) powder as a substitution (10 and 20%) for wheat flour was successfully developed and compared with non-substituted control (Lu et al., 2010). Specific flavan-3-ols were detected in the extract as gallocatechin (GC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin gallate (GTG) and epicatechin gallate (ECG) in a dose dependent manner from 10 to 30 % green tea powder. This result suggests that tea catechins were stable at a high temperature and could be used as functional food ingredients. Crust and crumb colour, sweetness, flavor, texture and overall acceptability of cake prepared with 10 and 20 % GTP were similar to those of white bread (Lu et al., 2010). However, the addition of 30 % GTP in bread produced an unfavorable crust and crumb colour, sweetness, flavour, texture and overall acceptability when compared with white bread.

Sharma and Zhou studied the effects of tea extract addition in biscuit making (Sharma and Zhou, 2011). Tea extracts were added at the levels of 150, 200 and 300 mg per 100 g flour. In this system, the percentage of ECG was higher than EGCG as the former is more stable (Zhu et al., 1997). EGCG and ECG were stable during dough development but decreased as the baking took place. Before baking, the levels of EGCG and ECG were 65% and significantly reduced to 20%. The presence of alkaline-inducing agents such as sodium bicarbonate and baking powder could increase the pH and this will cause a loss of EGCG. Hence, it could be concluded that heat treatment during baking and an increase in pH lead to the loss of EGCG. This study also showed that catechins were stable at acidic pH

(Sharma and Zhou, 2011). A recent product in which tea encapsulated with maltodextrin in bread and showed similar bread volume, crumb firmness, taste and colour when compared with GTE alone and control bread (Pasrija et al., 2015). This may protect tea (poly)phenols from being degraded during baking and may also prevent starch retrogradation (Wang et al., 2016).

Table 1-9. Tea in food products.

Product	Amount (mg or g per 100 g flour)	Formulation	Reference
Bread	2.0 g/ 100 g flour	Freeze-dried microencapsules	(Pasrija et al., 2015)
Bread	50, 100 and 150 mg/ 100 g flour	GTE powder	(Wang and Zhou, 2004)
Cake	10, 20, and 30 g/100 g flour	GT powder	(Lu et al., 2010)
Biscuit	150, 200 and 300 mg/100 g flour	GTE	(Sharma and Zhou, 2011).

GTE: green tea extract; GT: Green tea.

1.8 Physiological effects of tea (poly)phenols

A systematic review showed that long-term (8 to 24 weeks) intake of tea and tea products in different forms (capsule, extract and beverage) is associated with a reduced BMI and waist circumference (Amiot et al., 2016). Central obesity (elevated waist circumference) is one of the established risk factors for metabolic syndrome (Alberti et al., 2009). Other metabolic syndrome factors are elevated blood triglycerides, reduced high-density lipoprotein, systemic hypertension and elevated fasting glucose. Three abnormal changes of these parameters give a diagnosis for the metabolic syndrome. One animal study showed black tea (3.13 and 62.6 mg/ kg body weight) dose-dependently reduced glucose levels at 30, 60 and 90 min when compared with control (Satoh et al., 2015).

1.8.1 Tea (poly)phenols metabolism and absorption

The breakdown of flavan-3-ols begins in the mouth (**Table 1-11**). Drinking one cup (1.2 g tea extract in 200 mL) of green tea increased peak saliva levels of (–)-epigallocatechin (EGC), (–)-epigallocatechin-3-gallate (EGCG) and (–)-epicatechin (EC) (Yang et al., 1999). The enzyme esterase (extracted from the oral cavity) converts EGCG to EGC (removal of gallate) and this lead to the compounds being absorbed through the oral mucosa. An *in vitro* study showed that the salivary mucosa contains 74.8 to 99.5% intact flavan-3-ols from total tea with EGCG and catechin being the dominant forms (Tenore et al., 2015).

Table 1-10. Salivary, plasma and urine concentrations of tea (poly)phenols after green tea extract ingestion (200 mL).

	Peak occurrence time (h)	**EGC**	**EGCG**	**EC**	**Reference**
Saliva (μg/mL)	0.2 - 0.3	11.7 – 43.9	4.8 - 22	1.8 – 7.5	(Yang et al., 1999)
Plasma (ng/mL)	1	82 - 206	46 - 268	48 - 80	(Lee et al., 1995)
Urine (mg/24 h)	3- 6	2.8 to 3.2	Not detected	1.6 – 2.3	(Lee et al., 1995)

Acute consumption of green tea (3 g tea in 300 mL) led to the appearance of flavan-3-ols and their metabolites in plasma from 29 to 126 nM, and a peak time between 1.6 and 2.3 h, an indication of absorption in the small intestine (Stalmach et al., 2010). Flavan-3-ol aglycones are readily absorbed in the small intestine when compared with (poly)phenols containing esters, glycosides or polymers, which require catabolism by the intestinal enzymes or by the colonic microflora before they can be absorbed (**Figure 1-9**) (Manach et al., 2004; Henning et al., 2008). Stalmach et al. showed that flavan-3-ols were conjugated with sugars (Stalmach et al., 2010). The linked sugars can be glucose, rhamnose, galactose,

arabinose, xylose, and glucoronic acid (Harborne, 1994). Therefore, removal of sugars by glycosidases enzymes is important in order to make them bioavailable in the small intestine (Scalbert and Williamson, 2000).

EC and (–)-epicatechin gallate (ECG) were absorbed more efficiently than EGCG and (+)-gallocatechin gallate (GCG) (Auger et al., 2008). Henning et al. showed that non-gallated flavan-3-ols and theaflavins (TF) were more bioavailable than their gallated counterparts (Henning et al., 2008). In an *in vitro* system, EC was more bioavailable than ECG and TF were more bioavailable than theaflavin-3´-gallate. A similar study showed that catechin recovery after gastric digestion was less than 20%, while EGC and EGCG recovery was less than 10% (Green et al., 2007). Lamothe et al. demonstrated that under simulated digestion, the antioxidant activity of tea (poly)phenols was reduced by 50% during transition from the stomach to small intestine. Rashidinejad et al. demonstrated that an interaction between tea (poly)phenols and proteins reduced the antioxidant activity (Rashidinejad et al., 2015). The presence of galloylated compound in flavan-3-ols as in ECG, EGC and EGCG possessed higher affinity for protein interactions compared with non-galloylated catechin and epicatechin (Le Bourvellec and Renard, 2012; Bordenave et al., 2014).

Apart from proteins, tea (poly)phenols may also form interactions with starch when co-ingested. In humans, co-ingestion of 452 μmol EGCG with glucose or white bread had little effect on (poly)phenols recovery in ileal fluid and urine (Auger et al., 2008). This suggests that the (poly)phenols did not form complexes in the stomach, being absorbed in the small intestine, metabolised in the liver and excreted in the urine. (Poly)phenols, when occurring at natural levels in flours or starches, may reduce starch digestibility by inhibiting enzyme activities of α-amylase or α-glucosidase.

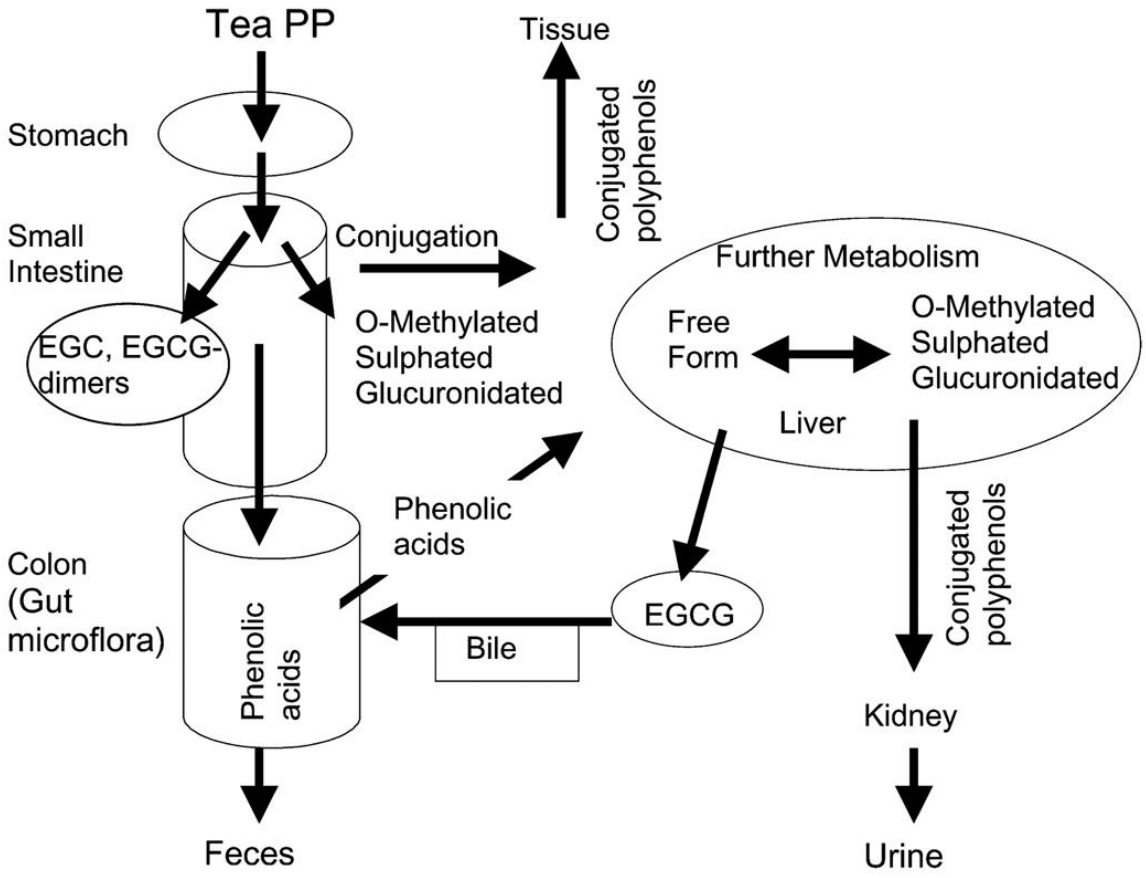
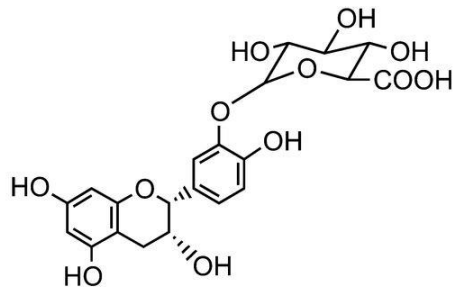
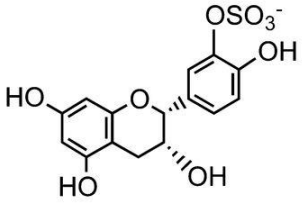


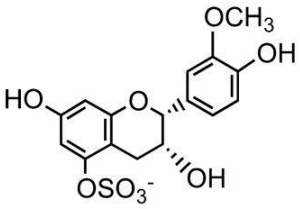
Figure 1-9. Tea flavan-3-ol absorption metabolism in the human body (Henning et al., 2008)



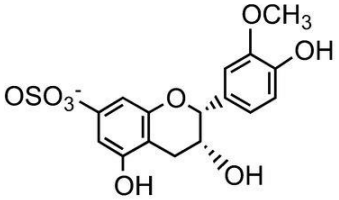
(–)-Epicatechin-3'-*O*-glucuronide



(–)-Epicatechin-3'-*O*-sulfate



3'-*O*-Methyl-(–)-epicatechin-5-*O*-sulfate



3'-*O*-Methyl-(–)-epicatechin-7-*O*-sulfate

Figure 1-10. Structures of main (-)-epicatechin conjugates in plasma (Clifford et al., 2013)

After absorption, tea (poly)phenols may undergo range of conjugations (**Figure 1-9**). Following conjugation, flavan-3-ols re-appear in plasma as glucuronidated, methylated and sulphated forms (**Figure 1-10**). Concentration of monomer flavan-3-ols (46 – 268 ng/mL) (1.2 g in 300 mL green tea) was higher in plasma when compared with large molecular weight theaflavins (700 mg theaflavins, ≈ 30 cups of black tea) (1 to 4.2 ng/mL) (Mulder et al., 2001; Lee et al., 1995). A lower concentration of theaflavins in the plasma suggests that it is not absorbed and may be further metabolised in the colon by the gut microbiota to phenolic acids (Chen et al, 2012; Manach et al., 2004) (this is discussed in the next section of this chapter).

Glucuronidation is the first step of conjugation and the reaction is catalysed by the presence of glucuronosyltransferase in the intestinal mucosa of both the small intestine and the colon (Piskula and Terao, 1998). Specifically, the presence of uridine-5'-diphospho (UDP)-glucuronosyltransferases (UGT) in the endoplasmic reticulum catalyses the mobilisation of glucuronic acid from UDP-glucuronic acid to an acceptor compound (flavan-3-ol) with the formation of a β-D-glucoronide product (Manach et al., 2004; King

et al., 2000). Piskula and Terao demonstrated that 90% of flavan-3-ol was glucuronidated in the intestinal mucosa of rats and appeared in blood circulation as glucuronised form (Piskula and Terao, 1998). Sulphation occurs mainly in the liver by the action of phenolsulfotransferases that transfers sulfate from 3'-phosphoadenosine-5'-photosulfate to a hydroxyl group on (poly)phenols (Manach et al., 2004; Piskula and Terao, 1998). Within 30 min, 50% of flavan-3-ols metabolites were detected in rats' plasma as the sulphated form (Piskula and Terao, 1998). Methylation involves the transfer of methyl group from *S*-adenosyl-L-methionine to (poly)phenols by the action of catechol-*O*-methyl transferase (Manach et al., 2004). The enzyme catechol-*O*-methyl transferase is present in various tissues but the highest amount was observed in the kidneys and the liver (Piskula and Terao, 1998). Forty percent of the plasma flavan-3-ols were present in the methylated form.

Currently, there is limited study focus on the concentration of black tea (poly)phenols that reach the tissue. (Poly)phenols metabolism is complex (**Figure 1-9**) and it could not be ascertained whether conjugated or free (poly)phenols that will reach the target tissues. A study demonstrated that black tea catechins (EGC, EC, EGCG and ECG) accumulated in the kidney and lung of guinea pigs in the range of 13 – 45 ng/g tissue and 15 – 90 ng/g tissue, respectively after administration of black tea (5% w/v) (Ganguly et al., 2016).

1.8.2 Colonic fermentation of tea (poly)phenols

In humans, 50 to 70% of flavan-3-ols reached the colon after drinking a cup of green tea containing 634 µmol flavan-3-ols (Stalmach et al., 2010; Roowi et al., 2010). A similar study showed that 43 - 73% of flavan-3-ols passed from the small intestine to the large intestine after the ingestion of 452 µmol flavan-3-ols (green tea extract, Polyphenon E) (Auger et al., 2008). Free and conjugated tea (poly)phenols may be fermented in the colon. These (poly)phenols were metabolised by the colonic microbiota to phenolic acids and subsequently re-absorbed for further metabolism in the liver or excreted in the urine (8%) or faeces (Stalmach et al., 2009).

In vitro incubation of (–)-epicatechin, (–)-epigallocatechin and (–)-epigallocatechin-3-*O*-

gallate with faecal slurries produced ~ 40% of 4-hydroxyphenyl acetic acid, (–)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, urolithin A-3-*O*-glucuronide, and urolithin A-8-*O*-glucuronide (**Figure 1-11**) (Roowi et al., 2010; Clifford et al., 2013). The human gut microflora metabolised tea (poly)phenols to 3-(3'-hydroxyphenyl)propionic and 3-(4'-hydroxyphenyl)propionic acids (Goodwin et al., 1994; Phipps et al., 1998). Phenyl propionic acids might be further metabolised to hydroxyphenyl-γ-valerolactones and phenolic acids through ring fission at the central C-ring (Scheline, 1999; Hollman and Katan, 1997). These metabolites are reabsorbed from the colon and metabolised into benzoic acid by means of β-oxidation in the liver before being excreted as hippuric acid in urine (Meselhy et al., 1997; Curtius et al., 1976; Bennet et al., 1992). In general, hippuric acid production is expected to increase after the consumption of (poly)phenols-rich diets. However, hippuric acid could also derive from quinic acid, aromatic amino acids (tryptophan, tyrosine and phenylalanine) and also benzoic acid (naturally present or as in food preservatives) (Scheline, 1999).

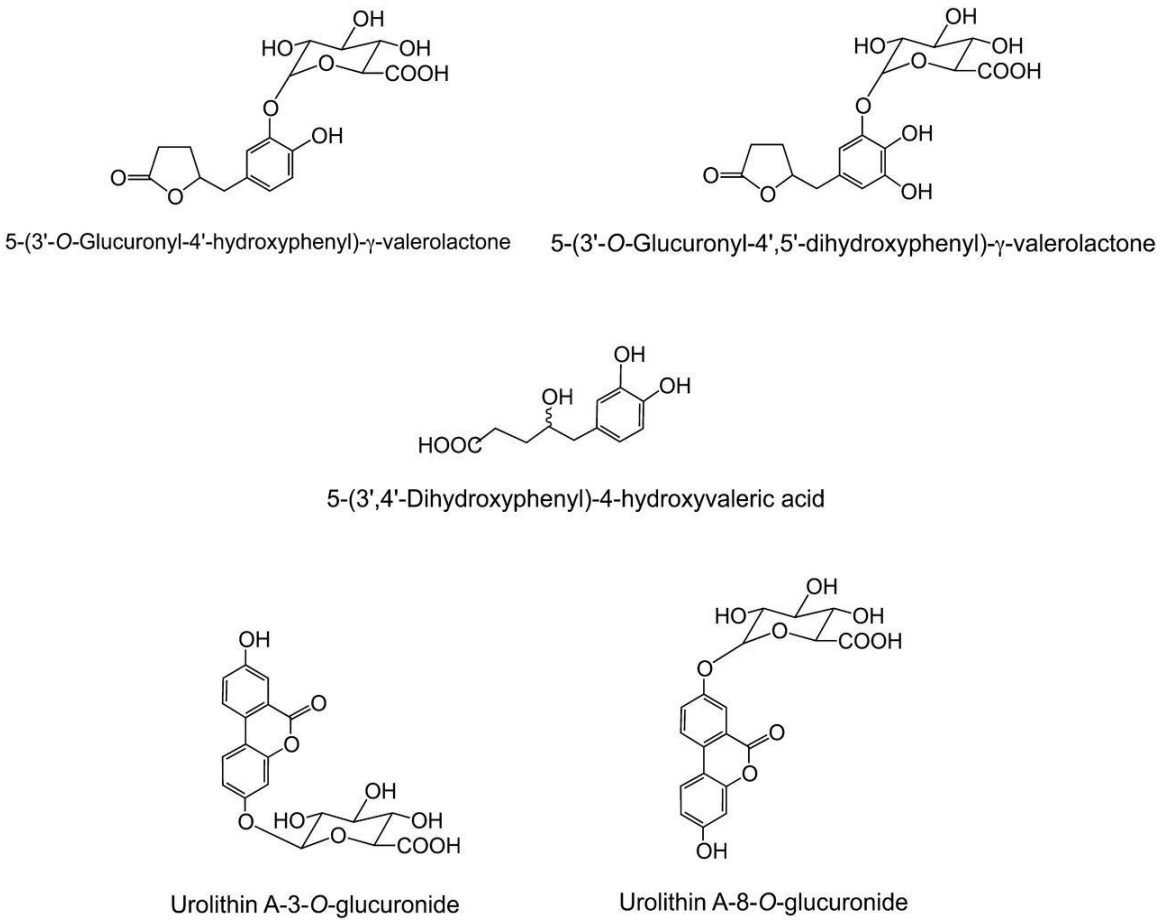


Figure 1-11. Structures of phenolic acids from the catabolism of green tea flavan-3-ols (Clifford et al., 2013)

In humans, black tea consumption resulted in hippuric acid (in urine) formation ranging from 334 to 1141 mg/day (Clifford et al., 2000). Another study showed that approximately 45% of ingested flavan-3-ols (5.4 mmol/24 h) from tea consumption were converted to hippuric acid (2.3 mmol/ 24 h) (Mulder et al., 2005). Both black and green tea intake resulted in significant production of hippuric acid in urine with 3.8 mmol/24 h and 4.2 mmol/24 h, respectively, when compared with control (Mulder et al., 2005). Therefore, both black tea and green tea intake had similar effects on urinary excretion of hippuric acid.

1.8.3 Effects of tea intake on postprandial glucose and insulin levels

Tea (poly)phenols are highly absorbable in the intestine, metabolised in the liver, reaching

the colon for colonic fermentation (43 – 73 % detected in the colon) and finally excreted in urine or faeces (Stalmach et al., 2009; Stalmach et al., 2010; Piskula and Terao, 1998). In rats, the presence of black tea (poly)phenols in the small intestine inhibited α-amylase and/or α-glucosidase enzymic activity on the apical side of the enterocyte (Satoh et al., 2015). Black tea extract at a dose of 62.5 and 250 mg/kg body weight (≈ nine cups of black tea) significantly reduced plasma glucose levels of experimental rats at 30 and 60 min after an OGTT (Satoh et al., 2015). This study demonstrated that black tea extract inhibited enzyme activity of α-glucosidase (inhibits conversion of disaccharides to monosaccharides) by 50% in the small intestine, resulting in reduced postprandial glycaemia (Satoh et al., 2015). Flavan-3-ols (ECG and EGCG) and theaflavin-3-3′-di-*O*-gallate are responsible for the inhibition of α-glucosidase activity. In rats, 5 mg/kg body weight of pu-erh tea (a type of black tea from the Yunnan province, China) reduced glucose levels by 7% at 30 min when compared with acarbose, a drug commonly used for glycemic control in type 2 diabetes (Deng et al., 2015). There was higher reduction (29%) when the tea dose was doubled to 10 mg/ kg body weight at the same time point.

In vitro α-glucosidase activity is measured as a concentration that induces 50% enzyme inhibition (IC_{50}). Lower IC_{50} value indicates higher inhibition towards enzyme activity. Koh et al. showed a higher inhibition of black teas on α-glucosidase activity when compared with green and oolong teas with IC_{50} of 0.56 mg/mL and 2.8 mg/mL, respectively (Koh et al., 2010). Moreover, theaflavin digallate showed a higher inhibition against α-glucosidase with IC_{50} = 165 μM when compared to theaflavin monogallate and teaflavins with IC_{50} = 310 and 400 μM, respectively. The authors postulated that the inhibition could be due to the presence of the galloyl (3,4,5-trihydroxybenzoyl) moiety. However, for flavan-3-ols, ECG showed a higher inhibition against α-glucosidase with IC_{50} = 330 μM when compared with EGCG with IC_{50} = 220 μM. The galloyl moiety is hydrophobic and this group can interact with a side chain of an amino acid of protein through non-covalent interaction (hydrogen bonding) and inhibit the enzyme activity (He et al., 2006). Oral administration of 10 mg/g body weight of theaflavin-3-*O*-gallate reduced plasma glucose levels in rats (Matsui et al., 2007). The observed effect was also related to the inhibition of α-glucosidase by theaflavin-3-*O*-gallate. The presence of a free hydroxyl

group at the 3´- position of this flavan-3-ol was responsible for the inhibitory activity. These mechanisms might be further explained by the interaction of these flavan-3-ols with glucose transporters.

The sodium-dependent glucose transporter (SGLT1) is involved in glucose uptake from the apical surface of the intestinal lumen (follows concentration gradient of facilitated diffusion), while GLUT2 (a protein glucose carrier system) is involved in the exit of glucose (and also other sugars, eg. fructose) to the basolateral surface (Roder et al., 2014). An *in vitro* study utilising rat intestine showed that ECG and EGCG at a dose of 1 mM reduced glucose uptake by 53 and 35%, respectively, when compared with control (Kobayashi et al., 2000). The presence of galloyl moiety on ECG and EGCG is important for blocking glucose uptake. Besides, flavan-3-ols lack a carbon-carbon double bond between C2 and C3 of C-ring, and hence have less electron density. An *in vitro* system showed that the weaker electron density at C-ring was associated with inhibitory activity of flavan-3-ol with salivary α-amylase (Bandyopadhyay et al., 2012).

In vitro and animal studies showed positive effects of tea (poly)phenols on glucose homeostasis. However, the doses used in these studies are far higher than daily human consumption and this raises questions regarding their routine applications. Moreover, in human studies, there was heterogeneity in the methodology and hence the magnitude of effects differs from one study to the other. Human studies used different types of teas (green or black), dosages (e.g. 1 - 3 g in 250 - 300 mL hot water), with different formulations (eg. infusion, capsule, powder). One study used tea extract in powder form and participants were given a capsule that is equivalent to 3.5 cups.

Of the eight intervention studies, three showed a reduction in postprandial glucose response (**Table 1-12**). Acute tea supplementation either in the form of powder, extract or beverage reduced postprandial glucose response in the range of 18 to 52% (absolute response versus control) (Bryans et al., 2007; Park et al., 2009; Tsuneki et al., 2004). However, the reduction in glucose response was associated with increased insulin responses (or hyperinsulinaemia). GTE supplementation containing 500 mg EGCG reduced glucose levels at 30 and 60 min but increased glucose at 120 min by 29 % when

compared with control. The author suggests that GTE reduces early glucose (30 and 60 min) by decreasing glucose entering the intestine, while the presence of gallated catechin in blood circulation possesses a hyperglycaemic effect later at 120 min, by blocking glucose uptake into the tissues (Park et al., 2009). The author supported the results of an *in vitro* study, showing that cell lines treated with 1 and 10 μM EGCG (gallated catechin) decreased glucose (2-deoxy-[^{3}H]) uptake into hepatocytes (HepG2), adipocytes (3T3-L1), and beta cells (INS-1) (Park et al., 2009).

Three studies showed that tea supplementation did not reduce glucose response nor did it affect insulin release (**Table 1-12**). GTE at the levels of 0.4% or at high dose (3 capsules equivalent to 3.5 cups) was not associated with a reduced postprandial glucose response (Venables et al., 2008; Coe and Ryan 2016). Another one study showed how tea intake as a beverage had no effect on postprandial glucose response (Louie et al., 2008; Aldughpassi et al., 2008). These studies differed in brewing time (30 seconds to 3 min), and this time might be too short to efficiently extract the (poly)phenols (Sandip et al., 2013). Sandip et al. and Molan et al. showed that a minimum of 10 min is required to achieve maximum (poly)phenols content and antioxidant activity (Sandip et al., 2013; Molan and Meagher, 2009). Other factors such as extraction temperature, ratio of tea to extracting water and stirring might also have an impact on (poly)phenol content (Molan and Meagher, 2009). Moreover, these studies were conducted among healthy volunteers who may have an optimum dietary status, in which supplementation with tea may have no further effects (Cooper et al., 2008). One study showed a high dose of green tea (9 g in 300 mL) as a hot beverage increased glucose response compared with control (Josic et al., 2010). Possible explanations could be: 1) There is an intra individual variations in the metabolism and bioavailability of catechins in humans (Higdon and Frei, 2003. 2) *In vitro* work showed the presence of gallated catechin in the circulation increases blood glucose by blocking normal glucose entry into the tissues and hence secondary hyperinsulinaemia. The presence of gallated catechin in the intestinal lumen decreased glucose entry into the circulation and produced a lower blood glucose response (Park et al., 2009).

1.9 Effects of drinking tea on perceived satiety

A meta-analysis study showed that green tea has small positive effects on weight loss and weight management (Hursel et al., 2009). Tea catechin intake is associated with reduced body weight or maintained body weight, after a weight loss of ~1.3 kg (95% CI: -2.05, -0.57 kg). A summary of acute studies investigating the effects of tea on perceived satiety and energy intake in healthy subjects is shown in **Table 1-13**. Tea drinking is generally associated with an increase in perceived satiety and reduced energy intake at lunch. However, not all studies showed a positive outcome regarding weight loss and management, due to the differences in study design, ethnicity, types of tea mixtures, and concentrations of catechins (Hursel et al., 2013).

In a randomised controlled trial (n = 14), Josic et al. showed that green tea drinking (9 g in 300 mL) increased perceived satiety and fullness (Josic et al., 2010). Reinbach et al. studied the effects of green tea drinking at breakfast, lunch and dinner (Reinbach et al., 2009). They found that 350 mL of tea drinking was not associated with energy reduction at dinner (measured *ad libitum*). However, tea drinking reduced perceived hunger by 31% when compared with control. The difference in results between this study and the one by Josic et al. might be due to levels of catechin content (600 vs 200 mg, respectively). Studies among healthy older subjects showed that green tea supplementation for 12 weeks did not have significant effects on resting energy expenditure and body composition (Janssens et al., 2015).

A green tea beverage with soluble fibre dextrin reduced energy intake by 10% at lunch, and reduced perceived hunger by 24% when compared with soluble fibre alone (Carter and Drewnowski, 2012). As described in **Section 1.8.2**, tea (poly)phenols are absorbable in the small intestine and may pass to the colon for colonic fermentation and produce phenolic acids. Soluble dextrin is highly fermentable in the colon and produces SCFA (particularly propionate) and may increase short-term satiety in humans (Guerin-Deremaux et al., 2011).

In humans, the presence of flavan-3-ols inhibits catechol-*O*-methyl transferase (COMT), an enzyme responsible for cleaving norepinephrine in the synaptic cleft. It has been shown

that norepinephrine is a neurotransmitter responsible for the satiety signal in the brain and also plays a role in the control of thermogenesis and fat oxidation (Wellman, 2000; Dulloo et al., 2000). Using a respiratory chamber, Dulloo et al. demonstrated that a GTE capsule containing 90 mg EGCG intake significantly increased 24-h energy expenditure (EE) by 3.5% when compared with a placebo (Dulloo et al., 1999). This study also showed that the group fed with the GTE capsule had lower carbohydrate oxidation and higher fat oxidation when compared to the placebo, suggesting that there is a shift in substrate utilisation in favour of fat. GTE supplementation increased 24-h norepinephrine when compared with the placebo group. However, it must be noted that this is a pharmacological approach and may not be relevant to satiety study.

Using indirect calorimeter, Komatsu et al. demonstrated that oolong and green tea increased EE by 10% and 4% when compared with water (Komatsu et al., 2003). This percentage difference in EE may be due to the higher content of high molecular weight (poly)phenols TR and TF in oolong tea when compared with green tea. The cumulative increases of EE for 120 min significantly increased from 10% and 4% after the consumption of oolong tea and green tea, respectively. In a 12-week supplementation study, taking 9 capsules of GTE containing 1.36 g flavan-3-ols/day had no effects on resting energy expenditure (Janssens et al., 2015). Similar effects were obtained from a study regarding the consumption of tea capsules containing 1.21 g flavan-3-ols/day (Diepvens et al., 2006). Despite differences in dosage, tea may have positive short-term effects on energy expenditure.

Table 1-11. Acute human studies of teas on postprandial glucose and insulin levels in healthy subjects.

Study design	Subject	Study duration	Dose	Parameters	Major outcomes	Reference
Non randomised, non blind	Healthy subjects, n = 22 (age and gender not specified)	2 h postprandial	Green tea powder (1.5 g in 250 mL hot water) containing 108 mg total catechins	Plasma glucose and insulin (OGTT challenge)	↓ glucose at 30 min (17.8%) and 120 min (37.81%)	(Tsuneki et al., 2004)
Randomised, crossover, non blind	Healthy subjects, n = 14 (7M/7F), age 27 ± 3 years	2 h postprandial	Green tea (9.0 g in 300 mL hot water for 3 min) containing 202 mg catechin, taken with bread containing 50 g available carbohydrate	Plasma glucose and insulin (OGTT challenge)	↑ glucose at 120 min (63.8%) Insulin unchanged	(Josic et al., 2010)
Non-randomised, non blind	Healthy, n = 6 (all males), age 20 - 29 years	2 h postprandial	GTE containing 500 mg EGCG	Plasma glucose and insulin	↓ glucose at 30 min (23.0%) and 60 min (48.3%), but ↑ increased glucose at 120 min (29.1%) ↑ insulin	(Park et al., 2009)
Non randomised, crossover	Healthy subjects, n = 12 (all males), age 18 to 35 years	2 h postprandial	GTE (3 capsule), containing 340 mg total (poly)phenols and 136 mg	Plasma glucose and insulin (OGTT challenge)	Glucose unhanged ↓ insulin (16%) ↑ insulin sensitivity index* (13%)	(Venables et al., 2008)

			EGCG), (equivalent to approximately 3.5 cups green tea)			
Randomised, crossover	Healthy subjects, n = 16 (4M/12F), age 36 ± 2 years	2.5 h meal test (postprandial)	Black tea, 1 g in 250 mL hot water, containing 350 mg TP, 39 mg flavan-3-ols and 21 mg theaflavins	Plasma glucose and insulin (OGTT challenge)	↓ glucose (52%) in OGTT at 2 h ↑ insulin at 90 min	(Bryans et al., 2007)
Non randomised, crossover	Healthy subjects, n = 10 (6 F/4 M), age 31 ± 10 years	2 h postprandial	Black tea, 1 tea bag in 250 mL hot water for 30s Taken with bread containing 50 h carbohydrate	Plasma glucose response and OGTT challenge	No effect on postprandial blood glucose but reduced variability in blood glucose reading compared to water	(Aldughpassi et al., 2008)
Non randomised, crossover	Healthy subjects, n = 8 (5 M/ 3 F), age ranging from 26.3 ± 1.8 years	2 h postprandial	Black tea, 1 tea bag in 250 mL of hot water for 3 min	Plasma glucose and insulin response	No significant changes in glucose and insulin responses	(Louie et al., 2008)
Randomised, crossover	Healthy subject and slightly overweight, n = 13 (9 F/4 M), age 20 - 46 years	3 h postprandial	0.4 % GTE in bread containing 50 g available carbohydrate	Plasma glucose and insulin response	No significant changes in glucose and insulin responses	Coe and Ryan, 2016

OGTT challenge – oral glucose tolerance test with 75 glucose, AUC: area under the curve, TP: total (poly)phenols, EGCG: epigallocatechin gallate, GTE: green tea extract, Insulin sensitivity index: mathematical approach to estimate insulin sensitivity based on fasting plasma glucose and fasting serum insulin.

Table 1-12. Acute human studies of teas on perceived satiety and energy intake in healthy subjects.

Study design	Subject	Study duration	Dose	Parameters	Major outcomes*	Reference
Randomised, crossover, non blind	Healthy subjects, n = 14, 7 male and 7 female, 27 ± 3 years	2 h postprandial	Green tea (9.0 g in 300 mL hot water for 3 min) containing 202 mg catechin, taken with bread containing 50 g available carbohydrate	Hunger, fullness, desire to eat	↑ satiety ↓ desire to eat ↑ fullness ↑ AUC fullness at 0-90 min and at 0 – 120 min	(Josic et al., 2010)
Randomised, crossover, non blind	Healthy subjects, n = 27 (10 male and 17 female), age of 26.9 ± 6.3 years	3 times per day at breakfast, lunch and dinner	Green tea (350 mL) (598.5 mg catechins, 77 mg caffeine)	Energy intake, perceived satiety	No effects on energy (dinner) ↑ fullness ↓ hunger	(Reinbach et al., 2009)
Non-randomised, non blind	Healthy subjects, n = 74 (30 males, 44 females), 30.4 ± 7.8 years	3 times at 8:00 am, 10:00 an and 12:00 pm along with meal	Green tea extract (355 mL) containing 167 mg catechins (10 g soluble dextrin fibre and 100 mg caffeine)	Energy intake, perceived satiety	↓ energy intake (lunch) ↓ hunger ↓ desire to snacking	(Carter and Drewnowski, 2012)
Randomised, crossover	Healthy and slightly overweight, n = 13 (9 female and 4 male), age range 20-46	3 h postprandial	0.4 % GTE in bread containing 50 g available carbohydrate	Plasma and insulin response	No significant changes in satiety measures	Coe and Ryan, 2016

AUC – area under the curve (cm.min), * (asterisk) - comparisons were made with control drinks (without tea).

In this chapter, *in vivo* and *in vitro* studies utilising β-glucan or black tea showed promising health benefits (reducing postprandial glucose and insulin responses). Either β-glucan or black tea have been used for the development of functional foods (cake, bread and biscuit). However, there may be additional benefits when these two functional ingredients are added together in a product such as bread. β-Glucan is a viscous soluble fibre that might 'trap' some of the (poly)phenols during bread making. These breads might reduce postprandial glucose and insulin responses more than if only one functional ingredient was added. After digestion, some of the undigested (poly)phenol-linked β-glucan moved to the colon and then fermented to SCFA and particularly propionate which has the potential to increase satiety.

1.10 Hypothesis and aim

Previous studies showed the benefits of β-glucan and black tea on reducing postprandial glucose and insulin responses. However, it is not known whether there is a synergistic effect if these two ingredients are added together. On this basis, it was hypothesised that the bread can be developed using these two ingredients and has similar palatability compared with white wheat bread.

The overall aim of this research project is to study the effects of combining β-glucan and/or black tea in bread on starch functionality (*in vitro* starch hydrolysis), antioxidant properties and fermentability (mimicking human colon). One human study will be carried out to determine the palatability, perceived satiety and the effects on second energy intake at lunch. A second human study will focus on the effects of having this bread at breakfast on postprandial glucose, insulin and gut hormones.

Chapter 2: General materials and methods

This chapter describes the materials used for bread making and all individual analysis for the studies in **Chapter 3**, **4** and **5** (e.g. proximate analysis, *in vitro* antioxidant, *in vitro* batch fermentation, glucose and insulin assays, appetite hormone assays). Detail on study design, sample size calculation, inclusion and exclusion criteria, subjects' recruitment, study protocol and statistical analysis have been described in **Chapters 3, 4** and **5**.

OUTLINE:

The studies in this thesis are described as follows (**Figure 2-1**):

Study one: Development of functional breads with β-glucan and/or black tea, and determination of starch functionality, antioxidant activity and short-chain fatty acids production (**Chapter 3**).

Study two: Effects of functional breads on palatability, perceived satiety and energy intake at lunch (**Chapter 4**).

Study three: Effects of functional breads on blood glucose, insulin responses, and gut hormones (**Chapter 5**).

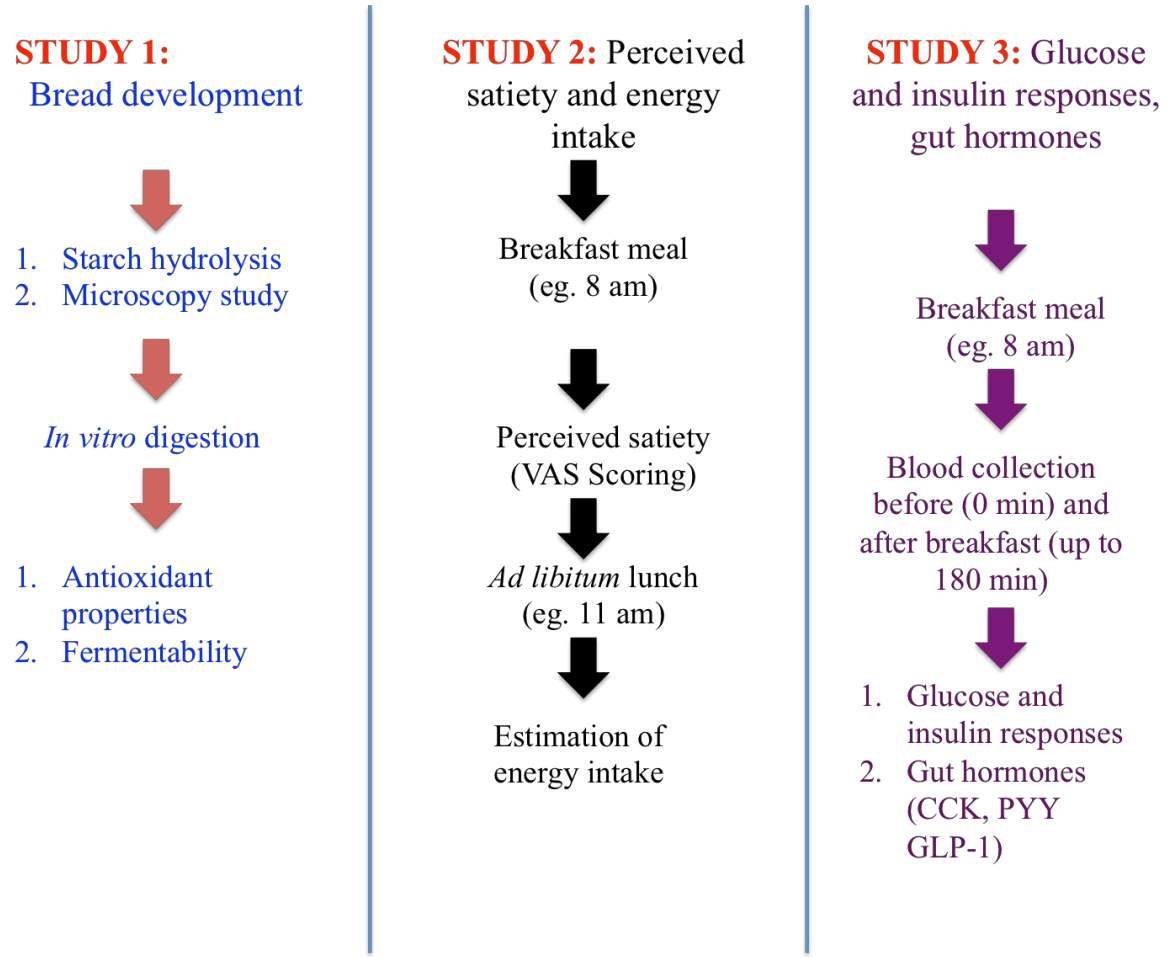


Figure 2-1. Flow chart of general methodology for studies 1, 2 and 3

2.1 Study one: Bread development, starch functionality, antioxidant activity and short-chain fatty acid production from *in vitro* fermentation

2.1.1 Bread development

2.1.2 Materials for bread making

White wheat flour and easy bake yeast were purchased from Allinson (Peterborough, United Kingdom), unsalted butter from Morning Fresh (Caerphilly, United Kingdom), dried skimmed milk powder from WM Morrisons Supermarkets PLC (Bradford, United Kingdom), barley β-glucan concentrate (Glucagel^{TM}) (containing ≥ 75% β-glucan, < 18% carbohydrate, < 10% moisture, 5% protein and other nitrogenous compounds, < 2% lipid and < 2% ash (according to manufacturer data) (DKSH, Quai du Rhône, France) and freeze-dried pure tea granules from Tata Global Beverages GB LTD (containing 452.17 ± 18.93 mg gallic acid equivalents/100 mL) (Greenford, United Kingdom). Black tea (2.5g freeze-dried) was added to 500 g of flours and one portion of black tea (BT) and black tea plus β-glucan (βGBT) bread contained 30% of (poly)phenols which would be ingested from a cup (250 mL) of black tea (Wang et al., 2004; Rothwell et al., 2012).

2.1.3 Bread preparation

Breads were prepared using a standard baking recipe (Burton-Freeman, 2010). All ingredients (**Table 2-1**) were weighed (in triplicate) on a digital kitchen scale (Brabanita, UK) into a baking pan and mixed manually before being placed in a domestic bread maker (Morphy Richards Ltd, South Yorkshire, UK). Breads were prepared using the Chorleywood Bread Process (Chamberlain et al., 1966). The programme of the baking times are as follows: kneading for 10 min, 1st proofing for 20 min, kneading for 15 min, 2nd proofing for 40 min and baking for 65 min (total time = 2 h 30 min). The addition of β-glucan competed with gluten for water; therefore, more water was needed to compensate for water uptake by β-glucan (Jacobs et al., 2008). Fully developed bread was allowed to cool for a total of 60 min, which included 30 min in the bread pan and 30 min at room temperature. The breads were individually weighed and sliced into 1.5 cm thickness. All breads were analysed in triplicate and used for each analysis. The structure of the breads was visualised using a digital still camera at 4x power (Sony Cybershot, Sony Corp, Japan). Fresh breads were used for each study.

Table 2-1. Ingredients for making the test breads

Ingredient (g)	White bread (WB)	Black tea bread (BT)	β-Glucan bread (βG)	β-Glucan + black tea bread (βGBT)
Strong white wheat flour	500.0	500.0	500.0	500.0
Sodium chloride (NaCl)	8.0	8.0	8.0	8.0
Sugar	6.0	6.0	6.0	6.0
Dehydrated yeast	8.0	8.0	10.0	10.0
Butter (unsalted)	6.0	6.0	6.0	6.0
Skimmed milk powder	7.0	7.0	7.0	7.0
β-glucan	0.0	0.0	35.0	35.0
Black tea	0.0	2.5	0.0	2.5
Water (mL)	300	300	540	540
Total	835.0	837.5	1112.0	1114.5

2.2 Proximate analysis

2.2.1 Protein content (Kjedahl method)

The Kjedahl method was developed by a brewer called Johann Kjeldahl in 1883 (McClements, 2003). The amount of protein is calculated from the amount of nitrogen concentration in the food. This method can be divided into three major steps, namely, digestion, neutralisation and titration.

1) Digestion: Preweighed sample was added into *digestion flask* with the presence of sulphuric acid (oxidising agent) and catalyst (potassium sulphate, copper and selenium). The digestion was initiated by heating to 450°C which, converts any nitrogen in the food into ammonia.

2) Neutralisation: Sodium hydroxide is then added to ammonium sulphate, which converts the ammonium sulphate into ammonia gas. The ammonia gas is then liberated and moves out to the receiving flask containing boric acid. At low pH, ammonia gas will be converted into the ammonium ion and at the same time boric acid converts to borate ion.

3) Titration: The nitrogen content is then estimated via the formation of ammonium borate with hydrochloric acid in the presence of an indicator (bromocresol green) to determine the end-point reaction.

The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen in the original food. In this study, one gram of bread was weighed in a digestion flask for protein analysis. The sample was digested at 450°C for 1 h using Foss Tecator Digestor (Foss Tecator AB, Höganäs, Sweden), then distilled and titrated in an automated Kjeltec 2300 Analyzer (Foss Tecator AB, Höganäs, Sweden). A specific nitrogen conversion factor for bread (Jones factor) (N x 5.70, for high protein content food) was used for the calculation of protein content.

2.2.2 Fat content (solvent extraction, Soxhlet method)

Fat content was estimated using a semi-continuous solvent extraction method (Soxhlet) (McClements, 2003b). In this method, one gram of dried and ground sample was weighed

into a porous thimble and carefully loaded into a Soxtec 2050 Automatic System (Foss Analytical AB, Höganäs, Sweden). This system consists of an extraction chamber, a flask and a condenser. This extraction chamber is located above the flask containing 70 mL petroleum ether and below a condenser. The flask was heated to 55°C, which allowed the evaporation of the solvent which then moves to the chamber where it is converted into a liquid form that fills into the extraction chamber containing sample. The solvent extracts the fat as it passes through the sample and carries it into the flask. The fat remains in the flask because of its low volatility. This process continues for 6 h and the flask was removed and evaporated to dryness. All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight.

2.2.3 Resistant starch (RS) and solubilised (digestible) starch

Resistant starch (RS) is the starch and starch degradation products that are not absorbed in the small intestine (Asp and Bjorck, 1992). Resistant starch (RS) was determined using a Resistant Starch kit (Megazyme International Wicklow, Ireland, AOAC Method 2002.02 and AACC Method 32-40, www.megazyme.com). This kit allows robust and reliable measurement and reflects *in vivo* conditions.

Non-resistant starch (solubilised starch) quantification is based on the determination of glucose in a free liquid after enzymic digestion with pancreatic α-amylase and amyloglucosidase (AMG) (**Figure 2-2**). Resistant starch (RS) was measured in the pellet after hydrolysis with potassium hydroxide (KOH) (**Figure 2-3**). This solution was neutralised with acetic acid followed by starch hydrolysis to glucose by AMG. D-glucose content in both non resistant and resistant starch (RS) starch was measured with oxidase/peroxidase reagent (GOPOD).

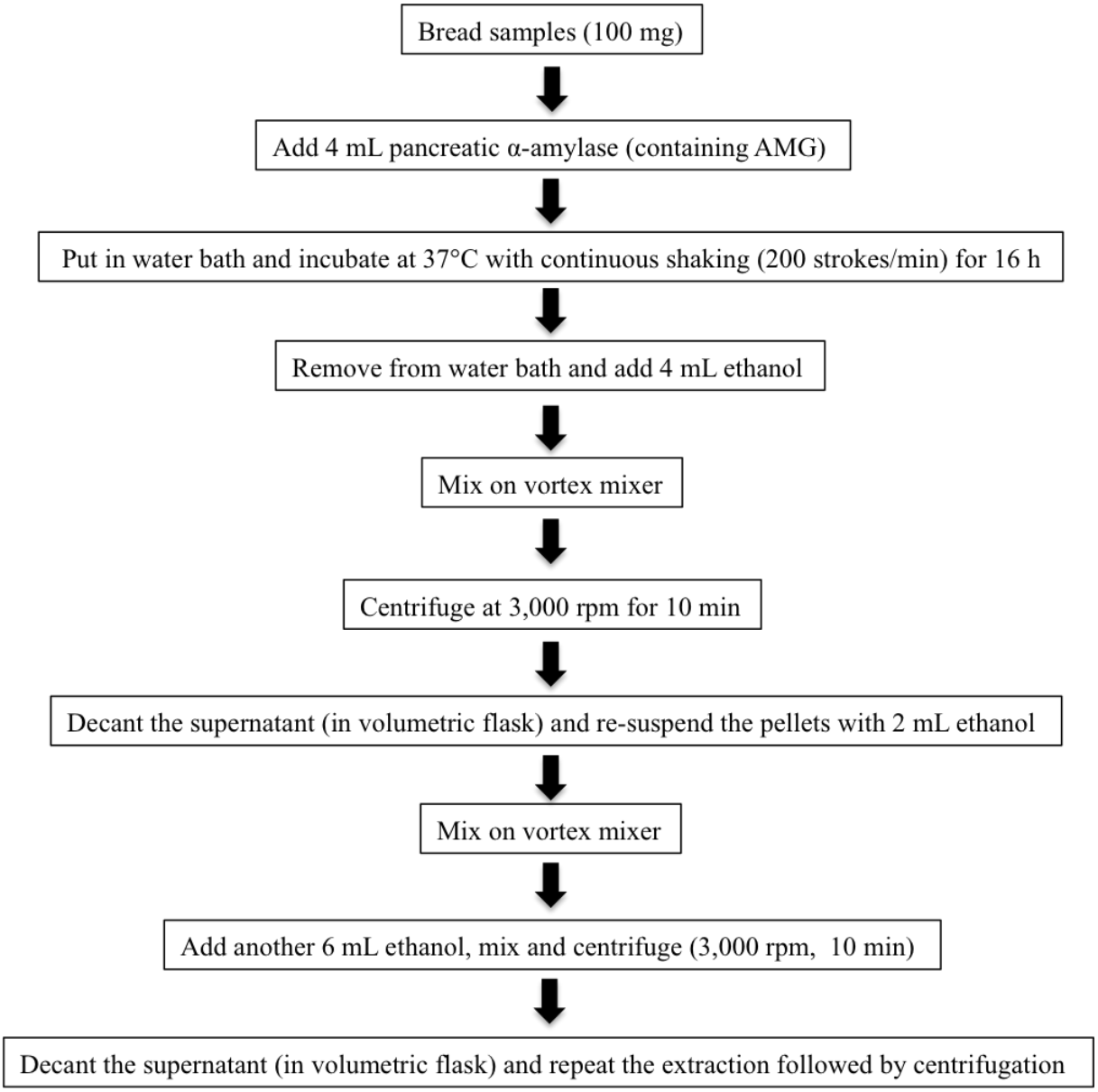


Figure 2-2. Flow chart for hydrolysis and solubilisation of non-resistant starch. AMG, amyloglucosidase

Two millilitres of 2 M KOH were added to each tube to re-suspend the pellets and stirred using a magnetic stirrer for 20 min in an ice water bath (**Figure 2.3**). Thereafter, 1.2 M sodium acetate buffer (8 mL; pH 3.8) was added to each tube and incubated with 0.1 mL of AMG (3300 U/mL) at 50°C for 30 min. An aliquot of the solution was transferred into glass test tubes and 3.0 mL of glucose oxidase/peroxidase (GOPOD) reagent added, incubated at 50°C for 20 min and absorbance measured at 510 nm against the reagent blank (BioMate 3, Thermo Electron Corporation, Madison, USA).

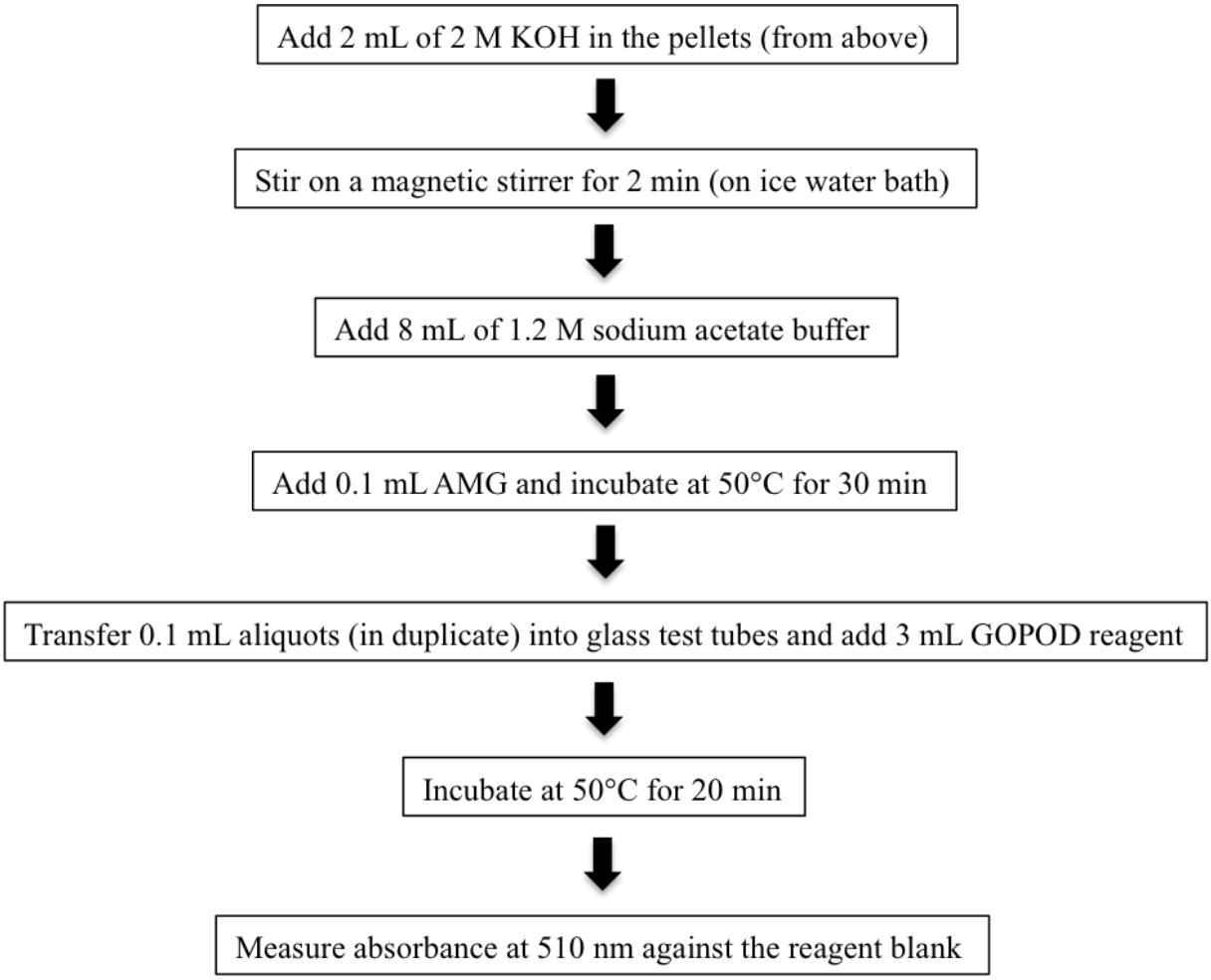


Figure 2-3. Flow chart for the measurement of resistant starch (RS).

GOPOD, glucose oxidase/peroxidase reagent

The supernatant obtained above (**Figure 2-2**) was pooled in a volumetric flask and 0.1 mL of this was incubated with 10 μL of dilute AMG (300 U/mL) solution in 100 mM sodium maleate buffer (pH 6.0) for 20 min at 50°C. Finally, 3.0 mL of GOPOD reagent was added, incubated at 50°C for 20 min and absorbance read at 510 nm against a reagent blank.

All analyses were done in triplicate and results are expressed as g/100 g fresh weight. Resistant starch and solubilised starch were calculated as follows:

$$= \Delta \text{ A x F x } \frac{10.3}{0.1} x \frac{1}{1000} x \frac{100}{W} x \frac{162}{180}$$

$$= \Delta \text{ A x } \frac{\text{F}}{\text{W}} x \ 90$$

Where:

Δ A	= absorbance (reaction) read against the reagent blank
F	= conversion from absorbance to micrograms (100 μg of D-glucose divided by absorbance of this 100 μg of D-glucose)
1/1000	= conversion from micrograms to milligrams
W	= fresh weight
100/W	= factor to present RS (or solubilised starch) as a percentage of sample weight
162/180	= factor to convert from free D-glucose, as determined, to anhydro-D-glucose as occurs in starch
10.3/0.1	= volume correction (0.1 mL taken from 10.3 mL) for samples containing 0 - 10% RS where the incubation solution is not diluted and the final volume is 10.3 mL)

2.2.4 β-Glucan content

A mixed-linkage β-Glucan kit (McCleary method) from Megazyme International Ireland (Wicklow, Ireland) was used for determination of β-Glucan. This assay is based on the breakdown of β-(1→3) and β-(1→4) linkages of β-Glucan. In principle, samples are first suspended and hydrated in a buffer solution of pH 6.5 followed by incubation with purified lichenase enzyme (**Figure 2-4**). An aliquot of the filtrate is then hydrolysed to D-glucose with purified β-glucosidase. The D-glucose formed is measured using glucose-peroxidase (GOPOD) reagent. All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight. β-Glucan content was calculated as described above in **Section 2.2.3**. The volume correction used was 9.4 mL instead of 100/0.1.

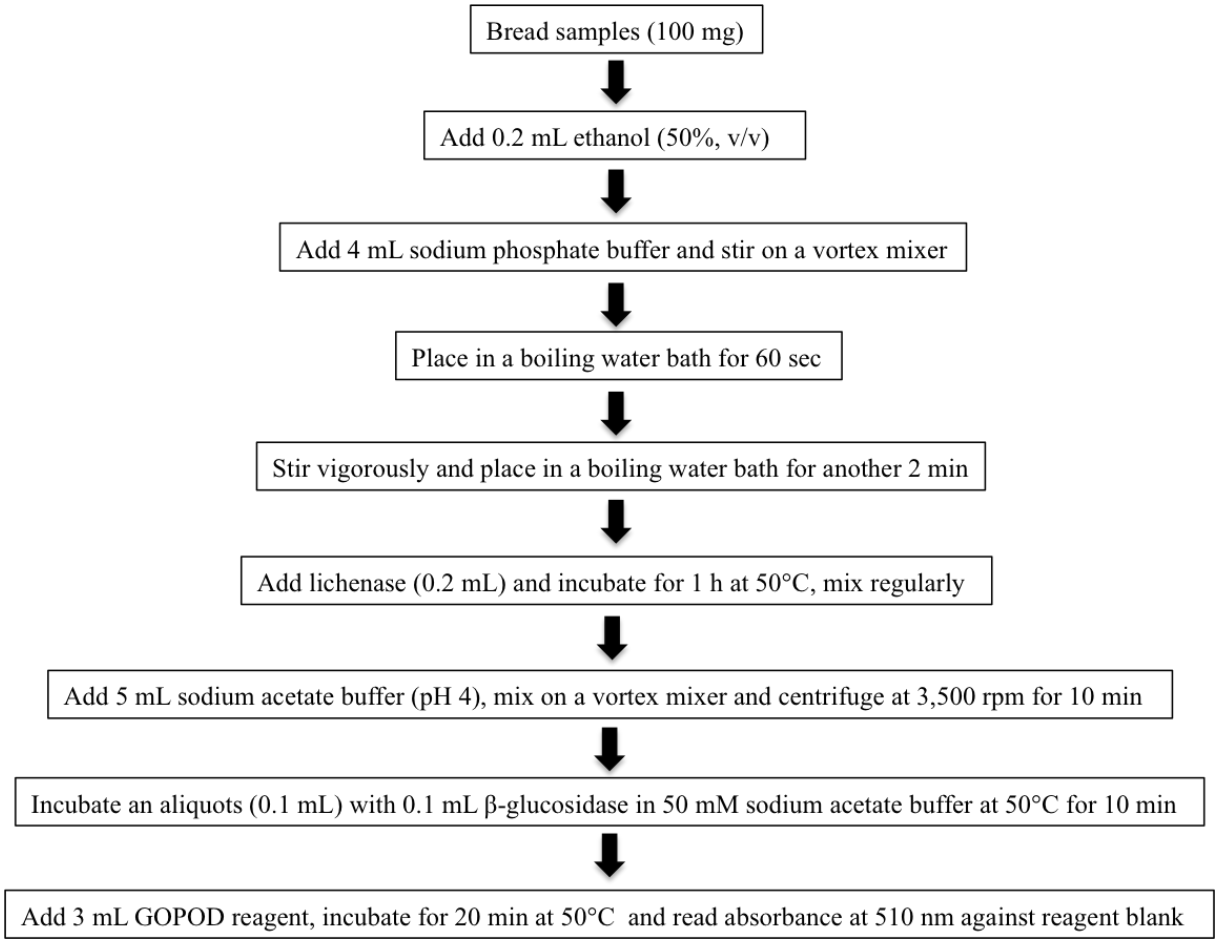


Figure 2-4. Flow chart for the measurement of β-glucan. GOPOD, glucose oxidase/peroxidase reagent

2.2.5 Starch hydrolysis of breads

Bread samples were grounded using a kitchen blender (Kenwood BL335, Havant, United Kingdom). The homogenised samples were used for the determination of starch hydrolysis. Starch hydrolysis was determined using a commercially available assay (Megazyme International, Wicklow, Ireland). Breads containing 50 mg available carbohydrate (resistant starch plus solubilised starch) were added to 4 mL pancreatic α-amylase (3 Ceralpha units/mg, 5 mg/mL). Tubes were incubated at 37°C and the reaction was stopped by adding 0.7 mL of ethanol (99% v/v) at different time points (0, 10, 30, 60, 90, 120, 150 and 180 min). Sodium acetate buffer (100 mM, pH 4.5) was added and incubated with 10 µl of dilute AMG solution for 20 min at 50°C. Finally, 3 mL of GOPOD reagent was

added, incubated for 20 min at 50°C and absorbance read at 510 nm against a reagent blank. The glucose content was calculated using Megazyme-Calc, a calculation sheet provided by the manufacturer. Glucose content was converted to starch using a 0.9 multiplying factor. Results are expressed as percentage (%) of total hydrolysed starch at different times.

2.2.6 Microscopic study of breads structures

Breads were sampled from the centre of the bread as this is more uniform area compared with the outer edge. The bread samples were processed using a standard protocol of 70% alcohol (1 h), 90% alcohol (1 h), absolute alcohol (6 h 30 min), xylene (2 h 30 min) cycles and fixed in paraffin. The sections were cut into 2.5 µm thickness with a microtome (Shandon Finesse E, Thermo Scientific, Runcorn, United Kingdom) and dried in an oven for 1 h at 60°C. The bread sections were stained with Lugol's iodine solution [(0.33% I_2, w/v) and 0.67% KI (w/v)] (Sigma Aldrich, Steinheim, Germany) for 2 min followed by 0.1% (w/v) Light Green (Gurr, BDH Ltd., Poole, United Kingdom) for another 2 min. The slides were visualised under light microscopy at a magnification power of 40x. Under light microscopy, amylopectin stains brown, amylose stains dark brown (appears in the centre of starch) and gluten stains light green.

2.2.7 *In vitro* digestion model

This *in vitro* digestion model was based on a pH controlled system (oral, gastric, small intestinal digestion). A collaborative study which reviewed the models available showed that there is no direct consensus on which digestion conditions will suit all underlying research questions but there is a set of conditions that are practical and close to the physiological environment (Minekus et al., 2014). The *in vitro* digestion method was used to remove starch and protein from breads prior to the determination of antioxidant activity and *in vitro* batch fermentation model (Aura et al., 1999). Dialysis tubing with a molecular cut-off point of 500-1000 (Spectrum Laboratories, California, USA) was used to remove digested products from the digestive mixtures. The resulting retentates were carefully transferred into a tube and freeze-dried prior to *in vitro* fermentation. The simulated *in vitro* digestion was divided into three phases: mouth, stomach, and small intestinal phase

(**Figure 2-5**). These are described below along with a summary of the conditions they are mimicking.

2.2.7.1 Mouth

The digestion of starch begins in the mouth with the mechanical force of 300-1000 N via chewing (Guyton, 1996a). At an average pH of 6.3 to 7.0, salivary α-amylase hydrolyses starch into maltose, maltotriose and α-limit dextrins (Aframian et al., 2006). However, only 5% of starch digestion occurs in the mouth and varies depending on the food structure.

In this study, samples containing 50 mg of available carbohydrate (resistant starch plus solubilised starch) (sample preparation described above in **Section 2.2.5**) were weighed into 100 mL beaker. Ten millilitre of distilled water was added followed by the addition of 7 mL sodium chloride (0.85%, w/v). α-Amylase (50 U/sample) (from *Aspergillus oryzae*, Sigma-Aldrich, Dorset, United Kingdom) in 0.02 M sodium phosphate buffer (pH 6.9) was added to the sample and incubated at 37°C for 5 min.

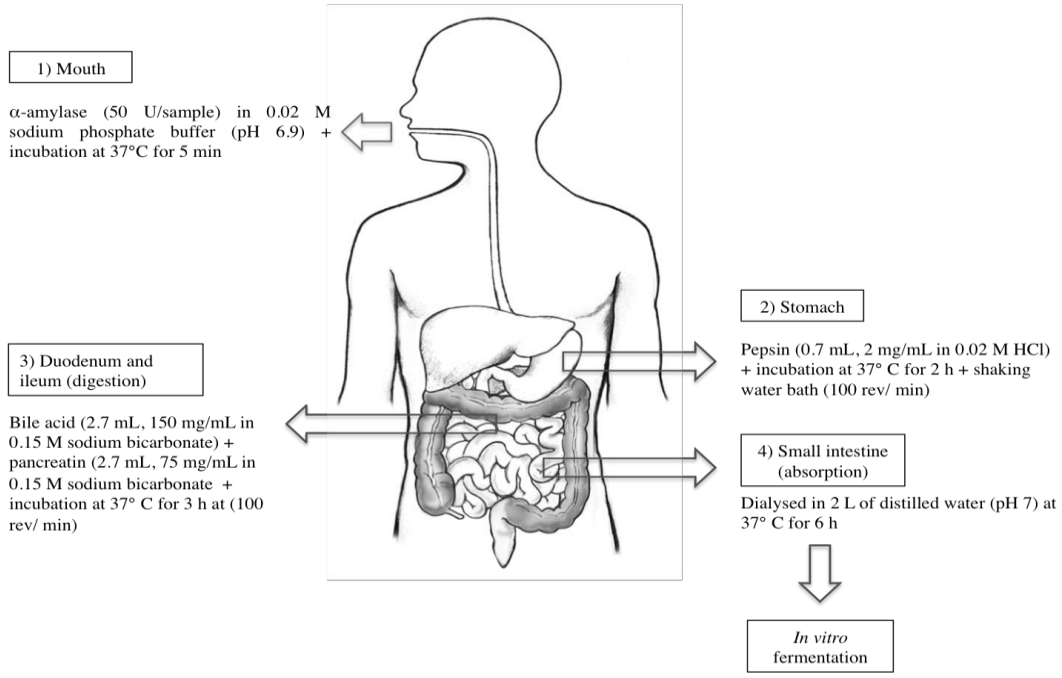


Figure 2-4. *In vitro* digestion procedure mimicking human digestion (Aura et al., 1999)

2.2.7.2 Stomach

The release of the hormone gastrin stimulates secretion of the enzyme pepsinogen. Pepsinogen is in non-active form and is activated to pepsin by hydrochloric acid and the pepsin then activates further pepsinogen. The optimum pH for pepsin is between 1.8 to 3.5. α-Amylase is inactive at low pH and hence limits carbohydrate digestion. Gastric movements disintegrate the stomach contents to produce semisolid chyme (Guyton, 1996a).

In this study, the pH of the stomach was simulated by hydrochloric acid (0.15 M, 3.0 mL, pH 2.5). Pepsin (0.7 mL, 2 mg/mL in 0.02 M HCl) (Sigma-Aldrich, Dorset, United Kingdom) was added and incubation was carried out at 37°C for 2 h in a shaking water bath (100 rev/ min). The residence time in the stomach varies from 30 min to 2 h depending on meals and particle size (Muir and O'dea, 1992; Lebet et al., 1998).

2.2.7.3 Small intestine (duodenum and ileum)

pH in the duodenum and distal ileum is between 6.2 to 7.9 (Gee et al., 1999). The release of bicarbonate ion and bile salts from the biliary gland of the liver and is stored in the gall bladder to be secreted during meal. The presence of bicarbonate ion (from pancreatic secretion) helps to neutralise the acid from the stomach and increases pH in the duodenum while bile salts play a major role in fat digestion (Guyton, 1996b). Available starch is digested rapidly once the chyme enters the duodenum and mixes with pancreatic juice and bile. Pancreatic juice contains multiple enzymes for protein (trypsin, chymotrypsin and carboxypeptidase) fat (lipase, co-lipase, cholesterol esterase and phospholipase) and carbohydrate digestion (amylase). Most starch is digested by pancreatic amylase and absorbed in the first 20% of the small intestine. The residence time of food in the duodenum and ileum is between 3 and 6 hours, during which most digestible nutrients are digested and absorbed.

In the model, the pH was adjusted to 6.5 to 7 using 6 M sodium hydroxide (NaOH). Bile acid (2.7 mL, 150 mg/mL in 0.15 M sodium bicarbonate) and pancreatin (mixture of amylase, proteases and lipase) (2.7 mL, 75 mg/mL in 0.15 M sodium bicarbonate) (Sigma-

Aldrich, Dorset, United Kingdom) were then added to the solution and incubated for another 3 h at 37°C (100 rev/ min).

Absorption from the small intestine was simulated in the *in vitro* digestion model using a dialysis tube with a small molecular weight (MWCO 500-1000 dalton) cut-off, 35 cm long with flat width of 31 mm and diameter of 20 mm (Biotech Cellulose Ester membrane MWCO, Spectrum Laboratories, California, USA). This allows removal of digested components (e.g. monosaccharide, disaccharides, polyphenol monomers) and retains the non-digestible carbohydrates in the retentates. After 3 h of incubation, the samples were filled into a dialysis tube and sealed with a clasp. The tube was dialysed in 2 L of distilled water (pH 7) at 37°C for 6 h. The digestive products were freeze-dried for 48 h and used for *in vitro* fermentation and antioxidant activity.

2.2.8 Determination of total (poly)phenols

Total polyphenol content was determined using a Folin-Ciocalteu assay, which measures formation of blue-green complexes between phenolic compounds and Folin-Ciocalteu's reagent (Singleton et al., 1999). Firstly, 20 µL of appropriately diluted samples (1:1) were added to 100 µL of diluted Folin-Ciocalteu reagent (Folin: water, 1:10) and 70 µL of distilled water. The mixture was then kept at room temperature for 5 min. Subsequently, 70 µL of 6% (w/v) Na_2CO_3 was added to it. The solution was left to stand at room temperature for 90 min. Absorbance was read at 765 nm using a spectrophotometer (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). Gallic acid in the range of 50-1000 µg/mL was used as the standard. Stock gallic acid solution (1000 µg/mL) was prepared in 10% (v/v) methanol. Total (poly)phenols are expressed as mg gallic acid equivalent per g retentate.

2.2.9 Determination of ferric reducing ability of plasma (FRAP) assay

Total antioxidant activity was measured using the ferric reducing ability of plasma (FRAP) assay (Benzie et al., 1996); modified for a measurement on a 96-well plate. This method is relatively inexpensive, highly reproducible and simple to prepare.

In principle, the FRAP assay measures the change in absorbance at 593 due to the

formation of blue coloured complex formed between ferrous ion (Fe^{2+}) and TPTZ. Prior to this, colourless ferric ion (Fe^{3+}) was oxidised to ferrous ion (Fe^{2+}) by the action of electron donating antioxidants. Freshly prepared FRAP reagent was warmed at 37 ºC in a water bath which gives the initial reading ($A_{initial}$; t = 0 min). This reagent was prepared by mixing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ and 0.3 M acetate buffer (pH 3.6) in the ratio of 1:1:10. For sample, 100 µL of each fraction were added to 100 µL of deionised water and 1.8 mL of FRAP reagent. The mixture was incubated at 37 ºC for 4 min. Absorbance was read at 593 nm using a spectrophotometer (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). FRAP value was calculated using the following equation:

$$\text{FRAP value} = A_{final} - A_{initial}$$

A_{final} - Final absorbance at 532 nm (4 min)

$A_{initial}$ - Initial absorbance at 532 nm (0 min)

A reducing ability was calculated with reference to the reaction of $FeSO_4.7H_2O$ (100 – 1000 µmol) and expressed as mol Fe^{2+} equivalents per g retentate.

2.2.10 *In vitro* batch fermentation

The colon has three main functional regions:

1) Caecum and proximal colon as the main site for carbohydrate fermentation
2) Transverse colon as the main site for absorption
3) Distal colon and rectum acts the reservoir of the residual waste material for excretion, water and SCFA absorption and protein fermentation (Edwards et al., 1997).

Fermentation and production of high amounts of SCFA takes place to a greater extent in the proximal ascending colon compared to the descending distal colon (Cummings et al., 1987). The SCFA reduce the pH in the ascending colon to 5.6 and increases the pH of the descending colon to 6.6. This buffering is due the absorption of SCFA and secretion of HCO_3^-. Nutrient content is depleted as the contents move from proximal to distal colon.

Nitrogenous compounds account for 6% of dry matter in all regional colonic content. Carbohydrate content decreases by half from 20% in the caecum to 11% in the sigmoid colon (Cummings and Macfarlane, 1991). The gradual depletion of carbohydrate from proximal to distal colon affects the fermentation levels, thus resulting in lower production of SCFA in the descending distal colon site. There is a shift in total SCFA detected in the blood circulation, decreasing from portal > hepatic > peripheral with 375, 148 and 79 μmol/L, respectively. There is a molar change in SCFA from the gut lumen to the portal blood (as determined in accidental death bodies). The proportion of acetate increases from 57 to 71% while the proportion of butyrate falls from 21 to 8%. This decrease in butyrate could be due to greater uptake by the colonic epithelium than acetate and propionate. The proportion of propionate decreases from 22% in the portal vein to 5% in the peripheral vein suggesting greater uptake by the liver (Cummings et al., 1987).

2.2.10.1 Sample and fermentation medium preparation

Faecal inoculum was obtained from healthy volunteers who had not had antibiotics, laxatives or gastrointestinal infections for at least two months before the study. Volunteers were supplied with a plastic container (a plastic bag inside) and a supporting bedpan to place on the toilet seat. To induce an anaerobic conditions, the pot was tightly sealed in a bag with an anaerobic gas kit (Anaerocult® A Merck KgaA 62471, Darmstadt, Germany). The faecal sample was kept cool in an insulated bag containing ice packs to slow down bacteria metabolism and was used within 2 h of passage (Edwards et al., 1996).

2.2.10.2 Fermentation medium preparation

All chemicals and reagents (analytical grade) used in this fermentation were purchased from Fisher Scientific (Leicestershire, UK). Freeze-dried digesta were used for *in vitro* batch fermentation according to Edwards et al. (1996). Freeze-dried sample was pre diluted in 1 mL of 0.85% sodium chloride and used as a substrate for fermentation. Fermentation medium was prepared as the following: tryptone 4.50 g/L, 224.5 μL micromineral (13.2 g $CaCl_2.2H_2O$, 10.0 g $MnCl_2.4\ H_2O$, 1.0 g $CoCl_2.6\ H_2O$, 8 g $FeCl_2.6\ H_2O$ and made up with distilled water to 100 mL), 450 mL macromineral (2.85 g Na_2HPO4, 3.1 g KH_2PO_4, 0.3 g $MgSO_4.7H_2O$ and made up to 500 mL with distilled water), 450 mL buffer (2 g NH_4CO_3,

17.5 g $NaHCO_3$ and made up to 500 mL with distilled water) and 2.25 mL resazurin (redox indicator 1%, w/v).

The fermentation medium was covered with a cotton cap and boiled on a hot plate to degas the solution. The medium was then cooled to 37°C in oxygen free nitrogen (OFN) (approximately 30 min). The pH was adjusted to 7 using 6 M HCl. The medium (42 mL) was then transferred to 100 mL McCartney bottle and sealed with an aluminium lid and again purge with OFN for 1 min before the final addition of 2 mL of reducing solution (312.5 mg cysteine hydrochloride, 1 M NaOH, 312.5 mg sodium sulphide and made up with distilled water up to 50 mL).

2.2.10.3 Fermentation preparation

Faecal slurry was prepared by mixing 96 g of homogenised faeces in 300 mL phosphate buffer using a hand blender (Braun™), giving a total concentration of 32% (w/v). The slurry was strained using a nylon stocking and 5 mL was transferred to each sterilised McCartney bottle containing fermentation medium and reducing solution. Thus, the initial fermentation reaction consisted of 42 mL fermentation medium, 2 mL reducing solution, 5 mL of faecal slurry and 1 mL of pre-diluted sample (final volume of 50 mL). The bottles were sealed using a crimper and purged using OFN. They were placed in a shaking water bath at 37°C with a speed of 60 strokes/min to mimic conditions in the colonic lumen. Aliquots of fermentation solution (3 mL) were taken at 0, 6 and 24 h for the measurement of pH and SCFA (acetate, propionate and butyrate). Sample (3 mL) was mixed with 1 mL of 1 M NaOH (1:3) to prevent the evaporation of SCFAs and stored at -20°C before further analysis. pH of the freshly fermented medium was measured using a portable digital pH meter (Hannah pH20 instruments, USA).

2.2.10.4 Determination of short chain fatty acid (SCFA)

The determination of SCFA in faecal slurry samples was done according to a previous method (Laurentin and Edwards, 2004). Internal standard (100 µL; 86.1 mM 3-methyl-n-valeric acid) was added to the previously stabilised fermented faecal slurry (800 µL) and 100 µL orthophosphoric acid. The mixture was mixed on a vortex mixer for 15 sec and

extracted twice with 3 mL of diethyl ether with rigorous mixing for 1 min. The upper organic phase was pooled in a 15 mL tubes.

Analysis of SCFA was performed using a TRACETM 2000 GC-FID gas chromatography (Thermo Quest Ltd, Manchester, UK). This GC-FID is equipped with a flame ionization detector (250°C) and a Zebron ZB-Wax capillary column (15 m x 0.53 mm id x 1 μm film thickness) (Phenomenex, Cheshire, UK). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. Samples (1 μL) were injected on to the column using an autosampler (230°C, splitless) onto the column. The temperature was gradually (15°C/min) increased form 80°C to 210°C. Both temperatures were held for 1 min. The resultant GC-FID chromatogram was analysed using Chrom-Card 32-bit software (version 1.07β5) (Thermo Quest, Milan, Italy). Individual SCFA identification was based on comparison with retention times of authentic standards. Concentrations of external standards were based on the expected values in healthy/normal faecal samples as follows: 166.5 mM acetic acid, 135.0 mM propionic acid, 113.5 mM isobutyric acid, 113.5 mM n-butyric acid, 97.9 mM isovaleric acid, 97.9 mM n-valeric acid, 86.1 mM n-hexanoic acid, 76.8 mM heptanoic acid, 69.3 mM n-octanoic acid.

Quantification was based on the averaged area ratio of each external standard. A set of five calibrated standards was extracted and analysed before and after the samples. Standards were extracted once and injected twice. Quantification was done batch-wise (in duplicate) with group analysis of each individual together to reduce inter-assay error. Samples were extracted twice and the results were averaged. Retention times of typical chromatograms found in faecal and standards are shown in **Table 2-2**.

Table 2-2. Retention times of individual SCFA in standard solution and faecal samples

No.	SCFA	Retention time (t_R)
1	Acetic acid (C2)	2.6
2	Propionic acid (C3)	3.2
3	Isobutyric acid (IC4)	3.4
4	Butyric acid (C4)	3.9
5	Isovaleric acid (IC5)	4.1
6	Valeric acid (C5)	4.6
7	2-ethylbutyric (Internal Standard)	4.8
8	Caproic acid (C6)	5.4
9	Enanthic acid (C7)	6.1
10	Caprylic acid (C8)	6.7

2.3 Study two: Effects of functional breads on palatability and satiety

This section describes the methodology for assessing palatability and satiety of breads prepared with β-glucan and black tea as a breakfast meal. This study did not involve any blood collection and was conducted with untrained panelists. Details of the study design, sample size calculation, inclusion and exclusion criteria, and statistical analysis are described in **Chapter 4**. This study was approved by the College of Medical, Veterinary and Life Sicences Ethics Committee, University of Glasgow (Project Number 200140006).

2.3.1 Breakfast meal preparation

The test breads were given with cheese to provide a standardised breakfast meal providing 400 - 450 kcal/meal (21% of total daily energy requirement). The breakfast meal and nutrient composition of breads are shown in **Table 2-3**. Breads were prepared according to the method detailed in **Section 2.0**. Participants were provided with one of the following breads in a randomised order at each experimental trial:

i. White bread (WB)
ii. Black tea bread (BT)
iii. β-Glucan bread (βG)
iv. β-Glucan plus black tea bread (βGBT)

The bread was eaten during breakfast time (e.g. 8:00 am) with cheese and unsalted butter spread, and with 300 mL water. Participants were instructed to eat the bread within 15 min and self-reported eating time was recorded using a stopwatch. Volunteers were provided with 300 mL water during breakfast and *ad libitum* lunch session. Volunteers were instructed to drink all of the water before meal completion and no additional water was permitted. During the postprandial period (3 h), volunteers were provided with extra water (500 mL), and no additional water was permitted.

Table 2-3. Breakfast meal and nutrient composition

Breads	White bread (WB)	Black tea bread (BT)	β-Glucan bread (βG)	β-Glucan + black tea bread (βGBT)
Breakfast meal (g/meal)				
Bread	111[a]	111[a]	148[b]	153[b]
β-glucan	0.3[a]	0.3[a]	7.0[b]	7.0[b]
Butter	15	15	15	15
Cheese	25	25	25	25
Total food serving (g)	151	151	195	200
Nutrient composition (g/serving)				
Total available carbohydrate	50	50	50	50
Fat	8	7	8	8
Protein	16	16	17	17
Moisture	41	40	71	73
Energy, kJ (kcal)‡	1740 (417)	1725 (413)	1740 (417)	1750 (419)

‡Energy was calculated based on the formula (Atwater factor) [total available carbohydrate (50 g) x 17] + [protein (g) x 17] + [fat (g) x 37].
*Energy density was calculated based on energy (kcal) / food amount (g).
Different superscript letters indicate statistically significant ($p < 0.05$) values within the same row.

2.3.2 *Ad libitum* lunch and energy intake estimation

The *ad libitum* lunch consisted of multiple food items as follows: pasta and tomato pasta sauce, white bread, unsalted potato crisps, roasted chicken slices, turkey ham, sandwich, cheddar cheese and plain sponge cake. This meal was served with 300 mL water (Vitaglione et al., 2009; Wiessing et al., 2012). A choice of food items was offered to participants to avoid the feeling of boredom of taste, and was provided in excess as such the leftovers always remained on the plate (Reinbach et al., 2009). Lunch was provided 3 h after breakfast (e.g. 11:00 am). The time gap between breakfast and lunch (3 h) was chosen based on the amount of nutrients being emptied from the stomach to small intestine (Jones et al., 2005; Brener et al., 1983). Nutrients are emptied at the rate of 2-4 kcal/min, thus as the energy of the breads were 413 - 419 kcal (**Section 2.2.1, Table 2.3**), 103 – 210 min was required to empty the nutrients from the stomach. Participants were advised to eat the lunch until they were comfortably full according to their satiety within 30 min. All food items were weighed before and after the meal to the nearest 1.0 g using an electronic kitchen scale (Salter ARC Electronic Kitchen scale, 1066 BKDR08, Kent, UK). The uneaten food was weighted, subtracted from the initial amount and the food consumed was used to calculate energy (kcal) intake. The WinDiets dietary programme (Robert Gordon University, Scotland, UK) was used to calculate energy and nutrient intake. Participants were allowed to read or use their own computer during the experiment except during lunch to avoid interfering with the eating session.

2.3.3 Satiety and palatability scoring (adaptive visual analogue scale, AVAS)

Satiety and palatability were measured using the Adaptive Visual Analogue Scale (AVAS) software (Marsh-Richard et al., 2009). This is free software available at www.nrlc-group.net, which allowed automated calculation of visual analogue scale scoring. The interface of this software contains a question above a horizontal line anchored with a response on the left and right. Participants were instructed on how to do the scoring before the session. They were directed to the 'training' mode, which allowed participants to practice before starting the real test. In the training session, participants had to place a cursor on any points on the horizontal line of the scale and click on them. For example, to

the question '*how hungry are you?*' the response on the left is '*not at all*', and on the right is '*as hungry as I ever felt*'. By placing the cursor on the very left of the line indicated that they did not feel hungry at all whilst placing the cursor at the very right end of the anchor indicated they were extremely hungry. The software automatically stored and calculated the responses in millimeter (mm).

After acclimatisation, participants completed an AVAS scoring, before receiving a test meal at breakfast (fasting state), and at different time points (30, 60, 90, 120 and 180 min) after breakfast. AVAS consists of five questions, as follows: 1) *Hunger, how hungry are you?* 2) *Fullness, how full are you?* 3) *Satiety, how satiated are you?* 4) *Desire to eat, how strong is your desire to eat?* 5) *Prospective consumption (quantity), how much do you think you could eat right now*.

The palatability study was completed using the AVAS (10-mm scale) between 0 – 5 min post-breakfast meal assessing visual appeal, smell, taste, after taste, palatability (how palatable the bread is) and overall acceptance (the overall acceptability of the bread) (Vitaglione et al., 2010). A higher score indicates a higher degree of acceptability of the breads. The raw data for palatability was translated and presented as a star diagram (British Nutrition Foundation, 2016a; Aldughpassi et al., 2008; Finocchiaro et al., 2012).

2.4 Study three: Effects of functional breads on postprandial glucose, insulin and gut hormones

This section describes the methodology for assessing the effects of breads prepared with β-glucan and black tea as breakfast meal on postprandial glucose, insulin and gut hormones. This study involved blood collection and was conducted with healthy volunteers. Details of the study design, sample size calculation, inclusion and exclusion criteria, and statistical analysis are described in **Chapter 5**. This study was approved by the College of Medical, Veterinary and Life Sicences Ethics Committee, University of Glasgow (Project Number 200140006).

2.4.1 Blood collection

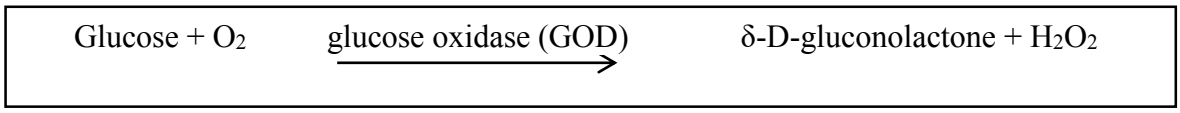
Blood was taken by a trained phlebotomist. After resting for 10 min, fasting venous blood was obtained from an antecubital vein of a non-dominant arm while seated using intravenous cannula (size 20 GA, 1.0 x 32 mm) (BD Venflon™, Bectson-Dickinson™, Helsingborg, Sweden). A 3-way stopcock (10 cm and 360° rotation angle) (BD Connecta™, Bectson-Dickinson™, Helsingborg, Sweden) was immediately placed after venipuncture and 9 mL of resting venous blood was collected before the test (0 min) and at 15, 30, 45, 60, 90, 120 and 180 min thereafter. The site of cannulation was covered with sterile plaster (Tegaderm™, Neuss, Germany). The cannula was flushed with 3 mL of normal saline (0.9% sodium chloride, w/v) infusion (B. Braun, Melsungen, Germany) after each blood collection. The saline was cleared from the catheter before each venous blood sample by withdrawing 1 mL of blood into a syringe and discarded.

The blood (9 mL) was transferred in two different tubes as follows: 1) five millilitre in an ice-cooled tube containing dipeptidyl peptidase-IV inhibitor (250 KIU aprotinin) (BD Vacutainer®, Bectson-Dickinson™, Plymouth, UK) for measurement of gut hormones (GLP-1, PYY and CCK) and 2), four millilitre in a tube containing anti-clotting agent (lithium heparin) (Vacuette®, Greiner Bio-One, Monroe, USA). The blood samples were immediately centrifuged at 4°C at 3,000 rpm for 15 min. Plasma was carefully separated, aliquoted (100 µL in duplicates) into microfuge tubes and stored at -80°C until further use.

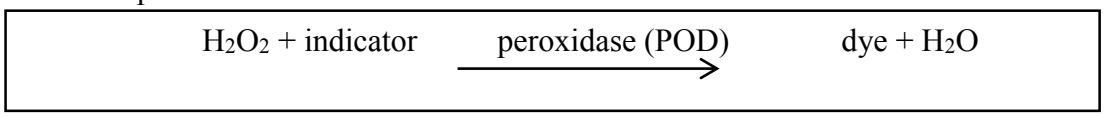
2.4.1.1 Glucose determination

Glucose was measured using Reflotron Glucose kit (ROCHE Diagnostics GmbH, Mannheim, Germany). This assay is based on the calorimetric measurement of dye formation after an enzymic reaction of glucose as follow:

First step:



Second step:



The dye formed from this reaction is measured at 642 nm at 37°C. The amount of dye formed is directly proportional to the glucose concentration in the sample.

Fresh heparinised plasma sample (30 uL) was carefully applied on the glucose test strip and directly placed in the Reflotron® analyser within 15 sec (Reflotron, Mannheim Boehringer GmBH, East Sussex, United Kingdom). Glucose levels are expressed as mmol/L. Fasting glucose value was in the range of 3.3 to 6.05 mmol/L. Inter-assay variation was less than 6%. Incremental area under the curve ($iAUC_{0\text{-}180\ min}$, mmol/L x min) for glucose were estimated using a trapezoidal rule (Vitaglione et al., 2009).

2.4.1.2 Insulin determination

Insulin was measured using Mercodia Insulin ELISA kit (product code 10-1113-10) (Mercodia AB, Uppsala, Sweden). This kit is based on the colorimetric method from the dye developed after the reaction of two-site enzyme immunoassay. It is based on the direct sandwich technique from two monoclonal antibodies tagged against separate antigenic determinants on the insulin molecule. Insulin reacts with the peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bind to the microplate during incubation at room temperature. Unbound labelled antibody is removed after simple washing steps. The bound conjugate is then reacted with 3,3',5,5'-tetramethylbenzidine (TMB) and the

reaction is stopped by adding sulphuric acid. Colorimetric endpoint is measured photometrically at 450 nm (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). The amount of TMB detected is directly proportional to the amount of insulin bound to them.

Frozen samples were removed from -80°C freezer and transferred to a 4°C fridge and were allowed to thaw overnight before analysis. All reagents and samples were brought to room temperature before analysis according to manufacturer instruction. All analyses were done in duplicate. Twenty five microlitres of samples or standard (3.0, 9.74, 29.8, 104 and 207 mU/ L) was pipetted into appropriately labelled wells. One hundred microlitres of enzyme conjugate was added followed by incubation on a plate shaker (800 rpm) for 1 h at room temperature (18 – 25°C). The reaction volume was discarded by inverting the plate over a sink. Wash buffer (350 µL) was added to each well, discarded and tapped against absorbent paper to remove excess liquid. This step was repeated five times and precaution was taken to avoid prolong soaking during washing step. Substrate TMB was added (200 µL) followed by 15 min incubation at room temperature. Finally, the reaction was stopped by adding sulphuric acid (50 µL) and was mixed on a shaker for 5 sec. The plate was read within 30 min at 450 nm on a spectrophotometer (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). Results are expressed as mU/L. Inter-assay variations of this assay was less than 11%. Incremental area under the curve ($iAUC_{0\text{-}180\ min}$, mU/L x min) for insulin were estimated using a trapezoidal rule (Vitaglione et al., 2009).

2.4.2 Total cholescystokinin (CCK) determination

Total cholecystokinin was measured using Human Cholecystokinin-33 (CCK-33) EIA kit (EK-069-02) (Phoenix Pharmaceuticals Inc., California, USA). This assay is based on the competitive binding of the biotinylated peptide with the standard peptide or samples to the peptide antibody (primary antibody). This immunoplate kit was pre-coated with secondary antibody, blocking the nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide body) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptides in the samples. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. The yellow colour

development is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of the peptide in the standards solution or the samples. A standard curve is then established from known concentrations. The unknown concentration in the samples can be determined by extrapolation of this standard curve. This kit measures total CCK_{33} and CCK_{1-21} in plasma.

Frozen samples were removed from -80°C freezer and transferred to a 4°C fridge and were allowed to thaw overnight before analysis. All assay reagents, chemicals and samples were equilibrated to room temperature before beginning the assay. All analyses were conducted in duplicate. Initially, 50 µL of assay buffer was added into wells as total binding (TB). Peptide standards (50 µL) were added in to respective wells in reverse order of serial dilution (from lowest to highest concentrations). Rehydrated positive control (50 µL) was added into respective wells. Samples (50 µL) were added into respective wells followed by the addition of 25 µL rehydrated primary antibody to each well, except the blank well. Immediately after, 25 µL of rehydrated biotinylated primary antibody was added into each well except the blank well. The immunoplate was sealed and incubated for 2 h at room temperature (20-23°C) on a plate shaker (low speed, 300 rpm).

The plate content was discarded and washed 4 times with 350 µL of assay buffer and blotted dry on absorbent paper. Diluted SA-HRP (1:1000) (100 µL) was added into each well. The plate was sealed and incubated at room temperature for 1 h on a plate shaker (low speed, 300 rpm). The plate was washed again 4 times with 350 µL of assay buffer and blot dry. TMB substrate (100 µL) was added into each well, covered with an aluminium foil and place on a plate shaker at low speed (300 rpm) for 1 h at room temperature. Stop solution (2N HCl, 100 µL) was added in to each well after 1 h of incubation. The colorimetric change from blue to yellow was read within 20 min at 450 nm on a spectrophotometer (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). Results are expressed as ng/mL. Inter-assay variation for this assay was less than 6%. The positive control of this assay was in the range of 0.5 to 0.6 ng/mL (manufacturer's data is 0.4 to 0.7 ng/mL). Incremental area under the curve ($iAUC_{0\text{-}180\ min}$, ng/ mL x min) for CCK were estimated using a trapezoidal rule (Vitaglione et al., 2009).

2.4.3 Active glucagon-like peptide (GLP-1) determination

Active glucagon-like peptide 1 (GLP-1) was measured using the Glucagon-Like Peptide-1 (Active) ELISA kit (EGLP-35K) (EMD Millipore, Missouri, USA). This assay is based, sequentially, on the capture of active GLP-1 from samples by an immobilised monoclonal antibody on a microwell plate that binds specifically to the N-terminal region of active GLP-1 molecules. The unbound materials are removed by washing with assay buffer. This reaction step is followed by the binding of an anti-GLP-1-alkaline phosphatase detection conjugate to the immobilised GLP-1. Unbound conjugate is again removed by washing with assay buffer. Finally, the presence of methyl umbelliferyl phosphate (MUP) forms the fluorescent product umbelliferone with the bound conjugate in the presence of alkaline phosphatase. The amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown sample. The amount of fluorescence generated can be derived by interpretation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1. This kit measures only active GLP-1_{6-37} and GLP-1_{7-37} in plasma.

Frozen samples were removed from -80°C freezer, transferred to a 4°C fridge and allowed to thaw overnight for 24 h before analysis. All assay reagents, chemicals and samples were equilibrated to room temperature before beginning the assay. All analyses were done in duplicate. Wash buffer (300 uL) was carefully added using a multichannel pipette in each well. The plate was incubated at room temperature for 5 min before the content was discarded. The assay was initiated by adding 200 µL assay buffer to the non-specific binding wells (NSB) (in duplicate) and 100 µL to the wells designated to standards, positive control and samples. Standards, positive control and samples were carefully added and the plate was shaken for proper mixing. The plate was covered with a sealer and incubated at 4°C for 20 h.

The plate content was decanted and the excess was tapped out on absorbent towels. The plate was washed 5 times using 300 µL wash buffer and again tapped out on absorbent paper. Detection conjugate (200 µL) was immediately added in each well followed by incubation at room temperature for 2 h. The plate content was again decanted and washed 3 times with wash buffer (300 µL). Diluted substrate (200 µL) was then added, followed

by incubation at room temperature in the dark for 20 min. Immediately after, 50 µL of stop solution was added to each well in the same order as the substrate was added, followed by incubation for 5 min at room temperature to arrest phosphatase activity. The bottom of the plate was wiped with a delicate tissue paper to remove any residue prior to reading the plate. The plate was read with an excitation/emission wavelength of 355 nm/460 nm on a fluorescence plate reader (Spectra Max M2^{e} Molecular Devices, California, USA). Results are expressed as pM. Inter-assay variations was less than 3%. The concentration of positive control was in the range of 42 to 45 pM (manufacturer's data are 30 to 63 pM). Incremental area under the curve ($iAUC_{0\text{-}180\ min}$, pM x min) for GLP-1 were estimated using a trapezoidal rule (Vitaglione et al., 2009).

2.4.4 Total peptide YY (PYY) determination

Total peptide YY (PYY) was determined using Human PYY (Total) ELISA kit (EZHPYYT66K) (EMD Millipore, Missouri, USA). PYY is one of the key GI hormones regulating appetite and energy balance. The blood PYY level is low after fasting and elevates significantly after meal. This assay is based on direct binding of human PYY molecules (both 1-36 and 3-36) in the sample by rabbit anti-human PYY IgG and immobilisation of the resulting complex to the wells of a microtiter plate coated by a pre-tittered amount of anti-rabbit IgG antibodies. The second step involves the binding of a second biotinylated antibody to the PYY. The unbound materials are washed away followed by conjugation of horseradish peroxidase to the immobilised biotinylated antibodies. This second washing step removes any free enzyme. The quantification of immobilised antibody-enzyme conjugates is based on the formation of horseradish peroxidase (HRP) activities in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine (TMB). The enzyme activity is measured at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human PYY (both 1-36 and 3-36) in the unknown sample, the concentration of total PYY can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human PYY. This assay kit measures total $PYY_{1\text{-}36}$ and $PYY_{3\text{-}36}$ in plasma sample.

Frozen samples were removed from -80°C freezer, transferred to a 4°C fridge and allowed

to thaw overnight before analysis. All reagents, chemicals and samples were equilibrated at room temperature before analysis. All analyses were done in duplicate. The plate was washed 3 times with diluted wash buffer (300 µL), decanted and blotted dry on absorbent paper. Matrix solution (20 µL) was added to the blank, standard and quality control wells. Assay buffer (20 µL) was then added to the blank and sample wells. Standards (20 µL) were carefully added into designated wells in reverse order of serial dilution (lowest to highest concentrations). Positive controls (20 µL) (low and high positive control PYY) were added into appropriate wells. Samples (20 µL) were added into the remaining wells. Twenty microlitres of blocking solution was added in each well, covered with a sealer and incubated for 30 min at room temperature (20-23°C) on a plate shaker (low speed, 300 rpm). A mixture of capture and detection antibody (50 µL, 1:1) was added using a multi-channel pipette, sealed and plate was again incubated at room temperature for 1.5 h on a plate shaker (low speed, 300 rpm).

The plate was washed 3 times with 300 µL HRP was buffer per wash. Enzyme solution (100 µL) was carefully added to each well with a multi-channel pipette, before covering and incubating for 30 min on a plate shaker (300 rpm). The plate content was decanted and blotted on absorbent paper to remove residual fluid. The plate was washed 6 times with HRP wash buffer (300 µL per wash), decanted and tapped firmly after each wash to remove residual buffer. Substrate solution (100 µL) was added to each well, which were then covered with a plate sealer and shaken on a plate shaker for 20 min in the dark. A blue colour was formed with the intensity proportional to increasing concentrations of PYY standards. Finally, stop solution (100 µL) was added and shaken by hand to ensure complete mixing of solution in all wells. The blue colour turned to yellow after acidification and these coloured compounds were measured at 450 nm and 590 nm in a plate reader within 5 min (Multiskan® Spectrum, Thermo Labsystems, Vantaa, Finland). Results are expressed as pg/mL. Inter-assay variation of this assay was less than 15%. The concentrations of positive control were in the range of 656 to 820 pg/mL (manufacturer's data is 397 to 825 pg/mL). Incremental area under the curve ($iAUC_{0\text{-}180\ min}$, pg/mL x min) for PYY were estimated using a trapezoidal rule (Vitaglione et al., 2009).

Chapter 3: Combined Effects of β-Glucan and Black Tea on Starch Hydrolysis, Antioxidant Potentials and *In vitro* Batch Fermentation

OUTLINE:

This chapter describes the development of different breads with added β-glucan and/or black tea, and their effects on starch functionality and antioxidant capacity. The second section describes the fermentation characteristics of predigested (*in vitro*) breads in a simulated *in vitro* batch fermentation, using samples donated by healthy volunteers.

The first section of this chapter has been published in International Journal of Food Science and Nutrition, 2015, 66 (2): 159-165, titled 'Combined effects of added beta glucan and black tea in breads on starch functionality'.

The results from the second and third sections of this chapter were presented at the Nutrition Society Summer Meeting, 14–17 July 2014, Carbohydrates in health: friends or foes? University of Glasgow, Scotland, UK, as follows:

1. Antioxidant properties of breads prepared with β-glucan and black tea. Proceedings of the Nutrition Society (2015), 74 (OCE1), E31.

2. Interaction of β-glucan and tea during bread baking increased SCFA production in vitro. Proceedings of the Nutrition Society (2015), 74 (OCE1), E73.

3.1 Introduction

Ischaemic heart disease, stroke, diabetes mellitus, and hypertensive heart disease are among the top 10 causes of death in high-income countries (WHO, 2016c). These diseases are associated with modifiable behavioural risk factors including physical inactivity, tobacco use and unhealthy diet. These behaviours lead to four metabolic/physiological (intermediary risk factors) changes that increase the risk of non-communicable diseases including high blood pressure, overweight/obesity, hyperglycaemia and hyperlipidaemia.

An important public health goal is to reduce these diseases by decreasing the risk factors associated with them via preventive actions. One low cost solution would be to reduce common modifiable behavioural risk factors. The World Health Organization (WHO) delineated preventive strategies aiming to improve eating behaviour, these include: 1) reducing sugar and salt intake 2) limiting total fat intake 3) increasing consumption of fibre through intake of fruit, vegetables, pulses, wholegrain and nuts (WHO, 2016d). However, achieving effective diet modification is challenging because of the many factors that influence eating behaviour. These include physical, social and environmental factors, such as cost, accessibility and affordability, alongside personal factors, such as taste, preference, level of education and knowledge, and these are associated with low fruit and vegetable (FV) consumption (EUFIC, 2016b). Low FV consumption contributes to low fibre intake; the current dietary fibre intake in the UK is 18 g/day, which is lower than the daily recommended intake of 30 g/day (Marlett et al., 2002; British Nutrition Foundation, 2016b; SACN, 2015).

Apart from dietary fibre, FV are also an important source of (poly)phenols in the diet (Rothwell et al., 2012; Zamora-Ros et al., 2015). *In vitro* and *in vivo* studies suggest a diet rich in (poly)phenols is associated with reduced risk of chronic diseases (Morand and Sies, 2016). Among the UK's 'health-conscious' group (predominantly vegetarians), coffee, tea and fruit were the main source of (poly)phenols (49 – 62 % flavonoids) in the diet. However, data for other sub-groups in the UK are not available.

Besides FV consumption, including other sources of isolated dietary fibre such as guar gum, β-glucan or polydextrose in a meal could be a useful strategy for helping the population meet the daily dietary fibre recommendation. Furthermore, there are other health benefits associated with increasing dietary-fibre intake: food is digested slower,

promotes earlier satiety and the meal is usually less calorically dense, lower in sugar and fat (Marlett et al., 2002). In a product such as bread, there is food-matrix interaction between gluten, soluble fibre (e.g. pectin) and (poly)phenols during baking (Sivam et al., 2011; Sun-Waterhouse et al., 2009). However, it is still unknown whether there is a synergistic effect on health when (poly)phenols is combined with dietary fibre in the same food matrix.

β-Glucan is a major component of barley and oat and can be produced commercially (Cleary et al., 2007; Panahi et al., 2007). It is a soluble dietary fibre with mixed β-(1→3) and β-(1→4) linkages. The effects of β-glucan on post-prandial glycaemic and insulinaemic responses in humans have been well established (Ostman et al., 2006). The European Food Safety Authority (EFSA) approved a health claim in which 4 g of beta glucan from either oats or barley per 30 g available carbohydrate is recommended to reduce glycaemic response without disproportionally increasing postprandial insulinaemic response (Agostoni et al., 2011). Hence, β-glucan can be used as an active ingredient in formulating products aimed at increasing dietary fibre intake and also positively regulating the postprandial glucose response.

Black tea is one of the most common beverages consumed around the world. Acute intake of tea reduces postprandial glycaemia in humans (Bryans et al., 2007). The health benefits of tea have been attributed to the presence of bioactive (poly)phenols (Rothwell et al., 2012). Tea (poly)phenols remain stable during bread making and thus may confer further health benefits when incorporated into food products (Wang and Zhou, 2004). Due to their thermal stability, tea has been successfully added to food products such as bread, cake and biscuits (Pasrija et al., 2015; Lu et al., 2010).

Bread is the most popular starchy food in Europe with an average intake of 50 kg bread per person per year (Bakers, 2014). Bread contains starch and gluten whose properties are directly influenced by different stages of bread making (mixing ingredients, proofing and baking) (Rosell, 2011). Breads are a good target for further development and improvement of functional properties (Hayta and Gamze, 2011). The addition of guar gum (guar galactomannan) to bread reduced *in vitro* starch hydrolysis by forming a physical 'barrier' to starch-alpha-amylase interactions (Brennan et al., 1996). The mechanism is due to the layer of galactomannan mucilage that coats the starch granules and bread matrix. Studies

have shown that starch hydrolysis was directly related to postprandial glycaemia and insulinaemic response in acute human feeding trials (Ekstrom et al., 2013). Hence, in this study we hypothesised that the addition of black tea and/or β-glucan will change the characteristics and functionality of wheat bread.

Food constituents can interact in several ways when eaten separately, but if cooked together in a product such as bread, these interactions may be more complex and influence other components during food processing. The addition of (poly)phenols and/or isolated dietary fibres to foods may directly or indirectly modify the properties of the starch components, their digestibility, and antioxidant properties (Juntunen et al., 2002; Juntunen et al., 2003; Rosen et al., 2011; Gawlik-Dziki et al., 2013). Barros et al. demonstrated the monomeric and polymeric proanthocyanidins (PAs) may have different behavior in modifying starch components (Barros et al., 2012). PAs increased the levels of resistant starch (RS) two-fold compared with monomeric (poly)phenols. An *in vitro* study showed that β-glucan from different sources, namely Glucagel (barley) and PromOat (oat) showed similar ability to form complexes with (poly)phenols (Simonsen et al., 2009). Secondly, we hypothesised that the addition of β-glucan could 'trap' some of the black tea (poly)phenols in between the starch granules and β-glucan and thus will remain intact during simulated *in vitro* digestion. This will later become a substrate for colonic bacterial fermentation and lead to increased production of short chain fatty acids.

3.2 Materials and methods

The objectives of this chapter were described as follows (**Figure 3-1**):

1. To develop the breads using home baking techniques and determine their *in vitro* starch hydrolysis and starch structure.
2. To determine the antioxidant potential of breads after an *in vitro* digestion model mimicking human gastric and duodenal phase.
3. To determine the fermentability of breads using *in vitro* fermentation model mimicking human colon using faecal samples collected from healthy volunteers

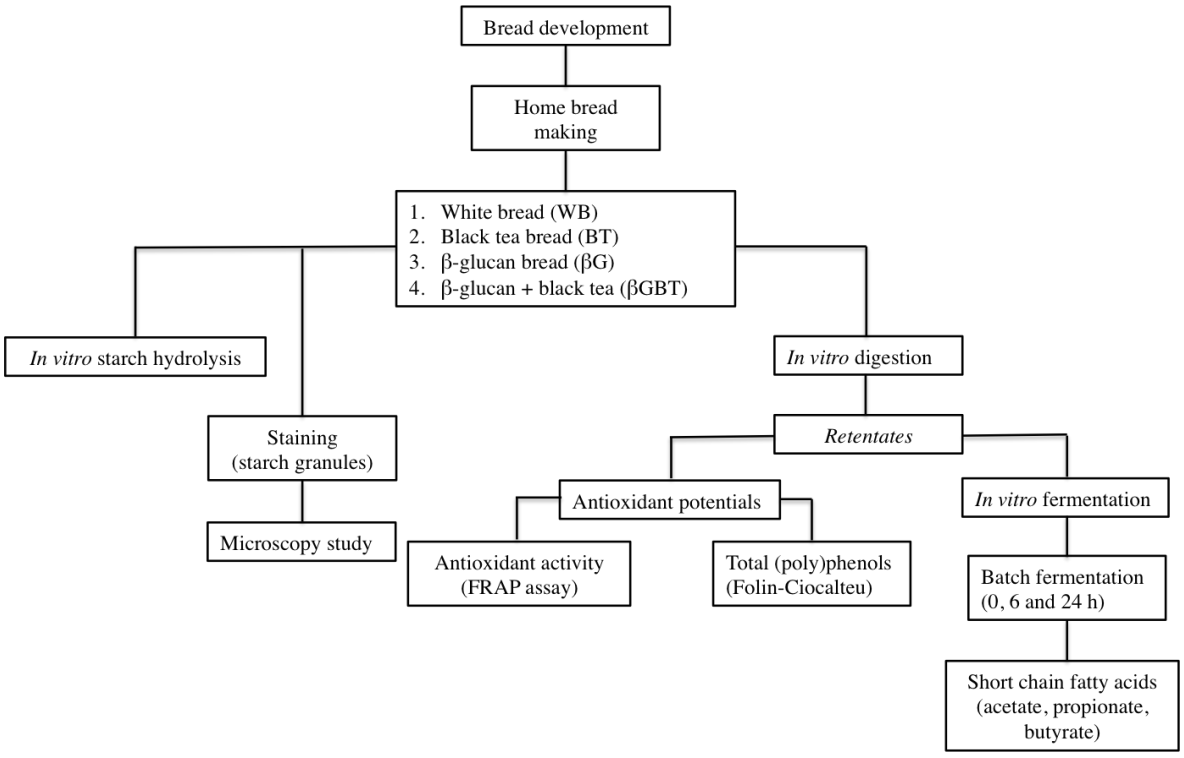


Figure 3-1. Flow diagram of methodology for bread development, *in vitro* digestion and *in vitro* fermentation.

3.2.1 Materials and bread preparation

Materials for bread making were purchased from a local supermarket. Barley β-glucan concentrate (Glucagel^{TM}) was purchased form DKSH (Quai du Rhône, France) and commercial freeze-dried black tea from Tetley (Tata Global Beverages, Greenford, United Kingdom). The breads were prepared using a domestic bread maker (Morphy Richards Ltd, South Yorkshire, UK). Details regarding bread preparation were described in **Chapter 2**, **Section 2.1**.

3.2.2 Proximate analysis

Protein content was determined according to the Kjedahl method. The analysis involved three major steps as follows: 1) digestion with sulphuric acid 2) neutralisation with sodium hydroxide 3) titration with hydrochloric acid to determine the nitrogen content. All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight.

Fat content was determined using solvent extraction method (Soxhlet). Petroleum ether was used as solvent to extract the fat as it passes through the sample and carries them into a flask. All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight.

Details of protein and fat content analyses are described in **Section 2.2.1** and **2.2.2**.

3.2.3 Determination of resistant starch (RS) and digestible (solubilised) starch

Resistant starch (RS) is a type of starch that resists digestion in the intestine and being fermented in the colon. RS was determined using a Resistant Starch assay kit (Megazyme International, Wicklow, Ireland). The assay procedures are based on: 1) Hydrolysis, solubilisation and analysis of digestible (solubilise) starch 2) Measurement of RS in the pellet (residue).

1) *Hydrolysis and analysis of digestible starch*. Bread samples were incubated at 37°C for 16 h with pancreatic α-amylase containing amyloglucosidase (AMG) followed by centrifugation. The supernatant was decanted and used for the analysis of digestible starch. GOPOD reagent was added to 3 mL of the supernatant, incubated for 20 min and

absorbance read at 510 nm against a reagent blank.

2) *Measurement of RS*. Potassium hydroxide was added to re-suspend the pellet and incubated with AMG at 50°C for 30 min. GOPOD reagent was added to 3 mL of the supernatant, incubated for 20 min and absorbance read at 510 nm against a reagent blank.

All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight. Details regarding each analysis were described in **Section 2.2.3**.

3.2.4 β-Glucan content

β-Glucan content was analysed using a Mixed-Linkage Beta-Glucan kit (Megazyme International, Wicklow, Ireland). All analyses were done in triplicate and nutrient contents are expressed as g/100 g fresh weight. Detail of this procedure was described in **Section 2.2.4**.

3.2.5 Starch hydrolysis of bread

Starch hydrolysis of bread was measured using a commercially available assay (Megazyme International, Wicklow, Ireland). Pancreatic α-amylase was incubated with bread containing 50 mg available carbohydrate. Detail of this procedure was described in **Section 2.2.5**. Results are expressed as percentage (%) of total hydrolysed starch at different time.

3.2.6 Microscopic study of breads structures

Breads were sampled from the centre of the loaf and processed using a standard protocol of alcohol and xylene cycles followed by fixation in paraffin. Bread sections were cut with a microtome followed by drying in an oven and stained with Lugol's iodine and Light Green. Amylopectin stains brown, amylose stains dark brown (appears in the centre of starch) and gluten stains light green under light microscope. Details regarding this procedure were described in **Section 2.2.6**.

3.2.7 *In vitro* digestion model

This *in vitro* digestion system was performed to mimic conditions in the human GI tract, i.e. oral, stomach and small intestine (duodenum and ileum). The main objective of this analysis was to remove starch and protein. The retentates obtained from the digestion were

freeze-dried and used for the determination of antioxidant potential and *in vitro* batch fermentation. The *in vitro* digestion procedure was divided into three phases:

1) *Oral phase*: Samples containing 50 mg of available carbohydrate were hydrated in sodium chloride and incubated with α-amylase at 37°C for 5 min.

2) *Gastric phase*: pH of retentates were adjusted to 2.5 using HCL and incubated with pepsin at 37° C for 2 h on shaking water bath.

3) *Small intestine (duodenum and ileum)*: pH of retentates were adjusted to 7.0 using sodium hydroxide. Bile acid and pancreatin were added and incubated at 37° C for 3 h on a shaking water bath. Absorption in the small intestine was simulated using a dialysis tube and dialysed for 6 h in 2 L of distilled water.

The digestive products (retentate) were freeze-dried and used for the determination of antioxidant potential and *in vitro* fermentation. Details of *in vitro* digestion procedure were described in **Section 2.2.7**.

3.2.8 Determination of total (poly)phenols

The main objective of this assay was to determine the total (poly)phenols content released from breads after *in vitro* digestion. Freeze-dried samples were diluted in NaCl and added to Folin-Ciocalteu reagent and was kept at room temperature for 5 min. Sodium carbonate was added and kept at room temperature for 90 min and absorbance read at 765 nm against reagent blank. Total (poly)phenols are expressed as mg gallic acid equivalent per g retentate. Details regarding this analysis were described in **Section 2.2.8**.

3.2.9 Determination of ferric reducing ability of plasma (FRAP) assay

This assay measured antioxidant potential of sample based on the oxidation of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). Samples were added with FRAP reagent containing mixture of 2, 4, 6- Tris(2-pyridyl)-s-triazine, ferric chloride and acetate buffer, incubated at 37 ºC for 4 min. The reaction end point was measured after 4 min and absorbance was read at 593 nm against reagent blank. The values were expressed as mol Fe^{2+} equivalents per g retentate. Details regarding this analysis were described in **Section 2.2.9**.

3.2.10 *In vitro* batch fermentation

The objective of this procedure was to determine the fermentability of starch from the bread that resists digestion and (poly)phenols-β-glucan complex in the colon. This method was followed according to standardised *in vitro* batch fermentation procedure (Edwards et al., 1996). Faecal samples were obtained from four healthy volunteers and were used for batch fermentation within 2 h of passage. Details regarding this analysis were described in **Section 2.2.10**.

Samples (from *in vitro* digestion) were added to fermentation medium (42 mL) containing tryptone, micromineral, macromineral, buffer and resazurin in a sterilised glass fermentation bottle. Faecal slurry (5 mL) was added to each bottle, sealed and purged under oxygen-free nitrogen. The bottles were then placed in a shaking water bath. Aliquots of fermentation medium were taken at 0, 6, and 24 h and assayed for pH and short chain fatty acids (SCFA). Details regarding this analysis were described in **Section 2.2.10.1** to **2.2.10.3**.

SCFA in fermentation samples were extracted three times using diethyl ether and injected on GC-FID. Individual SCFA was identified based on comparison with retention times of authentic standards. The value of individual SCFA was expressed as mM. Details regarding this analysis were described in **Section 2.2.10.4**.

3.3 Statistical analysis

Data are expressed as the mean ± standard variation (SD). All data were analysed using SPSS software (SPSS version 22.0, SPSS Inc., Chicago, USA). The distribution of variables was evaluated using Shapiro-Wilk tests and the data considered to be normal if $p > 0.05$. Values with $p < 0.05$ were considered significant.

One-way ANOVA with Bonferroni *post hoc* correction were used to determine the mean differences between groups. A General Linear Model (Repeated Measures) was used to determine the effect of time interactions. Total area under the curve (TAUC) for starch hydrolysis was calculated from baseline to 3 h using the linear trapezoidal method (Vitaglione et al., 2009). Values were considered significantly different at the level of $p < 0.05$. Coefficient of variation (CV) was calculated as standard deviation/mean x 100.

3.4 Results

3.4.1 Bread characteristics

White bread (WB) and black tea bread (BT) had significantly ($p < 0.05$) lower bread volumes than β-glucan (βG) and β-glucan plus black tea (βGBT) breads (**Table 3-1**). βG and βGBT showed significantly ($p < 0.05$) higher weight compared with WB and BT. βG and βGBT showed significantly ($p < 0.05$) lower energy content compared with WB and BT. All breads showed similar length ranging from 12.1 to 13.4 cm. βG and βGBT breads had significantly lower height than WB and BT breads (8.1 - 8.7 cm and 13.0 - 13.6 cm, respectively; $p < 0.05$). A cross-section of the bread structure is shown in **Figure 3-2**. Both BT and βGBT appeared darker because of the black tea.

3.4.2 Nutrient composition

The total available carbohydrate of WB and BT (measured by total resistant and digestible starch) was significantly ($p < 0.05$) higher than that of βG and βGBT (**Table 3-1**). There was no significant difference in resistant starch content. Digestible starch was lower ($p < 0.05$) in βG and βGBT than WB and BT (dilutional effects of water). The protein content ranged from 7.0 - 9.1 g/100 fresh weight and was similar between the different types of bread. Fat content was significantly ($p < 0.05$) higher in WB bread than the other breads (dilutional effects of water). Moisture content of bread with added β-glucan was significantly ($p < 0.05$) higher compared with WB and BT. The addition of β-glucan significantly ($p < 0.05$) reduced the total energy content of βG and βGBT.

Table 3-1. Nutrient composition of breads

Nutrient (g/100 g fresh weight)	White bread (WB)	Black tea bread (BT)	β-Glucan bread (βG)	β-Glucan + black tea bread (βGBT)
Total available carbohydrate	45.4 ± 4.0[a]	45.4 ± 4.6[a]	34.8 ± 8.1[b]	32.8 ± 1.6[b]
Resistant starch	1.00 ± 0.2	0.9 ± 0.1	1.0 ± 0.3	0.8 ± 0.20
Digestible starch	44.4 ± 3.8[a]	44.5 ± 4.5[a]	33.8 ± 7.8[b]	32.0 ± 1.5[b]
β-glucan	0.3 ± 0.0[a]	0.3 ± 0.0[a]	4.7 ± 0.1[b]	4.6 ± 0.2[b]
Fat	0.8 ± 0.1[a]	0.6 ± 0. [b]	0.6 ± 0.1[b]	0.6 ± 0.1[b]
Protein	9.1 ± 0.1	8.9 ± 0.2	7.1 ± 0.1	7.0 ± 0.7
Moisture	37.0 ± 0.4[a]	36.0 ± 0.6[a]	49.7 ± 0.6[b]	47.8 ± 0.6[b]
Ash	1.6 ± 0.0	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
Weight (g)	722.3 ± 9.3[a]	729.0 ± 9.6[a]	866.7 ± 27.6[b]	835.3 ± 22.6[b]
Dimension (L x H x W)*	13.4 x 13.0[a] x 1.5	12.7 x 13.6[a] x 1.5	12.7 x 8.1[b] x 1.5	12.1 x 8.7[b] x 1.5
Energy, kJ (kcal)‡	957.3 (228.8)[a]	943.0 (225.4)[a]	735.1 (175.7)[b]	698.1 (166.8)[b]

*L-length, H-height, W-width

‡Energy was calculated based on the formula [total available carbohydrate (%) x 17] + [protein (%) x 17] + [fat (%) x 37]. Different superscript letters indicate statistically significant ($p < 0.05$) values within the same row.

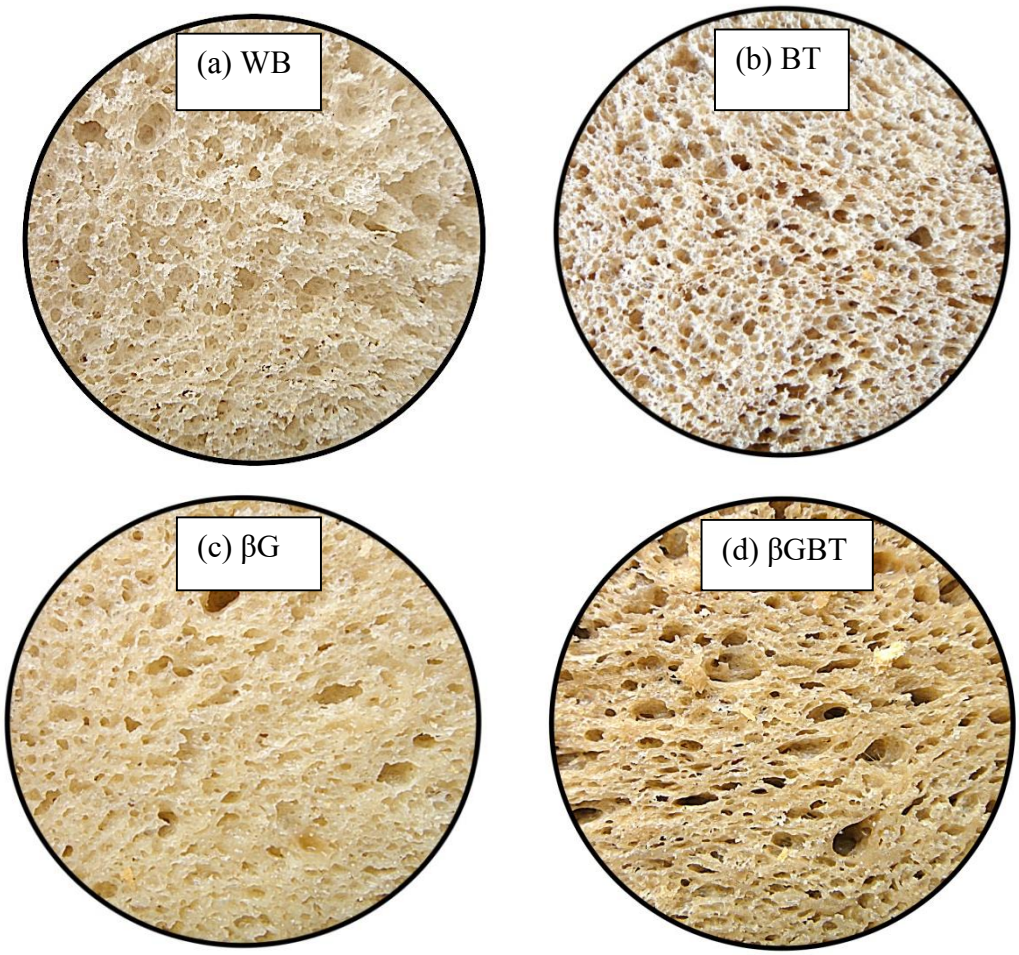


Figure 3-2. Detail of bread structure. The structure of bread was visualised at 4x power. (a) WB, white bread; (b) BT, black tea bread; (c) βG, β-Glucan bread; (d) βGBT, β-Glucan plus tea bread.

3.4.3 Microscopic study of bread structure

The bread microstructures, starch granules and protein (gluten) were studied under a light microscope (**Figure 3-3**). Amylopectin granules stained brown, amylose dark brown (leached in the middle of starch granule) and gluten light green. Starch granules in WB and BT were swollen and sheared into small circular structures. In contrast, starch granules in βG and βGBT were elliptical and closely packed to each other. The green-stained gluten area was embedded in between starch granules and the porous (irregular white structures) area of the breads. The gluten networks were more prominent and appeared as a more continuous matrix in βG and βGBT compared with WB and BT.

3.4.4 Starch hydrolysis of breads

Standard flour (SF) showed the lowest *in vitro* starch hydrolysis from 10 to 120 min (**Figure 3-4**). There was no significant difference in starch hydrolysis between WB and BT from 0 to 180 min. Both WB and BT showed significantly ($p < 0.05$) higher starch hydrolysis at 10 min compared with βG and βGBT. WB and BT showed significantly ($p < 0.05$) higher $TAUC_{0\text{-}180\ min}$ compared with βG and βGBT. Combination of β-glucan and black tea in βGBT significantly ($p < 0.05$) reduced early (10 min) starch hydrolysis by 25% compared with βG. However, $TAUC_{0\text{-}180\ min}$ of βGBT did not change significantly when compared with βG (**Figure 3-5**).

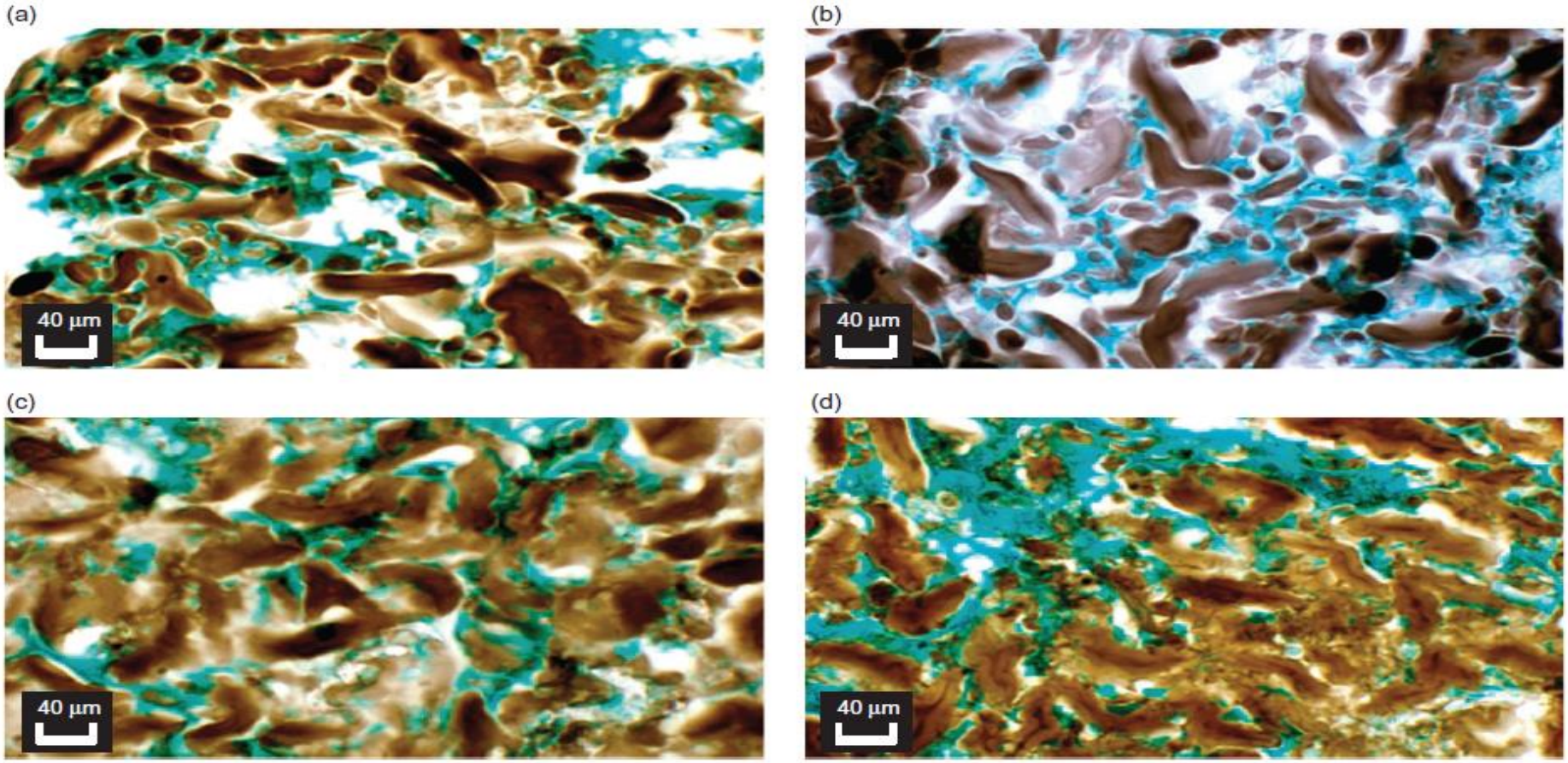


Figure 3-3. Microscopic figures of bread structures. (a) WB, white bread; (b) BT, black tea bread; (c) βG, β-Glucan bread; (d) βGBT, β-Glucan plus black tea bread. Amylopectin granule stains brown, amylose dark brown (appears in the middle of starch granule) and gluten light green.

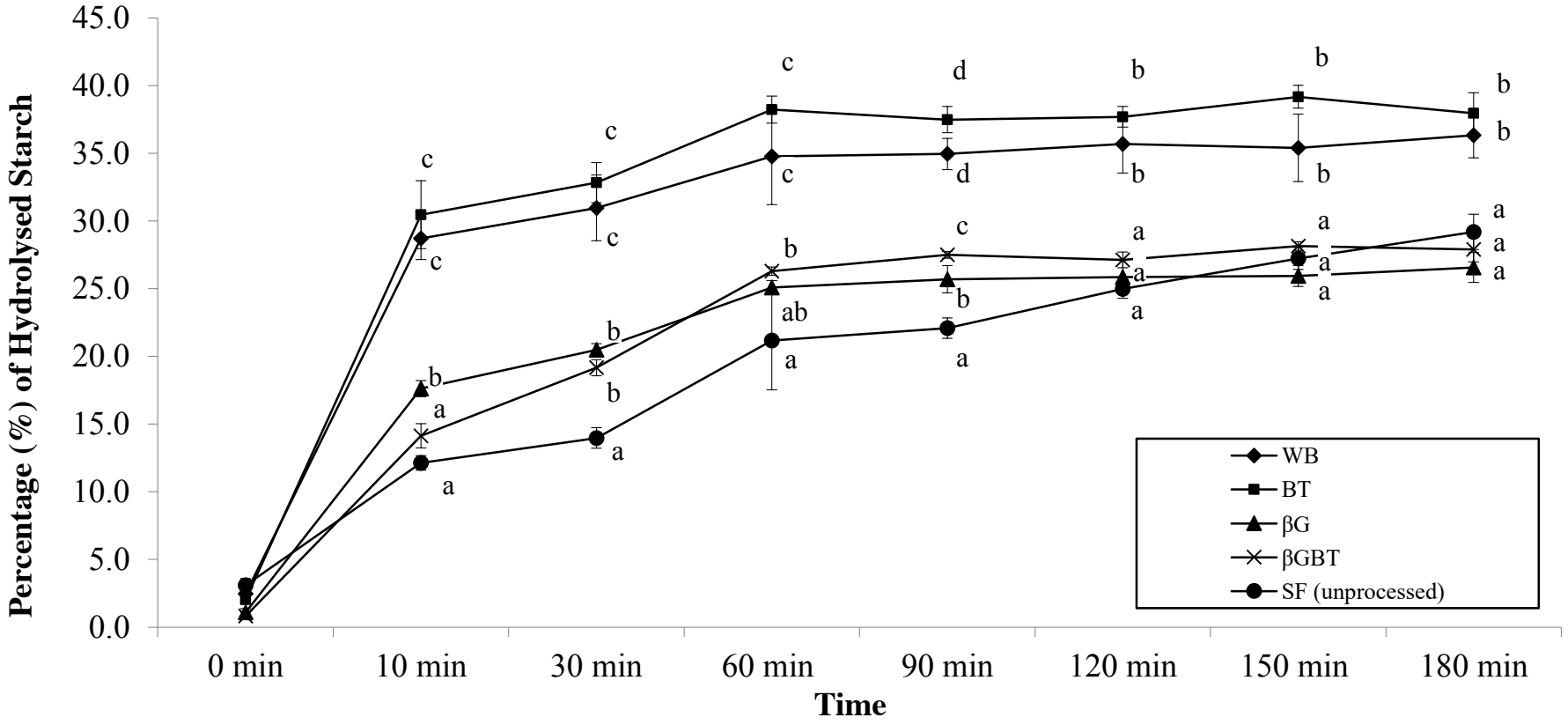


Figure 3-4. Starch hydrolysis based on percentage (%) of total hydrolysed starch of different breads. WB, white bread; BT, black tea bread; βG, β-Glucan bread; βGBT, β-Glucan plus black tea bread; SF, Standard flour (unprocessed, provided by supplier). Values are expressed as mean ± standard deviation. Values with different letters are significantly different at the level of $p < 0.05$ within same time point. There were significant ($p < 0.05$) time interactions. Coefficient of variation (CV) is less than 32.0%.

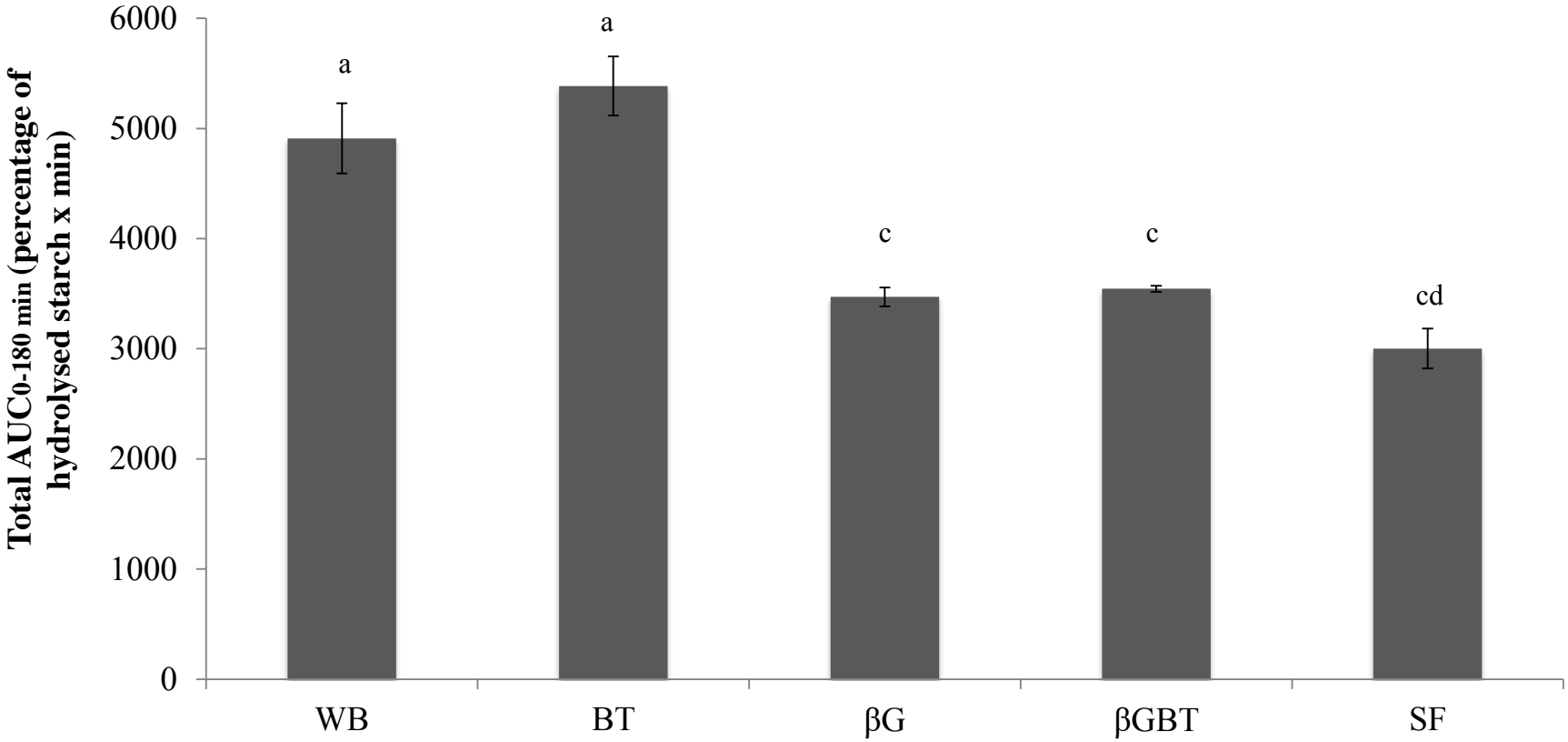


Figure 3-5. Total area under the curve ($TAUC_{0\text{-}180min}$) of percentage (%) hydrolysed starch x min. WB, white bread; BT, black tea bread; βG, β-Glucan bread; βGBT, β-Glucan plus tea bread; SF, Standard flour (provided by supplier). Values with different letters are significantly different at the level of $p < 0.05$. Coefficient of variation (CV) is less than 6.5 %.

3.4.5 Total (poly)phenols released and antioxidant activity of breads

3.4.5.1 Total (poly)phenols released after *in vitro* digestion

The total (poly)phenol content of digested breads is shown in **Figure 3-6**. (Poly)phenols released from the breads after digestion with α-amylase at 0 h were in the range of 0.23 to 0.361 mg GAE/ g retentate. BT bread showed significantly ($p < 0.05$) higher total (poly)phenol content after digestion with α-amylase compared with other breads. Black tea (positive control) showed significantly ($p < 0.05$) highest total (poly)phenols (0.47 mg GAE/ g retentate) compared with all breads and SF. The retentates were dialysed for 6 h after digestion with pepsin and pancreatin. (Poly)phenols content did not change significantly after dialysis in all breads (1.08 to 1.38 mg GAE/ g retentate) compared with WB.

3.4.5.2 FRAP antioxidant activity

BT breads showed significantly ($p < 0.05$) higher antioxidant activity (1.06 ± 0.07 mg Fe^{2+} equivalents/g retentate) when compared with other breads (0.24 to 0.61 mg Fe^{2+} equivalents/g retentate) after digestion with α-amylase (**Figure 3-7**). Black tea (positive control) showed the highest antioxidant potential when compared with other breads and SF (positive control). The retentates were dialysed for 6 h after digestion with pepsin and pancreatin. Antioxidant potential did not change significantly after dialysis in all breads in the range of 3.35 to 4.02 mg Fe^{2+} equivalents/g retentate.

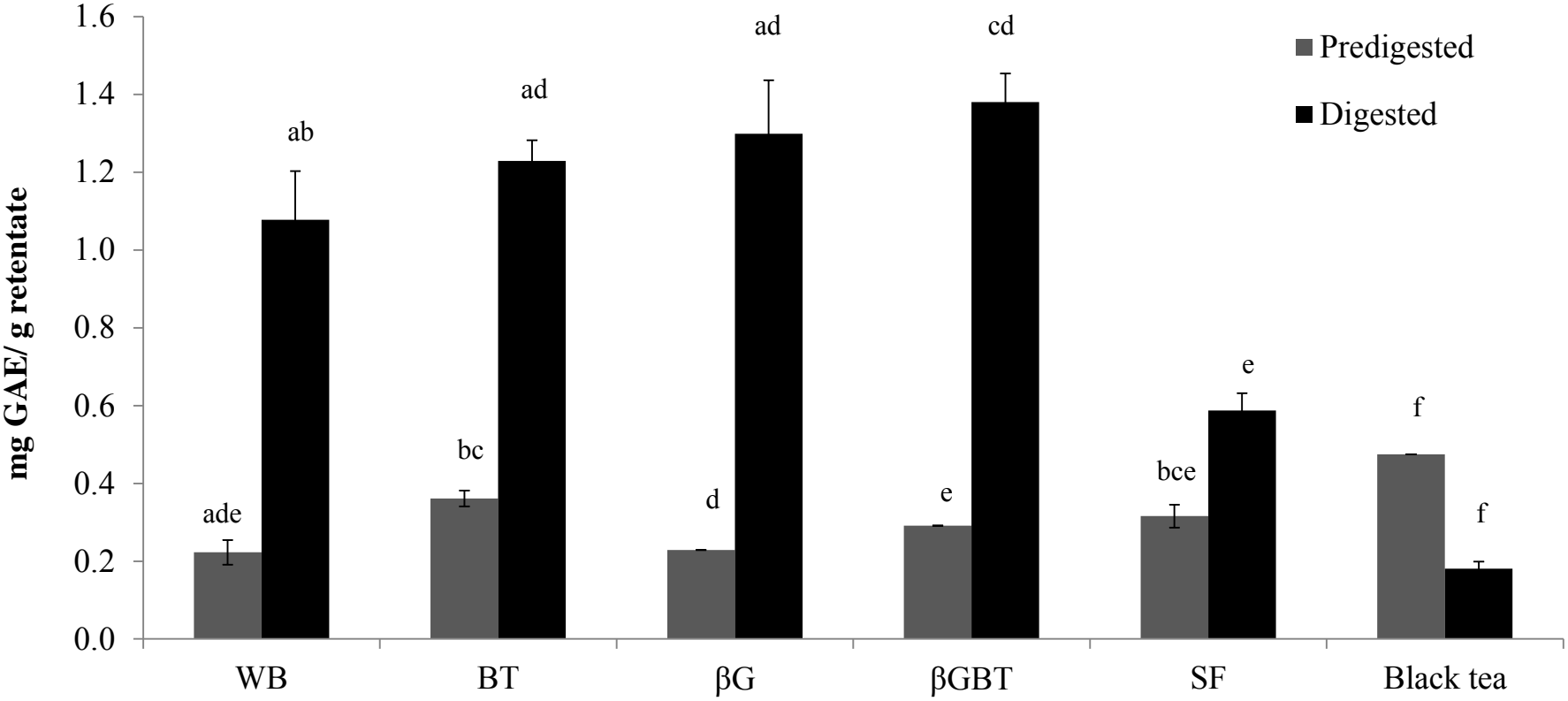


Figure 3-6. Total (poly)phenols released from breads after digestion with α-amylase (0 h) (predigested) and after digestion with pepsin (2 h), pancreatin (3 h) and followed by dialysis for 6 h (digested). Values are expressed mean ± SD. Different letters indicate significant ($p < 0.05$) differences between breads, SF and Black tea. There were significant ($p < 0.05$) differences between predigested and digested samples. WB, white bread; BT, black tea bread; βG, β-Glucan bread; βGBT, β-Glucan plus black tea bread; SF, standard wheat flour (positive control); Black tea (freeze-dried, positive control).

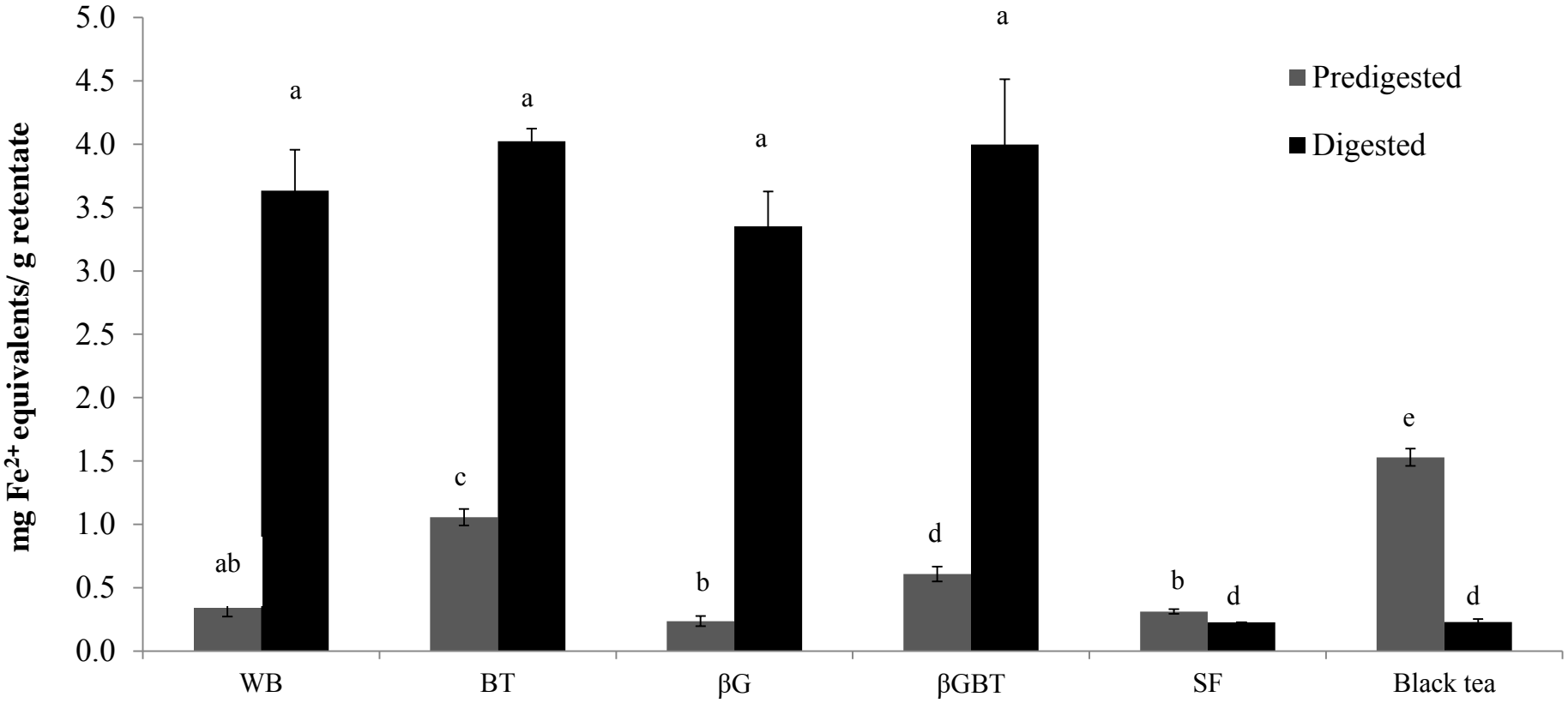


Figure 3-7. FRAP activity of breads after digestion with α-amylase (0 h) (predigested) and after digestion with pepsin (2 h), pancreatin (3 h) and followed by dialysis for 6 h (digested). Values are expressed mean ± SD. Different letters indicate significant ($p < 0.05$) differences between breads, SF and Black tea. There were significant ($p < 0.05$) differences between predigested and digested samples. WB, white bread; BT, black tea bread; βG, β-Glucan bread; βGBT, β-Glucan plus black tea bread; SF, standard wheat flour (positive control); Black tea (freeze-dried, positive control).

3.4.6 Short chain fatty acid production

3.4.6.1 pH

The fermentation fluid containing the breads showed similar baseline (0 h) pH in the range of 6.5 ± 0.1 to 6.6 ± 0.2 (**Table 3-2**). The pH was significantly ($p < 0.05$) reduced at 6 h for all breads compared with baseline in the range of 5.0 ± 0.4 to 5.5 ± 0.1. At 24 h after fermentation, the pH of fermentation culture remained significantly ($p < 0.05$) lower compared with baseline in the range of 5.2 ± 0.5 to 5.8 ± 0.2. The fermentation containing wheat flour (SF, unprocessed wheat flour as positive control) showed similar pH profiles as breads.

Table 3-2. The pH in fermentation fluid containing breads at 0, 6 and 24 h

Bread	0 h	6 h	24 h
WB	6.6 ± 0.1	5.5 ± 0.1	5.8 ± 0.2
BT	6.6 ± 0.2	5.4 ± 0.2	5.7 ± 0.43
βG	6.6 ± 0.2	5.1 ± 0.4	5.3 ± 0.5
βGBT	6.6 ± 0.2	5.0 ± 0.4	5.2 ± 0.5
SF	6.5 ± 0.1	5.2 ± 0.2	5.5 ± 0.2

3.4.6.2 Individual and total SCFA

Acetate production was similar in fermentations containing the breads at 0, 6 and 24 h **(Figure 3-8)**. Acetate production significantly ($p < 0.05$) increased from 0 to 6 h in all breads. There was no significant increase in acetate between 6 to 24 h. Breads showed similar propionate production at 0, 6 and 24 h (**Figure 3-9**). Propionate levels significantly ($p < 0.05$) increased at 6 h in all breads when compared with 0 h. There was no significant increase in propionate between 6 to 24 h. Butyrate production was similar between breads

at 0 h, 6 h and 24 h (**Figure 3-10**). There was significant ($p < 0.05$) production of butyrate from 0 to 6 and 24 h in all breads. Total SCFA as the sum of acetate, butyrate and propionate was similar in fermentations containing the breads (**Figure 3-11**). The ratio (acetate:propionate:butyrate) of each individual SCFA of WB, BT, βG and βGBT were similar in the ratio of 54:38:8, 55:37:8, 55:38:7, and 56:38:6, respectively.

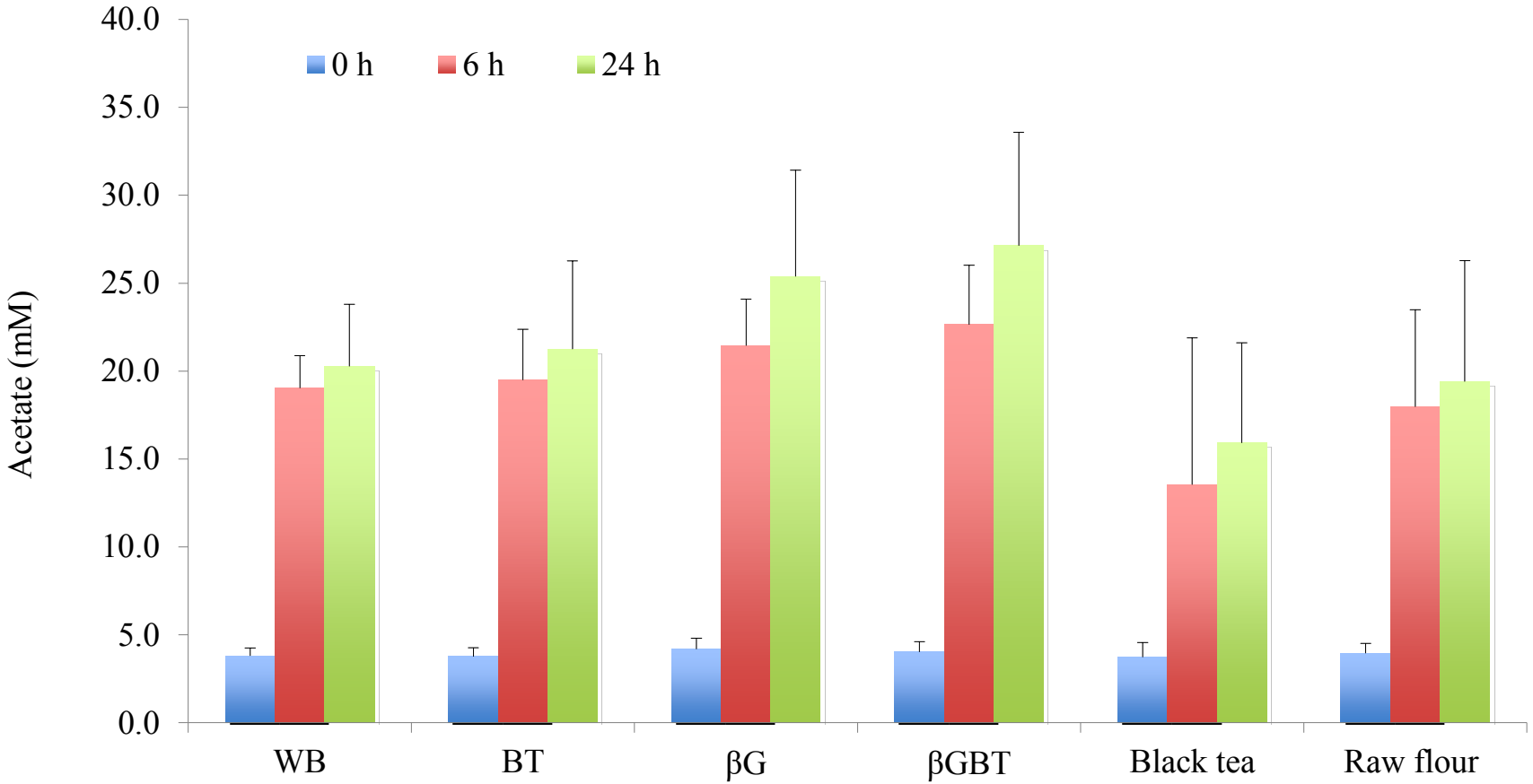


Figure 3-8. Acetate concentrations (mM) in fermentation slurry at 0, 6 and 24 h. Values are mean (standard deviation). No significant difference between breads (one-way ANOVA test). There were significant ($p < 0.05$) time-interactions (0 and 6 h) (ANOVA repeated measures).

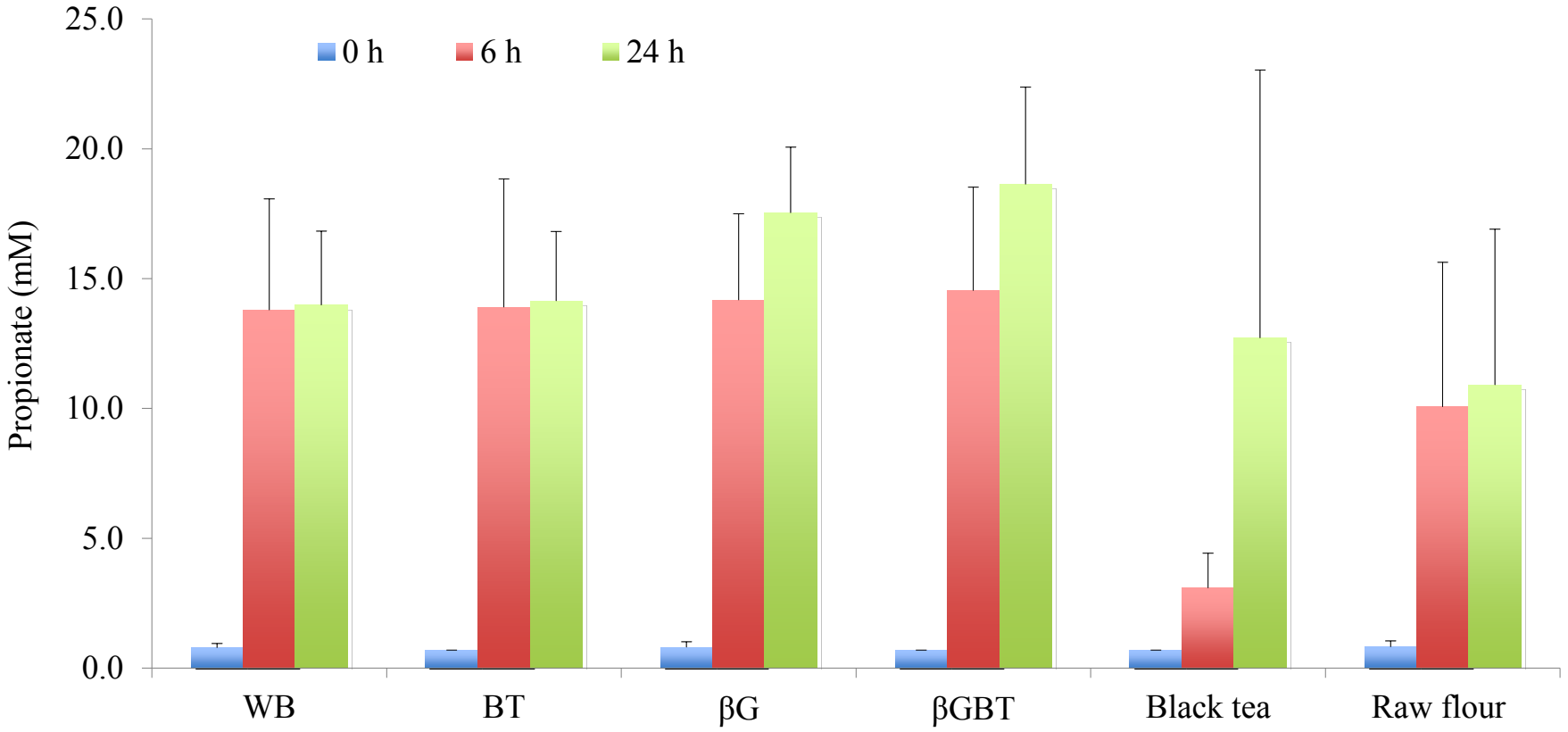


Figure 3-9. Propionate concentrations (mM) in fermentation slurry at 0, 6 and 24 h. Values are mean (standard deviation). No significant difference between breads (one-way ANOVA test). There were significant ($p < 0.05$) time-interactions (0 and 6 h) (ANOVA repeated measures).

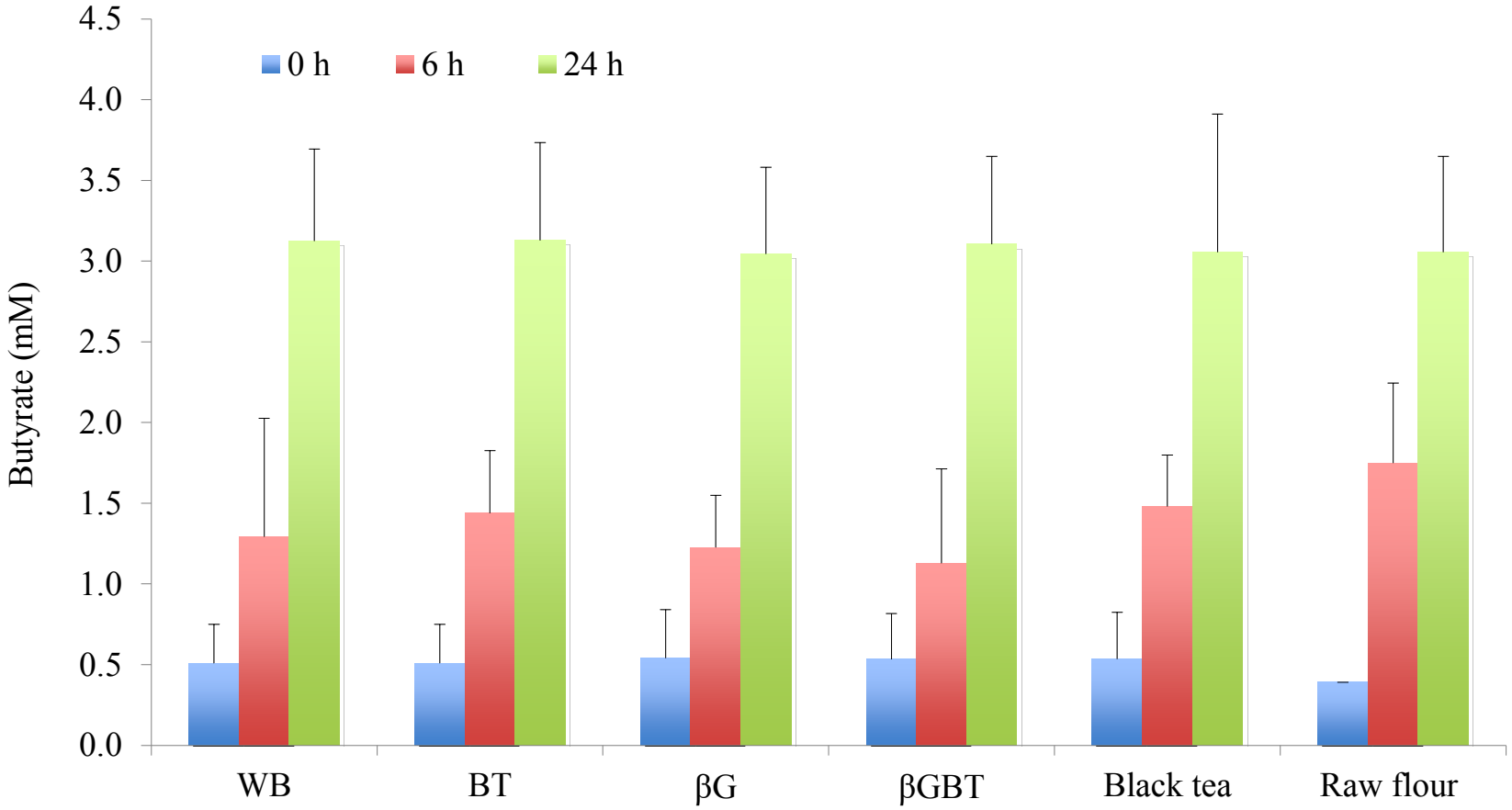


Figure 3-10. Butyrate concentrations (mM) in fermentation slurry at 0, 6 and 24 h. Values are mean (standard deviation). No significant difference between breads (one-way ANOVA test). There were significant ($p < 0.05$) time-interactions (0 h, 6 h and 24 h) (ANOVA repeated measures).

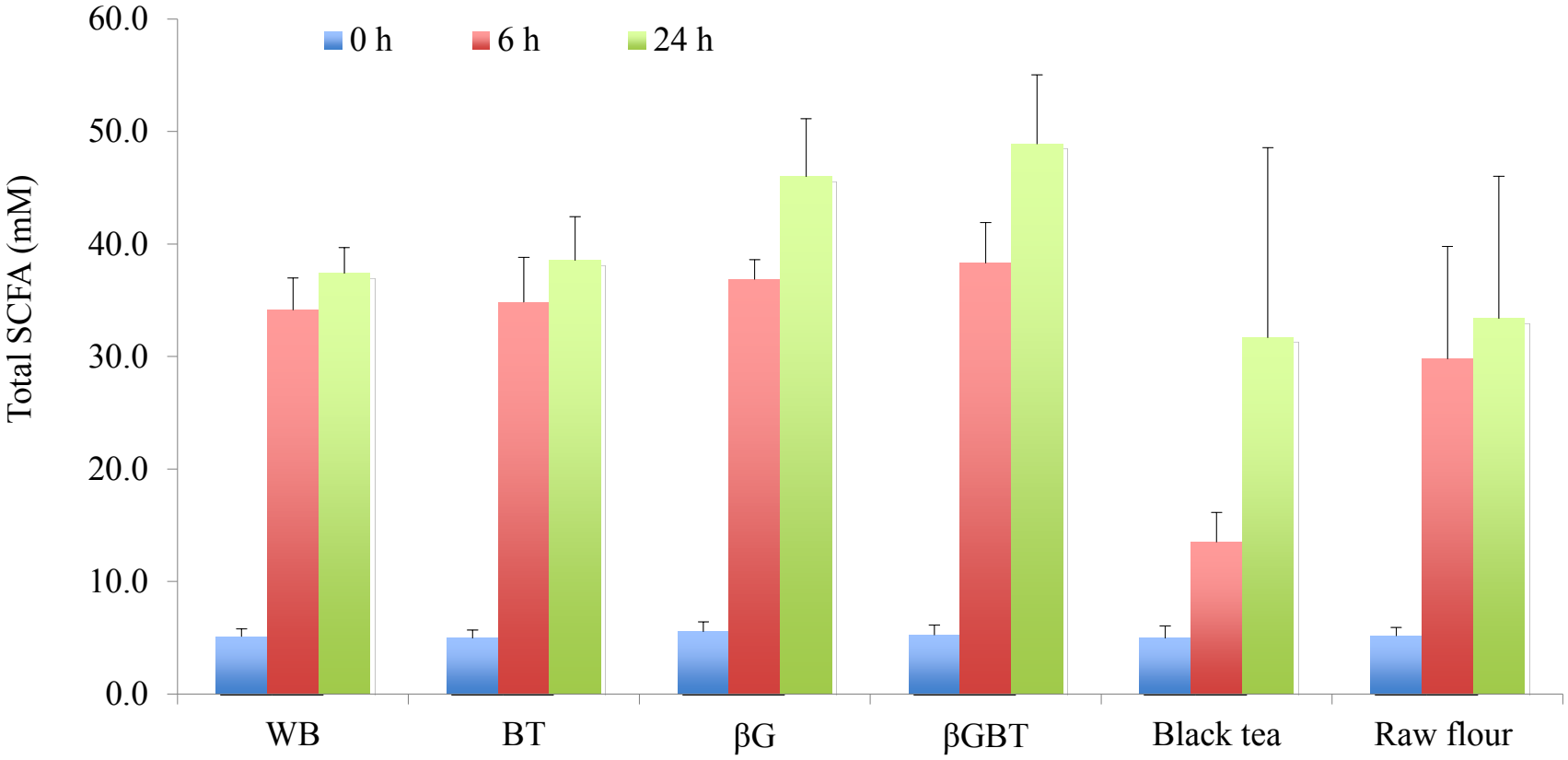


Figure 3-11. Total SCFA (acetate, propionate and butyrate) concentration (mM) in fermentation slurry at 0, 6 and 24 h. Values are mean (standard deviation). No significant difference between breads (one-way ANOVA test). There were significant ($p < 0.05$) time-interactions (0 h, 6 h and 24 h) (ANOVA repeated measures).

3.5 Discussion

The aim of this study was to develop breads and determine the food matrix interactions of two food components with established health effects. The effects of adding β-glucan and/or black tea on starch structures and the impact of these interactions on starch characteristics, (poly)phenol content, and antioxidant potential were determined. The second aim of this study was to determine the fermentability of the test breads using an *in vitro* batch fermentation mimicking the human large intestine.

The composition of protein (gluten), starch and water play an important role in making good quality bread (Flander et al., 2007). Gluten is responsible for dough formation while starch is important in textural properties and stability of the bread. Water hydrates and expands gluten forming a viscoelastic protein network. The added β-glucan competed with gluten for water and decreased the rising of the dough during proofing; therefore, more water was needed to compensate for water uptake by beta glucan; this was necessary to improve dough quality (Hager et al., 2010; Jacobs et al., 2008). In the present study, more water was added in β-glucan breads (βG and βGBT) and this led to diluted starch content (higher moisture content). This was similar when soluble fibre guar gum was incorporated in bread (Ellis et al., 1981). The presence of mixed linkage (1→3)(1→4)-β-D-glucans stabilises the air cells in bread dough and improves coalescence (Wang et al., 1998). Others have demonstrated that the addition of sorghum flour in flat bread lowers the proportion of rapidly digestible starch (RDS) and starch digestibility by 29% and 25% respectively (Yousif et al., 2012).

In vitro studies demonstrated that black teas reduced starch hydrolysis (Guzar et al., 2012; Zhang and Kashket, 1998). Mechanistically, a structural relationship of flavonoids and α-amylase activity has been described with hydrogen bonding as well as the formation of a conjugate that stabilised the interaction with the active site of α-amylase (Lo Piparo et al., 2008). This effect may have been mediated through inhibition of amylase activity compared with white bread. In our study, the addition of black tea increased (poly)phenol content and antioxidant potential of breads after *in vitro* digestion with α-amylase. However, given an increase in (poly)phenol content and antioxidant potential, our study

showed the addition of black tea in bread (BT) did not reduce starch hydrolysis. This may be explained by differences in experimental design. In our study, tea was added to breads as an ingredient during baking. Therefore, we would anticipate that this would lead to a different effect than that observed in previous studies because of the complex food matrix-interaction between gluten, black tea and starch in bread.

In bread, the addition of apple pectin and (poly)phenols extracts, from kiwi, blackcurrant or apple during dough development and bread baking, directly influenced the cross-linking of gluten polymers which could lead to more water holding and softer bread (Sivam et al., 2011). The type of (poly)phenols used are likely to have different mechanisms in forming complexes with bread protein (gluten). Highly polar phenolic acids (*i.e.* the caffeic acid present in kiwi) are more mobile in bread than low polarity (poly)phenols (*i.e.* anthocyanins and proanthocyanins in blackcurrant and apple). Caffeic acid is attracted to charged components in protein and/or directly incorporates into the protein network with less steric hindrance (Sun-Waterhouse et al., 2009; Sivam et al., 2011). This may disrupt the gluten network and hence lead to the softer bread. The present study showed that *in vitro* digestion of BT with α-amylase increased (poly)phenol content and antioxidant potential compared with WB and βGBT breads (**Figure 3-6** and **Figure 3-7**).

In guar gum wheat bread, the presence of galactomannan coated the starch granules and protein matrix during the bread making process and subsequently reduced starch hydrolysis (Brennan et al., 1996). Our study showed that black tea had an additional effect when added together with β-glucan. The incorporation of black tea and β-glucan significantly reduced short-term (10 min) starch digestibility in βGBT compared with βG bread. However, it must be noted that the change was only observed at one time point (10 min) and may or may not have any beneficial effects in human. The microscopy suggested that both β-glucan and black tea preserved the starch granule structure, which resulted in lower short-term starch hydrolysis. Black tea contains higher levels of high molecular weight theaflavins and thearubigins compared to the other types of teas (Shao et al., 1995). Black tea also contains theaflavin trigallate and tetragallate (Chen et al., 2012). We propose that there is a food-matrix interaction between black tea (poly)phenols, β-glucan

and starch granules in the bread. The presence of black tea (poly)phenols could form complexes with the gluten-network while β-glucan preserved the starch structure and/or decreased the surface area for starch digestion by α-amylase and hence reduced starch hydrolysis. *In vitro* digestion showed antioxidant potentials and total (poly)phenols of βGBT did not increased significantly after 10 min incubation with α-amylase compared with BT bread (**Figure 3-6** and **Figure 3-7**). This suggests that (poly)phenols are being 'trapped' in between starch granules and β-glucan and hence prevent them from being released during digestion with α-amylase. As discussed in **Chapter 1**, there is a strong hydrogen bond that governs the interaction between tea (poly)phenols and β-glucan (Wang et al., 2013).

The translation potential of these findings is of interest to human health. It was observed that βGBT bread showed blunted early *in vitro* starch hydrolysis, higher (poly)phenol content and antioxidant potential after digestion with α-amylase. The possible mechanisms in humans are as follows: first, it could reduce post-prandial glucose and insulin response *in vivo*. It has been previously demonstrated that the supplementation of guar bread reduced post-prandial insulin response in healthy individual (Ellis et al., 1991). Secondly, the (poly)phenol-linked β-glucan will be passed undigested into the large intestine and metabolised by gut microbiota to SCFA and phenolic acids. Tea has been shown to be metabolised by microbiota in the large intestine into phenolic acids (Stalmach et al., 2009; Stalmach et al., 2010). *In vitro* fermentation of β-glucan by intestinal microbiota significantly increased propionate giving an acetate:propionate:butyrate production ratio of 51:32:17, which was considered to be propionate-rich (Hughes et al., 2008).

Breads with β-glucan preserved some of the starch granules. This has implications for gut microbiota actions. The preserved starch granules, along with (poly)phenols and β-glucan have the potential to change the amount of SCFA produced by the action of the gut microbiota. I*n vitro* batch fermentations using human fecal samples demonstrated that βG and βGBT had increased production of acetate and propionate compared to WB, giving a production ratio of 55:38:6. Propionate production from β-glucan breads (βG and βGBT) was 17.53 and 18.63 mM respectively. This is similar to Nordlund et al. who showed how

predigestion of oat concentrate increased production of propionate (18 mM) (Nordlund et al., 2012). *In vitro* fermentation of β-glucan from different sources (oat concentrate, oat and barley) produced propionate in the range of 5.5 – 18 mM (Hughes et al., 2008; Kim and White, 2011; Nordlund et al., 2012). In addition, propionate levels were higher in βG and βGBT but were not statistically significantly different from white bread. Faecal samples were obtained from 4 individuals, which could be the source of intervariation due to differences in gut microbiota composition. The method used in this study was validated based on 40 donors from different laboratories and is considered as rapid and accurate for *in vitro* fermentation. However, this method showed the greatest intra-laboratory variation with those utilising samples from only four donors (Edwards et al., 1996). Apart from the *in vitro* fermentation method using faecal samples, there are also other methods to study colonic fermentation such as animals and human studies. Although these methods allow longer study duration, they are not easy to replicate, are time consuming, and relatively expensive compared with *in vitro* fermentation. The proximal colon is difficult to access *in vivo* so proxy measures of faeces in the fermentation chamber are often used which may not fully reflect colonic fermentation as most SCFA will have been absorbed.

In pigs, the supplementation of β-glucan concentrate for 14 days enhanced growth of lactobacilli and bifidobacteria in the colon (Metzler-Zebeli et al., 2011). This study also showed a higher production of propionate in the range 24-29 mM. Feeding pigs with barley and oats for 35 days increased production of propionate in the range of 13 – 43 mM (Jozefiak et al., 2006). In humans, Verbeke et al. showed propionate (measured in breath samples) production in groups supplemented with barley porridge but not in those supplemented with barley kernel (Verbeke et al., 2010). This study showed how barley porridge is rich in non-starch polysaccharides (NSP), whereas barley kernel is also rich in RS and NSP demonstrating that meals containing dietary fibre (measured as NSP) in combination with RS (barley kernel) resulted in a different SCFA profile when compared to having dietary fibre alone (barley porridge). The results showed that barley porridge and barley kernel increased the concentration of propionate and butyrate, respectively.

Short chain fatty acids, particularly propionate, play a role in hepatic glucose production

(acting as a precursor) and are involved in appetite regulation through the stimulation of hepatic vagal afferents (in ruminant studies) (Chambers et al., 2014). In humans, inulin-propionate ester (propionate bound to inulin through ester bond) increased subjective appetite, released appetite hormones (GLP-1 and PYY) from human colonic cells, and reduced energy intake among obese subjects (Chambers et al., 2015). However, the dosage used was much higher than the amount produced *in vitro* and animal studies (**Chapter 1**, **Table 1-10**). Propionate may act through activation of G protein-coupled receptor 41, located on colonic enteroendocrine L-cells (Psichas et al., 2015; Karaki et al., 2008). These cells secrete gut hormones GLP-1 and PYY and may act to suppress food intake through gut-brain axis (Karaki et al., 2008; Murphy and Bloom, 2006; Batterham et al., 2002).

3.6 Conclusions

The results of this study suggest that bread prepared with β-glucan and black tea appeared to preserve some of the starch granules and lowered short-term (10-min) starch hydrolysis. BT bread was softer and showed higher antioxidant potential and (poly)phenol content compared with other breads. Propionate concentrations were higher in fermentations with β-glucan breads but did not change significantly compared with white bread. The addition of black tea had no apparent effect on SCFA production. Reduced early starch hydrolysis could positively regulate postprandial glucose response, but the palatability of the breads needs to be determined before conducting human feeding studies. Higher propionate production ratio might have a positive effect on satiety in humans and this warrants further investigation.

Chapter 4: Palatability, Perceived Satiety and *Ad Libitum* Energy Intakes at Lunch after Intake of Functional Bread Prepared with β-Glucan and Black Tea

OUTLINE:

The breads were successfully developed with β-glucan and/or black tea but showed lower palatability compared with white bread (**Chapter 3**). The bread palatability needs to be more thoroughly tested before it can be used in feeding study. This chapter assesses the palatability of the breads based on texture, smell and overall acceptability using adaptive visual analogue scale (AVAS) in healthy volunteers. Perceived satiety was measured before and at 30 min up to 180 min after breakfast meal using AVAS. Perceived satiety consists of measurements of hunger, fullness, desire to eat, and prospective food intake.

The results of this study have been presented at the Nutrition Society Postgraduate Conference, Trinity Hall, University of Cambridge, 7-8 September 2015. The oral presentation was entitled 'Palatability and satiety effects of breads prepared from β-glucan and black tea in healthy volunteers'.

4.1 Introduction

In **Chapter 3**, breads prepared with β-glucan and black tea showed reduced *in vitro* starch hydrolysis. This may have potential in reducing postprandial glucose response but the palatability of the breads need to be tested first. These breads might have favourable effects on postprandial glucose, insulin and may also induce early satiety. Dietary fibre such as β-glucan in breads, biscuits and muesli positively regulate postprandial glucose responses and increase satiety in humans (Casiraghi et al., 2006; Granfeldt et al., 2008; Juntunen et al., 2002; Vitaglione et al., 2009; Vitaglione et al., 2010). In a randomised controlled trial, bread and biscuits prepared with β-glucan reduced food intake at lunch by 19 to 22% and increased perceived satiety by 43 to 55% when compared to white bread (Vitaglione et al., 2009, Vitaglione et al., 2010). A recent systematic review demonstrated that soluble β-glucan was amongst the fibres that increased perceived satiety (39%) and reduced energy intake (or reduced food intake) compared with control (Clark and Slavin, 2013).

As described in **Chapter 1**, an acute consumption of green tea beverage (9 g in 300 mL hot water, containing 151 mg catechins) together with white bread increased perceived fullness by 51 % when compared to control (Josic et al., 2010). The combination of green tea catechins and the soluble fibre dextrin (Nutriose) as a beverage consumed three times per day reduced perceived hunger (34%), increased fullness (57%), and reduced energy intake by 12% at lunch when compared with control (no beverage) (Carter and Drewnowski, 2012). Reinbach et al. showed green tea beverage (350 mL, 3 times per day) ingestion increased perceived fullness but had no effect on energy intake when measured in the evening (Reinbach et al., 2009). These studies showed that either green tea alone, or when eaten with food, increased perceived satiety. However, there may be different effects when black tea is included in a solid food such as bread. Black tea is one of the most commonly consumed beverages in European countries (Clifford et al., 2013). Black tea contains additional large molecular weight (poly)phenols, theaflavins and thearubigins along with monomers epicatechin, catechin, gallocatechin, gallocatechin gallate (Del Rio et al., 2004; Roowi et al., 2010; Stalmach et al., 2010). This is the distinctive character of black tea compared with green tea and may have additional health benefits but needs further investigations.

Solid food induces greater satiety as more time is needed for oral processing (eating time) and gastric emptying when compared with liquid foods (Cassady et al., 2012; de Graaf et al., 2012; Hennessy-Priest, 2014; Willis et al., 2011). Solid food induces higher oral-sensory exposure time and leads to early meal termination and/or higher satiety response (de Graaf et al., 2012). Moreover, the addition of fibre (oats) to solid food might reduce the interaction with enzymes in the stomach, increasing stomach distention, reducing gastric emptying time, and leading to higher satiety response (Willis et al., 2011). The role of satiety on food intake is important in controlling overeating because highly satiating food augments fullness, as an interval between eating occasions, and reduces subsequent food intake (Gerstein et al., 2004). In addition, perceived satiety diminishes the desire to eat more and regulates energy intake and body weight. In **Chapter 3**, it was shown that the combining β-glucan and black tea in a bread food matrix reduces the rate of *in vitro* starch hydrolysis (Jalil et al., 2015). This has the potential to slow postprandial glucose responses *in vivo*, but the relative palatability and satiety needs to be tested before conducting further metabolic studies. This is also important if such food is expected to be consumed by the general population.

Breads prepared with dietary fibres showed good nutritional quality (increased dietary fibre fractions and reduced starch hydrolysis), but lower sensory characteristics (Angioloni et al., 2011; Ellis et al., 1981; Ellis et al., 2001; Jenkins et al., 2002). Ellis et al. showed that guar gum inclusion (5 and 10%) in breads was acceptable but the dose of 15% was unacceptable among normal subjects (Ellis et al., 1981). By using fourier-transform infrared spectrometry, Sivam et al. demonstrated that the addition of soluble dietary fibre pectin and (poly)phenols together in a bread caused conformational changes in the gluten network during bread making, resulting in softer bread (Sivam et al., 2011; Sivam et al., 2013). These changes will affect the texture, visual appeal, and palatability of the final products (Yuan et al., 2014). It is challenging to develop a good product with functional ingredients because there must be a balance between product acceptability and the amount required for any health benefits (Hall et al., 2010). An early study by Ellis et al. demonstrated how bread added with guar gum had no apparent effects on satiety, but showed reduced palatability compared with control breads (Ellis et al., 1981). Jenkins et al. measured the palatability of a high fibre diet (β-glucan) to identify whether or not their

dietary approaches were feasible for the general population (Jenkins et al., 2002). In this context, it is important to investigate the product's palatability to ensure that products will be consumed and accepted before testing possible health benefits. Hence, in this study, the β-glucan and/or black tea breads were compared with white bread for palatability and effect on perceived satiety and energy intake at lunch.

4.2 Experimental design

This study was a randomised, cross-over controlled experimental design and was divided into three sections as follows:

1. **Section 1** (palatability): This study used an adaptive visual analogue scale (VAS) to study the palatability of breads in healthy male and female volunteers.
2. **Section 2** (satiety): This study used an adaptive visual analogue scale (AVAS) to determine perceived satiety, hunger, fullness and desire to eat in healthy male and female volunteers.
3. **Section 3** (estimation of next meal effects on energy intake): The impacts of having breads as breakfast on energy intake was estimated based on *ad libitum* buffet lunch (3 h after breakfast). The food items were pre-weighed before being presented to the participants and the leftovers were weighed and estimated for energy intake.

4.2.1 Study design

Participants received one of 4 treatments, based on a single blind, randomised-controlled crossover design (Latin square design). Participants were randomly allocated using an online website (www.randomization.com) and coded into sequences of 4 treatments, with each of the treatments separated with a one week washout period (**Figure 4-1**).

4.2.2 Sample Size

The sample size was calculated with G*Power (version 3.1.9) statistical software (Faul et al., 2009). The parameter used for sample size calculation was based on a study investigating the effect of β-glucan bread on energy intake as primary outcome (Vitaglione

et al., 2009). With an alpha error of 0.05 and power of 80% to detect a 19% reduction in energy intake, 12 participants were needed for each gender group. Hence, the total sample size was 24 participants. Each participant completed 4 trials; the total numbers of trials completed was 96.

4.2.3 Inclusion and exclusion criteria

The study involved healthy males and females, aged between 18-50 years old, with a body mass index (BMI) ≥ 18.5 to 29.9 kg/m^2. Participants were excluded if they had any of the following conditions: Gastrointestinal disease (e.g. coeliac disease), smokers, vegetarian, regular use of dietary supplements, food intolerance, allergy to gluten, on dietary restrictions, significant changes in body weight for the past year (± 4 kg), known chronic illnesses, diabetes or impaired glucose tolerance, being prescribed with medication known to affect blood glucose and those with systolic/diastolic blood pressure more than 139/89 mmHg.

4.2.4 Subject recruitments

Participants were recruited using flyers and posters advertised in public areas in Glasgow, such as department notice boards at the University of Glasgow, library, churches, sports complex, University of Glasgow Student Union (Student Voice), word of mouth and also through a social network (Facebook). Interested participants were contacted by phone or email for a face-to-face interview with the researcher at Human Nutrition, Glasgow Royal Infirmary or at any convenient place. This interview took place to check inclusion and exclusion criteria, to discuss the study and to ask for consent.

4.2.5 Study protocol

Participants were instructed to fast for 10-12 h before the experimental trial. They were allowed to drink plain water at night and in the morning before the experiment. Participants were asked to complete this protocol for each visit and to maintain their regular lifestyle activities throughout the study. They were asked to avoid alcohol and excessive physical activity one day before the experiment. Female participants visited the lab during the same follicular phase of their menstrual cycle, and hence each female

subject spent 2-3 months completing the trial.

Participants came to the laboratory early in the morning after 10-12 h of fasting with light clothing and shoes were removed. Height and weight were measured using The Leicester Height Measure (maximum 210 cm) (SECA Ltd, Birmingham, UK) and SECA 2562 weighing scale (maximum weight 200 kg, accuracy of 0.1 kg) (SECA Ltd, Germany), respectively.

4.3 Materials and methods

4.3.1 Breakfast meal preparation

Breads were given as a standardised breakfast meal providing 400 - 450 kcal/meal (21% of total daily energy requirement). The breakfast meal and nutrient composition of breads are shown in **Table 2-3** (**Section 2.0**). Breads were prepared according to the method detailed in **Section 2.0**. Participants were provided with one of the following breads in a randomised order at each experimental trial:

i. White bread (WB)
ii. Black tea bread (BT)
iii. β-Glucan bread (βG)
iv. β-Glucan plus black tea bread (βGBT)

The bread was eaten during breakfast time (e.g. 8:00 am) and self-reported eating time was recorded using a stopwatch. The palatability study was completed using AVAS (100-mm scale) after breakf ast meal assessing visual appeal, smell, taste, after taste, palatability and overall acceptance. Details regarding this procedure were described in **Section 2.3.1.**

4.3.2 *Ad libitum* lunch and energy intake estimation

The *ad libitum* lunch consisted of multiple food items and was served with 300 mL water. Lunch was provided 3 h after breakfast (e.g. 11:00 am). Participants were advised to eat the lunch until they were comfortably full according to their satiety within 30 min. All food items were weighed before and after the meal. The remaining food was weighed, subtracted from the initial amount and the food consumed was used to calculate energy

(kcal). Details regarding this procedure were described in **Section 2.3.2**.

4.3.3 Satiety and palatability scoring (adaptive visual analogue scale, AVAS)

Satiety and palatability were measured using the Adaptive Visual Analogue Scale (AVAS) software. Participants were directed to the 'training' mode, which allowed participants to practice before starting the real test. After acclimatisation, participants completed an AVAS scoring, before receiving a test meal at breakfast (fasting state), and at different time points (30, 60, 90, 120 and 180 min) after breakfast. AVAS consists of five questions regarding perceived hunger, fullness, satiety, desire to eat and prospective food intake. Details regarding this procedure were described in **Section 2.3.3**.

4.4 Statistical analysis

All data were analysed using SPSS software (SPSS version 22.0, SPSS Inc., Chicago, USA). The distribution of variables was evaluated using Shapiro-Wilk tests and the data considered to be normal if $p > 0.05$. Values with $p < 0.05$ were considered significant.

Data for the time (min) spent eating breakfast and energy intake (kcal) during *ad libitum* lunch were normally distributed and were analysed using a General Linear Model (GLM). Data regarding eating rate (kcal/min) were not normally distributed and became normally distributed after log-transformation. Values with $p < 0.05$ were considered significant. Gender was used as a between-subject factor and BMI as covariates. The data for GLM test met the following assumptions:

i. The dependent variable is measured at a continuous level (e.g. energy intake measured as kcal, which is continuous data).
ii. The independent variable should consist of at least two categorical "related groups" or "matched pairs" (This study is a cross-over study hence subjects are present in both groups).

Data for bread palatability measures were not normally distributed after log transformation and were analysed using the Friedman non-parametric test (Altman, 1999). Total area under the curve (TAUC) for perceived hunger, fullness, satiety, desire to eat and

prospective food intake ratings were calculated from baseline to 3 h using linear trapezoidal method (Vitaglione et al., 2009). Data for perceived hunger, fullness, satiety, desire to eat and prospective food intake and total area under the curve ($TAUC_{0\text{-}180\ min}$) for each of satiety measures were not normally distributed. Both data sets (palatability and perceived satiety) were log transformed to improve normality. However, these data were still not normally distributed after log transformation and hence were analysed using the Friedman non-parametric test (Altman, 1999). This test is equivalent to GLM repeated measures for normally distributed data. The data meet the following four assumptions for the Friedman test:

i. One group that is measured on three or more different occasions
ii. Group is a random sample from the population
iii. The dependent variable is measured at the ordinal or continuous scale
iv. Data do not normally distributed

Values with $p < 0.05$ were considered significant. Any significant data were further tested using Wilcoxon matched pair signed-rank sum test. This test is equivalent to paired t-test for normally distributed data. Palatability ratings are presented as median in star diagram. Perceived satiety measures are presented as median and interquartile (IQR) (quartile 1 to 3, Q1 to Q3). Gender and BMI could not be added in the model analysis because the data were not normally distributed. Instead, the data were split into male and female to determine difference between gender.

The relationships between energy intake at lunch and individual perceived satiety measures (total area under the curve 0 – 180 min, $TAUC_{0\text{-}180min}$) were determined using Spearman rank-order correlation coefficient (Spearman's correlation, r_S).

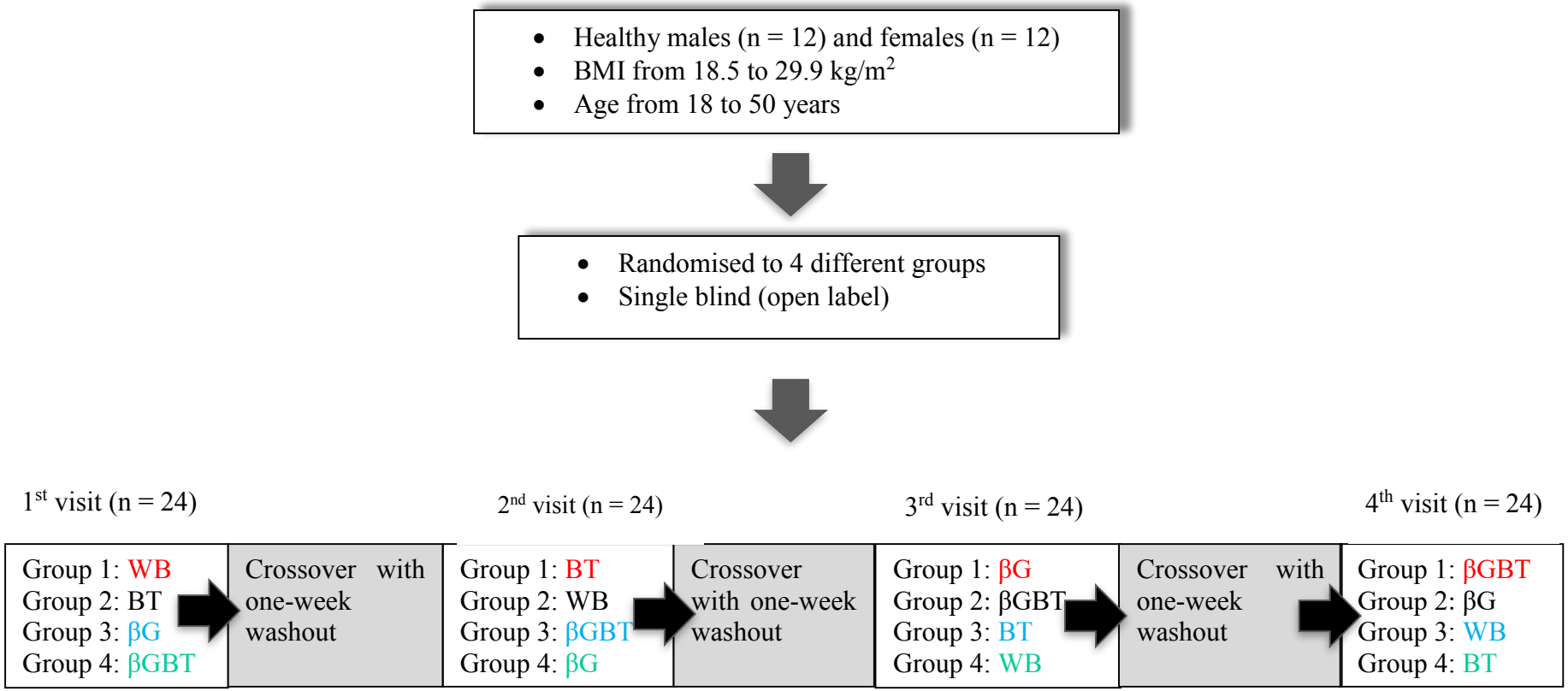


Figure 4-1. Study protocol for palatability and satiety study. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread.

4.5 Results

Subject characteristics are shown in **Table 4-1**. Twelve healthy males and eleven females with an age range of 26.7 ± 6.8 years completed all four-arm trials. One female subject showed unreliable perceived satiety responses (outlier) and was subsequently excluded from statistical analysis. One male subject dropped-out from the study and was replaced by another male subject.

Visual appeal, aroma, smell and aftertaste were similar between breads (**Figure 4-2**). Median taste rating for WB was significantly higher compared with βGBT ($p = 0.018$). Median texture ratings for WB and BT were significantly higher compared with βG and βGBT ($p = 0.002$ and $p = 0.023$, respectively; $p = 0.030$ and $p = 0.042$, respectively). Palatability ratings for WB and BT were higher compared with βG and βGBT ($p = 0.008$ and $p = 0.011$, respectively; $p = 0.037$ and $p = 0.019$, respectively).

WB showed significantly higher overall acceptability of breads when compared with βG and βGBT (**Figure 4-2**). There were gender differences in median ratings for taste, texture and palatability of βG and βGBT compared with WB. Females scored lower ratings for taste of βG and βGBT compared with WB ($p = 0.003$ and $p = 0.021$, respectively). Females scored lower ratings for texture of βG and βGBT compared with WB ($p = 0.006$ and $p = 0.013$, respectively). Palatability of βG and βGBT were lower compared with WB in female subjects ($p = 0.004$ and $p = 0.026$, respectively).

Eating time for βG and βGBT were significantly higher when compared with WB and BT (**Table 4-2**). Eating rate for βG and βGBT were significantly slower when compared with WB and BT. There were no significant effects of gender (within-subject factor) and BMI (covariates) on eating time and rate.

Table 4-1. Baseline characteristics

Characteristics	**Mean ± S.D**	**Range**
Age (years)	26.7 ± 6.8	18 - 43
Weight (kg)	65.2 ± 13.1	46.9 – 87.6
Height (cm)	168.8 ± 8.3	154.9 – 184.4
Body Mass Index (BMI, kg/m^2)	22.9 ± 3.4	17.8 – 29.8
Systolic blood pressure (SBP, mmHg)	112.4 ± 11.7	88 – 126
Diastolic blood pressure (SBP, mmHg)	68.2 ± 9.8	53 - 94
BMI category (%)		
<18.5	4 (17.4)	
18.5 – 24.9	10 (43.5)	
25.0 – 29.9	9 (39.1)	
Gender (male/female) (%)	12/11(52.2/47.8)	
Ethnicity (%)		
Asian	15 (65.2)	
Caucasian	8 (34.8)	

Table 4-2. Bread eating time (mean ± S.E.M) at breakfast in healthy volunteers (n = 23)

Breads	Eating time (min)	Range	Eating rate (kcal/min)	Range
WB	8.5 ± 0.7^{a}	4.5 – 15.1	55.9 ± 4.2^{a}	27.6 – 92.1
BT	8.4 ± 0.5^{a}	4.2 – 12.0	53.5 ± 3.6^{a}	34.4 – 97.4
βG	10.7 ± 0.7^{b}	5.4 – 17.0	43.5 ± 3.1^{b}	24.5 – 77.8
βGBT	11.0 ± 0.7^{b}	5.1 – 18.0	41.5 ± 3.1^{b}	23.3 – 83.0

Different superscript letters indicate significant ($p < 0.05$) differences between breads.
Eating time: βG vs WB, $p = 0.026$; βG vs BT, $p = 0.003$; βGBT vs WB, $p = 0.002$; βGBT vs BT, $p = 0.001$
Eating rate: βG vs WB, $p = 0.013$; βG vs BT, $p = 0.004$; βGBT vs WB, $p = 0.002$, βGBT vs BT, $p = 0.001$

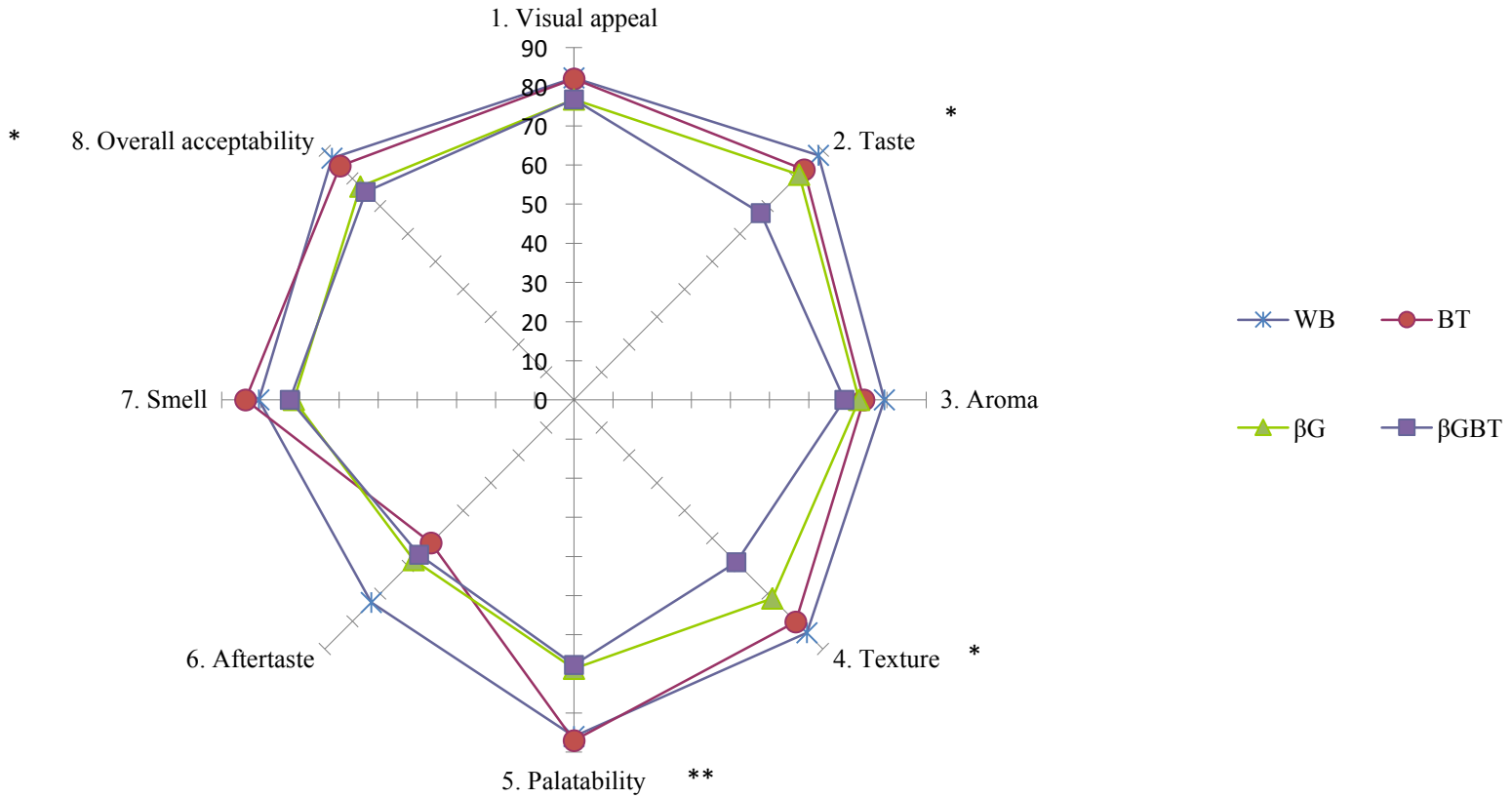


Figure 4-2. Star diagram of breads acceptability and palatability based on 100-mm visual analogue scale in healthy volunteers. Values are expressed as median. Asterisk (*) indicates significant ($p < 0.05$) differences between WB *vs* βGBT. Double asterisks (**) indicate significant ($p < 0.05$) difference between WB and BT *vs* βG and βGBT. Palatability (how palatable the bread is), overall acceptability (the overall acceptability of the bread)

Data for perceived satiety measures (hunger, fullness, satiety, desire to eat and prospective food intake) were not normally distributed and were analysed using the Friedman non-parametric test. Any significant results were further analysed using Wilcoxon matched pairs signed-rank sum test to determine the difference between pairs. Total area under the curve (TAUC) for satiety measures was preferable because single satiety data (individual time points) are not physiologically or statistically independent (Blundell et al., 2010).

Perceived hunger ratings were similar at baseline (0 min) and decreased postprandially ($p < 0.05$) at 15 min for all breads (**Table 4-3**). Perceived hunger gradually increased ($p < 0.05$) for all breads from 15 min to 180 min. Total area under the curve ($TAUC_{0\text{-}180min}$) were significantly different between breads, χ (3) = 14.2 ($p = 0.003$) (**Table 4-3**). Both WB and BT were significantly different compared with βG with $Z = -2.616$ ($p = 0.009$) and $Z = -2.616$ ($p = 0.016$), respectively. No significant difference was found between βGBT and other breads.

Perceived fullness ratings were similar at baseline (0 min) and increased postprandially ($p < 0.05$) at 15 min for all breads (**Table 4-4**). Perceived fullness gradually decreased ($p < 0.05$) for all breads from 15 min to 180 min. $TAUC_{0\text{-}180min}$ for fullness were significantly difference between breads, χ (3) = 11.5, $p = 0.010$ (**Table 4-4**). $TAUC_{0\text{-}180min}$ of perceived fullness significantly increased after eating βGBT bread compared with WB ($Z = -2.516$, $p = 0.012$) and BT ($Z = -2.321$, $p = 0.020$). No significant difference was found between βG and other breads.

Perceived satiety ratings were similar at baseline (0 min) and increased postprandially ($p < 0.05$) at 15 min for all breads (**Table 4-5**). Perceived satiety started to decrease ($p < 0.05$) at 15 min and reached the lowest point at 180 min for all breads. $TAUC_{0\text{-}180min}$ for perceived satiety were significantly difference between breads, χ (3) = 10.8, $p = 0.013$ (**Table 4-5**). βGBT bread significantly increased $TAUC_{0\text{-}180min}$ perceived satiety compared with WB ($Z = -2.9$, $p = 0.004$) and BT ($Z = -2.4$, $p = 0.016$). βG bread significantly increased perceived $TAUC_{0\text{-}180min}$ satiety compared with WB ($Z = -2.1$, $p = 0.042$).

Perceived desire to eat was similar at baseline and decreased significantly ($p < 0.05$) at 15 min after eating breads at breakfast (**Table 4-6**). Perceived desire to eat significantly ($p <$

0.05) increased starting from 15 to 180 min for all breads. $TAUC_{0\text{-}180min}$ of perceived desire to eat was lower for βG compared with WB (Z = - 2.0, p = 0.045).

Breads showed similar perceived prospective food intake at baseline and significantly ($p < 0.05$) reduced at 15 min after eating BT, βG and βGBT when compared with WB (**Table 4-6**). Prospective food intake remained lower after eating βG and βGBT when compared with WB at 30 to 180 min. However, only βG showed overall ($TAUC_{0\text{-}180min}$) reduction in prospective food intake when compared with WB and BT (Z = - 2.5, p = 0.014 and Z = - 2.0, p = 0.045).

Energy intake at lunch is shown in **Figure 4-3**. Total energy intake at lunch was 15.5% lower (p = 0.024) after consumption of BT bread at breakfast when compared with WB. Energy intake of βG did not change significantly when compared to WB due to higher inter-individual variation between subjects. Based on GLM analysis, gender (within-subject factor) and BMI (covariates) were not associated with energy intake at lunch. Correlation between perceived satiety measures ($TAUC_{0\text{-}180min}$) and energy intake at lunch (kcal) was determined using Spearman's correlation for non-parametric data. $TAUC_{0\text{-}180min}$ satiety was negatively correlated with WB energy intake at lunch with r_S = - 0.57 (p = 0.004). No significant correlations were found between BT, βG and βGBT in relation with other perceived satiety measures.

Table 4-3. Baseline and postprandial perceived hunger (median (IQR)) after consuming breads at breakfast in healthy volunteers (n = 23)

Time	**Fasting**	**15 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**	**$TAUC_{0\text{-}180min}$ (mm.min)**	**Time average***
Breads	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
WB	77.2 (56 – 95)	14.7 (6 – 20)	21.6 (4 – 51)	27.9 (11 – 43)[a]	33.4 (14 – 52)	38.9 (18 – 56)	52.4 (26 – 73)	58.6 (49 – 75)	6636.0 (4788 – 10471)[a]	36.9 (26.6 – 58.2)[a]
BT	89.5 (69 – 93)	7.5 (5 – 19)[a]	12.9 (7 – 28)	24.9 (10 – 44)[a]	25.5 (12 – 44)	32.6 (21 – 55)	48.2 (28 – 66)	57.0 (44 – 74)	6419.0 (4309 – 8997)[a]	35.7 (23.9 – 50.0)[a]
βG	76.7 (67 – 92)	3.0 (1 – 9)[b]	6.9 (32 – 14)	12.8 (8 – 25)[c]	16.0 (10– 33)	21.4 (14 – 42)	39.9 (25 – 55)	47.9 (24 – 60)	4784.0 (3145 – 7283)[b]	26.6 (17.5 – 40.5)[b]
βGBT	83.7 (66 – 92)	3.3 (1 – 15)	8.3 (4 - 21)	10.2 (5 – 21)[c]	15.9 (8 – 33)	19.1 (15 – 47)	41.0 (27 – 66)	15.9 (8 – 33)	4737.9 (3572 – 7466)[ab]	26.3 (19.8 – 41.5)[ab]

Data are presented as median (interquartile, IQR). Different superscripts indicate significant ($p < 0.05$) differences between breads at the same time point (Wilcoxon's matched pairs signed-rank sum test). There were significant ($p < 0.05$) time interactions for all breads. Asterisk (*): Time average was calculated as $TAUC_{0\text{-}180min}$/180 min.

Table 4-4. Fasting and postprandial perceived fullness (median (IQR)) after consuming breads at breakfast in healthy volunteers (n = 23)

Time	**Fasting**	**15 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**	**$TAUC_{0\text{-}180min}$ (mm.min)**	**Time average***
Breads	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
WB	4.6 (2 – 26)	84.3 (72 – 94)	77.9 (64 – 91)[a]	70.11 (48 – 86)[a]	60.9 (45 – 84)	58.4 (42 – 80)	49.0 (29 – 70)[a]	42.1 (24 – 61)	11140.5 (8926 – 13954)[a]	61.9 (49.6 – 77.5)[a]
BT	5.5 (2 – 19)	85.5 (74 – 94)	79.3 (64 – 89)[ab]	70.9 (50 – 84)[a]	72.3 (49 – 85)	50.8 (32 – 75)	48.6 (40 – 67)	38.9 (26 – 54)[a]	10457.0 (9042 – 13778)[a]	58.1 (50.2 – 76.5)[a]
βG	6.4 (2 – 21)	94.5 (75 – 98)	92.4 (75 – 94)[c]	81.4 (65 – 88)	82.8 (63 – 86)	68.3 (49 – 82)	63.5 (45 – 76)[b]	49.2 (30 – 61)	12468.0 (11360 – 13995)[ab]	69.3 (63.1 – 77.8)[ab]
βGBT	8.7 (2 – 22)	93.3 (83 – 98)	87.6 (80 – 94)	84.6 (71 – 92)[b]	81.7 (57 – 89)	74.5 (53 – 81)	61.1 (40 – 71)	53.3 (34 – 69)[b]	13119.5 (11438 – 14588)[b]	72.9 (63.5 – 81.0)[b]

Data are presented as median (interquartile, IQR). Different superscripts indicate significant ($p < 0.05$) differences between breads at the same time point (Wilcoxon's matched pairs signed-rank sum test). There were significant ($p < 0.05$) time interactions for all breads. Asterisk (*): Time average was calculated as $TAUC_{0\text{-}180min}$/180 min.

Table 4-5. Fasting and postprandial perceived satiety (median (IQR)) after consuming breads at breakfast in healthy volunteers (n = 23)

Time	**Fasting**	**15 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**	**$TAUC_{0\text{-}180min}$ (mm.min)**	**Time average***
Breads	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
WB	11.26 (5 – 27)	77.9 (64 – 89)^{a}	74.3 (54 – 88)^{a}	69.7 (59 – 84)	56.1 (41 – 79)^{a}	53.6 (40 – 72)^{a}	47.8 (31– 60)	37.5 (27 – 56)	10500.0 (8803 - 13283)^{a}	58.3 (48.9 – 73.8)^{a}
BT	7.6 (2 – 19)	86.7 (73 – 95)	81.0 (62 – 93)^{a}	74.3 (51 – 82)^{a}	69.7 (49 – 81)^{a}	53.1 (36 – 78)^{a}	50.0 (35 – 68)^{a}	38.4 (38 – 50)	10814.0 (8463 - 13284)^{ab}	60.1 (46.9 – 73.8)^{ab}
βG	12.9 (2 – 30)	90.9 (79 – 96)	87.8 (77 – 92)^{b}	81.8 (64 – 89)	77.9 (63– 86)^{b}	65.3 (46 – 77)	63.6 (45 – 69)^{b}	47.4 (31 – 62)	11995.0 (10609 - 13872)^{bc}	66.6 (58.9 – 77.1)^{bc}
βGBT	12.9 (2 – 24)	90.2 (82 – 95)^{b}	89.7 (77 – 93)^{b}	84.8 (70 – 89)^{b}	80.7 (54 – 86)^{b}	71.0 (54 – 80)^{b}	50.0 (34 – 72)	48.1 (48 – 67)	13226.0 (11840 - 14641)^{cd}	73.5 (65.8 – 81.3)^{cd}

Data are presented as median (interquartile, IQR). Different superscripts indicate significant ($p < 0.05$) differences between breads at the same time point (Wilcoxon's matched pairs signed-rank sum test). There were significant ($p < 0.05$) time interactions for all breads. Asterisk (*): Time average was calculated as $TAUC_{0\text{-}180min}$/180 min.

Table 4-6. Fasting and postprandial perceived desire to eat (median (IQR)) after consuming breads at breakfast in healthy volunteers (n = 23)

Time	Fasting	15 min	30 min	60 min	90 min	120 min	150 min	180 min	$TAUC_{0\text{-}180min}$ (mm.min)	Time average*
Breads	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
WB	90.11 (67 – 96)	23.8 (8 – 62)^{a}	21.4 (9 – 49)^{a}	30.0 (14 – 51)^{a}	46.4 (20 – 54)^{a}	48.5 (28 - 60)^{a}	47.8 (36 – 77)^{ad}	58.2 (51 – 79)	7636.0 (5105 – 10097)^{a}	42.4 (28.4 – 56.1)^{a}
BT	85.3 (75 – 97)	12.4 (7 – 26)^{b}	19.7 (10 – 40)^{ab}	22.8 (16 – 39)	29.2 (15 – 55)^{ab}	50.6 (24 – 66)^{a}	50.7 (31 – 65)^{ab}	61.8 (50 – 76)	7605.0 (4988 - 9576)^{ab}	42.3 (27.7 – 53.2)^{ab}
βG	84.8 (71 – 96)	7.5 (3 – 31)^{b}	7.5 (3 – 17)^{bc}	16.7 (9 – 32)	22.5 (11 – 31)^{bc}	28.5 (18 – 49)	33.7 (21 – 55)^{c}	54.0 (37 - 72)	5548.0 (3734 – 7202)^{b}	30.8 (20.7 – 40.0)^{b}
βGBT	90.6 (69 – 96)	6.3 (4 – 36)^{b}	8.5 (5 – 29)^{cd}	14.4 (7 – 30)^{b}	19.0 (9 – 37)^{bc}	28.3 (18 – 45)^{b}	47.9 (23 – 66)^{cd}	52.4 (32 – 66)	5369.0 (3834 - 8464)^{ab}	29.8 (21.3 – 47.0)^{ab}

Data are presented as median (interquartile, IQR). Different superscripts indicate significant ($p < 0.05$) differences between breads at the same time point (Wilcoxon's matched pairs signed-rank sum test). There were significant ($p < 0.05$) time interactions for all breads. Asterisk (*): Time average was calculated as $TAUC_{0\text{-}180min}$/180 min.

Table 4-7. Fasting and postprandial perceived prospective food intake (median (IQR)) after consuming breads at breakfast in healthy volunteers (n = 23)

Time	**Fasting**	**15 min**	**30 min**	**60 min**	**90 min**	**120 min**	**150 min**	**180 min**	**$TAUC_{0\text{-}180min}$ (mm.min)**	**Time average***
Breads	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
WB	75.2 (69 – 96)	21.2 (11 – 47)^{a}	26.7 (9 – 50)^{a}	28.5 (11 – 57)^{a}	44.5 (19 – 56)^{a}	49.4 (28 – 57)^{a}	51.4 (40 – 71)^{a}	65.2 (50 – 78)^{a}	8691.0 (5178 – 9809)^{a}	48.2 (28.8 – 54.5)^{a}
BT	84.1 (76 – 96)	13.7 (7 – 30)^{b}	21.2 (10 – 35)^{ab}	24.3 (14 – 48)	29.8 (17 – 53)	47.6 (28 – 64)^{a}	56.7 (41 – 70)^{a}	62.9 (52 – 73)^{a}	7690.0 (5059 - 9838)^{a}	42.7 (28.1 – 54.7)^{a}
βG	83.7 (65 – 96)	8.7 (3 – 27)^{b}	8.3 (5 – 15)^{c}	17.1 (9 – 34)^{b}	25.6 (11 – 38)^{b}	30.1 (18 – 50)	40.5 (23 – 52)^{b}	56.7 (33 – 69)	5507.0 (3778 - 7345)^{b}	30.6 (21.0 – 40.8)^{b}
βGBT	83.2 (73 – 94)	11.6 (4 – 23)^{b}	9.9 (6 – 32)^{bc}	15.9 (9 – 42)^{b}	17.7 (10 – 39)^{b}	31.5 (20 – 47)^{b}	50.0 (31 – 69)	36.6 (37 – 69)^{b}	5514.0 (4429 – 7738)^{ab}	30.6 (24.6 – 43.0)^{ab}

Data are presented as median (interquartile, IQR, Q1 – Q3). Different superscripts indicate significant ($p < 0.05$) differences between breads at the same time point (Wilcoxon's matched pairs signed-rank sum test). There were significant ($p < 0.05$) time interactions for all breads. Asterisk (*): Time average was calculated as $TAUC_{0\text{-}180min}$/180 min.

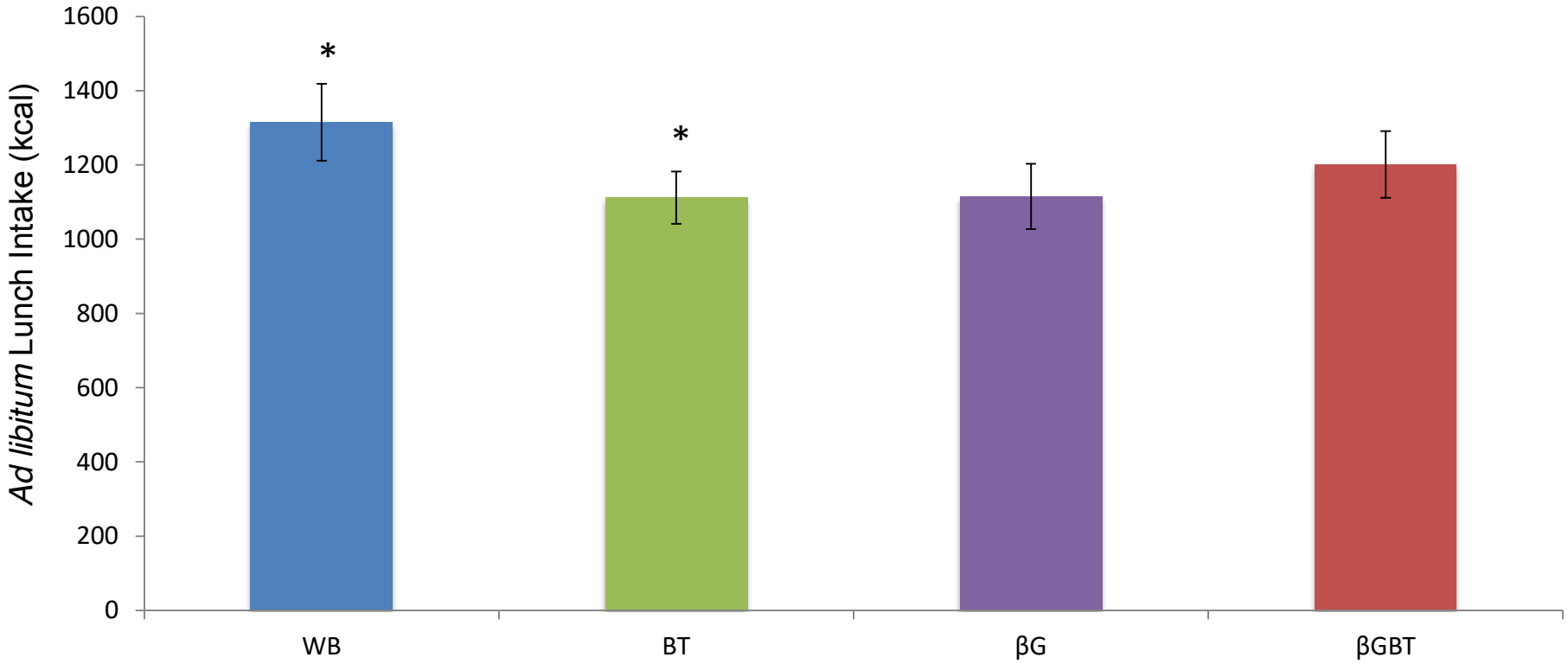


Figure 4-3. Energy intake (kcal) during an *ad libitum* lunch. Data are expressed as mean ± S.E.M. Asterisk (*) indicates significant ($p < 0.05$) differences between WB and BT.

4.6 Discussion

Eating behaviour is determined by three main factors: 1) metabolic processes that drive hunger and satiety 2) sensory factors that drive food choice 3) the cognitive factors that shape eating habits (Blundell et al., 2010). These three domains are interrelated due to a learning process. Based on this concept, sensory factors determine *what* one eats while metabolic factors determine *how much* one eats. Macronutrient composition, energy density, physical structure and sensory qualities are the external cues that could modulate satiation and satiety (Mela, 2006). In **Chapter 3**, breads with β-glucan and/or black tea were developed and showed different characteristics. Breads with the addition of black tea (BT and βGBT) were darker and bread with β-glucan (βG) was more chewy when compared with to white bread; this might have implications for palatability. Hence, this chapter aimed to determine the palatability and perceived satiety after eating bread with β-glucan and/or black tea and compared with white bread.

A previous study showed the addition of β-glucan in bread at the level of 2.5 and 5% had negative effects on dough quality and reduced loaf height by 19 and 36%, respectively (Brennan and Cleary, 2007). Ellis et al. showed that guar gum inclusion at the dose of 15% was unacceptable among normal subjects (Ellis et al., 1981). Guar gum is viscous in nature and its addition in bread (semi-moist) might have negative effects on texture when compared with low-moist products such as biscuits (Ellis et al., 2001). β-glucan has similar characteristics as guar gum but is less viscous, therefore it is anticipated that this would have a negative effect on bread texture. In **Chapter 3**, the addition of β-glucan reduced loaf height by 35% when compared with white bread (WB) (Jalil et al., 2015). This might partially explain why both βG and βGBT showed significantly lower overall acceptability when compared with WB based on 100-mm scale (Median of 77.1 mm and 75.2 mm vs 87.4 mm, respectively). Korczak et al. showed reduced overall acceptability of bars prepared with barley and oats (Korczak et al., 2014). Another study indicated that products with overall acceptability above 5 based on a scale of 10 were considered acceptable (neither like nor dislike) (Hennessy-Priest, 2014). In bread prepared with 10% (w/w) guar gum, Ellis et al. demonstrated the predictive hedonic value of 5.3 which is close to a neutral response of 5 (from a scale of 1 being *like very much* and 9 being *dislike very*

much) among untrained volunteers (Ellis et al., 1981). In our study, βG and βGBT had an overall acceptability of 75 to 77-mm compared with WB (87-mm) from a scale of 100-mm.

Texture and palatability ratings of βG and βGBT were significantly lower when compared with WB and BT breads. There were gender differences in bread palatability. Female participants showed a lower preference for taste, texture and palatability of βG and βGBT. Cho et al. demonstrated gender differences towards traditional Korean rice cake (*seolgitteok*) among consumers (Cho et al., 2016). *Seolgitteok* is chewy and has a plain flavour, both characteristics which are similar to β-glucan breads. In this study, females showed lower overall acceptability towards *seolgitteok* when compared with males. Females have higher chances of rejection and of aversion to unfamiliar foods than males. In addition, females have lesser preference than males towards unfamiliar and novel foods (Backstrom et al., 2003).

The second objective of the study was to determine the satiety effects of bread prepared with β-glucan and/or black tea breads. Bread with β-glucan and/or black tea synergistically reduced hunger, increased fullness and satiety, and reduced desire to eat when compared with control breads. The 'Satiety Cascade' was coined 25 years ago by Blundell et al. and describes the conceptual framework for investigating the effects of foods on satiation (meal termination or intra-meal satiety) and satiety (inter-meal satiety) (Blundell et al., 2010). Sensory and cognitive factors are two major determinants of satiation and satiety. Meal termination (satiation) and inter-meal satiety (satiety) is highly dependent on nutrient composition, energy density and the physical structure of the food products (Mela, 2006). Modifying food energy density and physical structure with the addition of soluble fibre increased eating time when compared with control food (Pentikäinen et al. 2014). Our study showed that breads prepared with β-glucan had lower energy density when compared with control breads due to higher water content. Participants spent more time (11 min) eating β-glucan breads when compared with control bread (8.5 min). Eating rate (kcal/min) was slower (28.5 to 35%) for β-glucan breads when compared with control white bread. Zijlstra et al. found that participants consumed more test products (e.g. chocolate custard) when oral processing time was reduced from 9 s to 3 s (Zijlstra et al., 2009). A systematic

review from 22 studies demonstrated that slower eating rate associated with lower energy intake (Robinson et al., 2014). Oat bran addition in biscuits might result in longer oral processing time and thus stronger oro-sensory cues for perceived satiety (Wanders et al., 2011). When energy content and eating rate was held constant, higher oro-sensory (small sip) (more time interval between the food entering the mouth and swallowing) exposure reduced intake of sweet drinks when compared with higher oro-sensory exposure (large sip) (Weijzen et al., 2009). Forde et al. demonstrated that oral-sensory time could contribute to higher satiation when subjects consumed isocaloric meals (Forde et al., 2013).

A recent meta-analysis showed that a slower eating rate reduced energy intake (Robinson et al., 2014). Physiologically, a higher amount of food present in the mouth leads to an increase in chewing and higher oral processing time, because more time is needed for enough saliva to be added to form a uniform bolus for swallowing (Engelen et al., 2005; Zijlstra et al., 2010). This also leads to early meal termination and higher satiety response. The sense of taste acts as a nutrient sensor and triggers the brain and the gut regarding the inflow of nutrients through cephalic phase response (de Graaf et al., 2012; Forde et al., 2013). Li et al. demonstrated how increasing chewing frequency (40 chews vs 15 chews of 10 g food) resulted in lower energy intake and evoked higher satiety hormones (GLP-1 and CCK) (Li et al., 2011). An increase in eating time from 5 to 30 min (physiologically moderate phase) leads to higher perceived fullness ratings and higher satiety hormone peptide YY (Kokkinos et al., 2010). However, Shah et al. showed no effects of slow eating time (30 min vs 10 min) during breakfast meal on appetite hormone responses (Shah et al., 2005). Hence, in our study, we anticipated that having breads with β-glucan increased oral processing time and had a lower eating rate. This action could increase perceived satiety over the period of 3 h when compared with control breads.

Previous studies have demonstrated that β-glucan increases satiety when ingested as a part of solid foods such as breads, biscuits or muffins. These studies showed increased perceived satiety in the range of 43 – 55%, perceived fullness by 19 – 25%, and reduced perceived hunger by 10 – 49% (Vitaglione et al., 2009; Vitaglione et al., 2010; Willis et al., 2009). However, these solid foods differed in physical structure, energy density, and

amount of β-glucan used and this does not allow one-to-one comparison regarding perceived satiety. A study showed addition of β-glucan in replacement bars for two consecutive days had no effects on perceived satiety but the amount of β-glucan (0.9 g) may have been too low to have any significant effects (Peters et al., 2009). Adjusting energy density by increasing the volume (e.g. adding water) without changing macronutrient content could be a useful strategy to enhance satiety and reduce subsequent energy intake (next meal effects) (Rolls, 2009). In our study, an *ad libitum* lunch was given to determine whether bread intake at breakfast could reduce subsequent energy intake. BT intake at breakfast reduced energy intake at lunch when compared with WB. βG showed lower and similar energy intake to that of BT but did not change significantly, due to high inter-individual variations. Breakfast meals were standardised based on 50 g of available carbohydrate. βG and βGBT contained more water per g of available carbohydrate and hence showed lower energy density (2.1 kcal/g) when compared with WB and BT (2.8 and 2.7 kcal/g respectively).

Energy density, dose, and meal volume are important factors in reducing energy intake after consuming β-glucan products. β-Glucan (3 g) prepared in bread (100 g) with an energy density of 2.6 kcal/g reduced energy intake at lunch by 19% (Vitaglione et al., 2009). Lower preload volume (60 g biscuit) of energy bar containing 0.9 g β-glucan had no effects on energy intake at lunch (Peters et al., 2009). Vitaglione et al. demonstrated small (38 g) and large (114 g) portions of biscuits with the same energy density of 17 kcal/g significantly reduced energy intake at lunch among women and not among men (Vitaglione et al., 2010). Another study demonstrated how males had more energy than females at lunch (Pedersen et al., 2013; Hess et al., 2011). In our study, gender had no effect on energy intake, i.e. males and females had eaten equally from an *ad libitum* lunch. The following steps were taken to prevent overeating during the lunch session: 1) foods were provided in small proportions specifically to prevent overconsumption of one individual food 2) they were advised to eat based on their satiety.

A correlation analysis was performed to further evaluate the effectiveness of perceived satiety measures in reducing energy intake at lunch. Based on Spearman's analysis, only WB negatively correlated with perceived satiety at lunch, i.e. less perceived satiety leads to

increased energy intake at lunch. No significant correlations were found between energy intakes and perceived satiety measures for other breads. Calame et al. demonstrated no correlation between perceived satiety ratings and energy intake at lunch when soluble dietary fibres (highly viscous) were given as beverage (Calame et al., 2011). Another study found a positive association between perceived satiety and energy intake at lunch in subjects supplemented with fibre-rich cereal (Freeland et al., 2009). However, it must be noted that these studies differ in the food matrix, fibre dose, and type of fibres; hence, they produced ambiguous results. The effectiveness of perceived satiety based on visual analogue scale (VAS) in predicting energy intake has received much attention in recent years. Some studies showed perceived satiety is a good *proxy* in assessing energy intake, but other studies failed to show any effects (Stubbs et al., 2000). Research on appetite is evolving and there is guidance on good practice in carrying out appetite research (Blundell et al., 2010). The use of VAS scoring under experimental condition is robust (but more artificial) than the real-life situation. This study has limitations related to the degree of accuracy in the measurement of perceived satiety and energy intake. Firstly, the assessment of satiety effect and palatability scorings of the breads were performed by untrained volunteers and there would be some extent of subjective inter-personal variability in rating the scorings (Solah et al., 2015). However, using a crossover study design reduced the inter-individual variations. Secondly, this a short-term intervention study and the dietary modifications may not be long enough to detect changes on subjective appetite and energy intake. A longer duration of treatment may be needed to detect changes in appetite as a result of dietary modification (Clark & Slavin, 2013). A post-hoc calculation using G Power software showed that the present study achieved a power of 90%. This value was 10% higher than our early calculation of 80% (as described in **Section 4.2.2**). Hence, the lack of results on energy intake may be due to other factors related to an *ad libitum* lunch session. Firstly, the choice of foods for the *ad libitum* lunch is not individual-specific, i.e. subjects might not eat what they not normally eat and hence make the *ad libitum* lunch insensitive. Secondly, given the *ad libitum* lunch is free, subjects might eat as much as possible and this might lead to overconsumption at lunchtime. The time interval of the availability of the food for the next eating session would also determine how subjects eat during the lunch. For example, subjects might eat more if they know that the food is

available in the next 2 h, and might eat less if they have food available in the next 20 min (de Graaf et al., 1999; Blundell et al., 2010). However, these are well documented limitations of feeding trials under experimental conditions. Thirdly, this is acute study (measured 3 h postprandially) and is not long enough to be affected by the colonic fermentation of β-glucan and tea (poly)phenols. A longer study (6 – 9 h) is needed to determine the colonic effects of these two components. Moreover, measurement of actual energy intake later in the day (second meal effects) may be relevant to determine the satiety effects of this bread.

4.7 Conclusions

Breads prepared with β-glucan and/or with black tea were palatable compared with white bread. The addition of black tea did not improve the taste, texture and palatability of β-glucan bread. This study suggests gender specific differences related to palatability of the experimental breads and these need to be considered in future intervention studies. Eating bread prepared with β-glucan and/or with black tea increased perceived satiety when compared with WB and BT. However, eating these breads did not have a significant effect on energy intake at lunch. There might be other mechanisms whereby these breads increased perceived satiety. Therefore, further investigations into mechanisms related to appetite hormones should be considered.

Chapter 5: Effects of Functional Breads Prepared with β-Glucan and Black Tea on Postprandial Glucose, Insulin and Appetite Hormones in Healthy Volunteers

OUTLINE

The studies in **Chapter 3** showed that the functional breads developed with β-glucan and/or black tea reduced *in vitro* starch hydrolysis. In **Chapter 4**, the functional breads with β-glucan and/or black tea increased perceived satiety when compared with white bread. Hence, this chapter describes whether these breads have a real effect on postprandial glucose and appetite-related hormones in healthy volunteers.

The results from this chapter were presented at the Nutrition Society Spring Conference, 21–22 March 2016, titled: 'Phytochemicals and health: new perspectives on plant based nutrition', at the Royal College of Physicians, Edinburgh, United Kingdom. The poster presentation was entitled: 'Acute effects of breads prepared with β-glucan and black tea on glucose and insulin responses in healthy volunteers'.

5.1 Introduction

In **Chapter 3**, the addition of β-glucan and black tea appeared to preserve some of the starch granules in the bread and reduced early *in vitro* starch hydrolysis. This indicates the potential to reduce postprandial glucose and insulin responses in humans. As discussed in **Section 1.5.3**, Jenkins et al. showed that viscous guar gum (as a drink) was effective in reducing postprandial glucose among healthy volunteers (Jenkins et al., 1978). However, the glucose lowering ability disappeared when partially hydrolysed guar gum (non viscous) was used. Another study showed that viscous β-glucan in biscuit or juice dose-dependently reduced glucose response (Pentikäinen et al., 2014). A recent study showed that guar gum bread reduced glucose and insulin responses between 0 to 120 min when compared with white wheat bread (Ekstrom et al., 2016).

Juvonen et al. demonstrated that adding β-glucan to a beverage had different effects on insulin response than when β-glucan was added to a semisolid food such as pudding (Juvonen et al., 2009; Juvonen et al., 2011). The incorporation of β-glucan in liquid reduced glucose responses without adversely increasing postprandial insulin responses. Wood et al. demonstrated 79-96% of changes in plasma glucose and insulin were due to the viscosity of the products (Wood et al., 1994). Reduction in plasma glucose and insulin concentrations was inversely related with the log [viscosity] of β-glucan. A possible explanation could be the complete solubilisation of β-glucan in liquid (Johansson et al., 2007). In solid or semisolid foods, β-glucan may form an 'intra' (within glucans) or 'inter' (e.g. with gluten, starch) hence reducing their solubility (Panahi et al., 2014). However, other factors such as molecular weight (low *vs*. high), the amount added to the food and different sources (oat bran, barley, barley kernel) of β-glucan are likely to have an effect on glycaemic and insulinaemic responses (Juvonen et al., 2009; Juvonen et al., 2011; Johansson et al., 2013; Vitaglione et al., 2009).

In **Chapter 3**, we postulated that (poly)phenol-linked β-glucans will pass to the colon and be metabolised by the gut microbiota to SCFA and phenolic acids. SCFA particularly propionate may have a beneficial effect in increasing satiety within humans (Chambers et al., 2015). However, there is still little information available on the effects of phenolic acids from black tea (poly)phenols fermentation on glucose response or satiety. However,

in **Chapter 4**, these functional breads increased perceived satiety among healthy subjects. There is a link between the gut and brain in regulating appetite and satiety (Chambers et al., 2013). The presence of macronutrients (protein, fat, and/or carbohydrates) or specific food components (e.g. dietary fibres) in the duodenum and ileum could modulate secretion of gut hormones such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (Chambers et al., 2013). These gut hormones may cross the blood-brain barrier, activating Y2 receptors (Y2R) expressed on neuropeptide Y (NPY) neurons in the hypothalamus, resulting in inhibition of food intake (Batterham et al., 2002). Hence, the second aim of this study was to determine whether these breads (β-glucan and/or black tea) have a real effect in increasing satiety by modulating the activity of appetite hormones (CCK, PYY and GLP-1).

5.2 Experimental design

5.2.1 Study design

Participants received 1 of 4 treatments based on a single blind (open label), randomised-controlled crossover design (Latin square design). Participants were randomly allocated using an online website (www.randomization.com) and coded into sequences of 4 treatments, and each of the treatments was separated with one week washout period (**Figure 5-1**). Each participant completed 4 trials and hence the total numbers of trials completed were 64 (16 participants x 4 trials).

5.2.2 Sample size

The sample size was calculated with G*Power (version 3.1.9) statistical software (Faul et al., 2009). The evidence for sample size calculation was based on EFSA claims on the effect of β-glucan on reducing glucose response (4 g of β-glucan per 30 g available carbohydrate) (Agostoni et al., 2011; Vitaglione et al., 2009). With an alpha error of 0.05 and the power of 80% in detecting 10% reduction in blood glucose, 14 participants were needed. Attrition rate was estimated to be 15% and therefore the total number of participants was 16.

5.2.3 Inclusion and exclusion criteria

This study involved healthy males and females, aged between 18-50 years old, body mass index (BMI) ≥ 18.5 to 29.9 kg/m^2. Participants were excluded if they had the following conditions: antibiotic medication (for the past 3 months), gastrointestinal disease (e.g. coeliac disease), smokers, vegetarian, regular use of dietary supplements, food intolerance, allergy to gluten, on dietary restrictions, significant changes in body weight over the past year (± 4 kg), known chronic illnesses, diabetes or impaired glucose tolerance and being prescribed with medication known to affect blood glucose. Those with systolic/diastolic blood pressure more than 139/89 mmHg were excluded.

5.2.4 Subject recruitment

Participants were recruited through flyers and posters in public areas e.g. department notice boards (University of Glasgow), library, church, sports complex, University of Glasgow Student Union (Student Voice) and also through social networks (Facebook). Interested participants were contacted by phone or email for an in-person interview at the Human Nutrition Unit, Glasgow Royal Infirmary or any convenient place regarding inclusion and exclusion criteria, informed consent sheet and consent form. Female participants visited the lab during the same follicular phase of their menstrual cycle, and hence each female subject spent 2-3 months to complete the whole trial.

5.2.5 Study protocol

Participants were asked to refrain from eating (poly)phenol-rich foods two days (48 h) before the experimental trial, on the day of the experiment and 24 h after receiving the experimental meal (total: 4 days for each trial). This was done to eliminate any interfering effects of metabolites produced from (poly)phenols before the intervention. Participants came for another 3 trials on different occasions. **Figure 5-2** shows details of the procedures on the day of each visit. This required participants to refrain from all (poly)phenol-rich foods including fruits, vegetables, tea, coffee, cocoa, red wine, beer, whole grains and cereals. Examples of foods to include during each study session were provided to the participants and they were instructed to contact the researchers using the contact details on

the information sheet if they had any further questions.

Following 48-h of the low-(poly)phenol diet, participants were asked to fast 10-12 h before the experimental trial. Participants were allowed to drink plain water at night and in the morning before the experiment. Participants were asked to follow this protocol for each visit and to maintain their regular lifestyle activities throughout the study. They were asked to avoid alcohol and excessive physical activity 24h before the experiment.

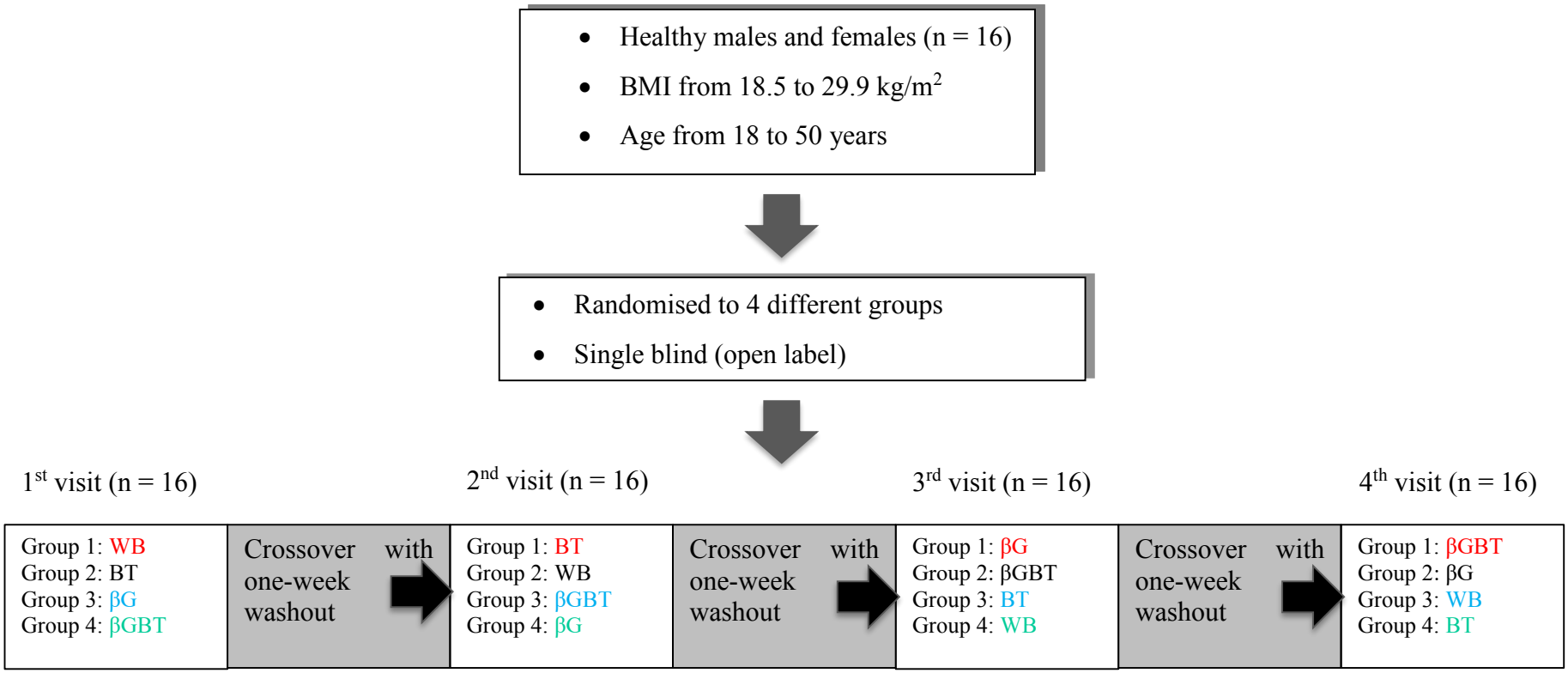


Figure 5-1. Study design for the effects of breads containing β-glucan and black tea on blood glucose and insulin responses, and gut hormones. WB, white bread; BT, black tea bread; βG, β-Glucan bread; βGBT, β-Glucan plus black tea bread.

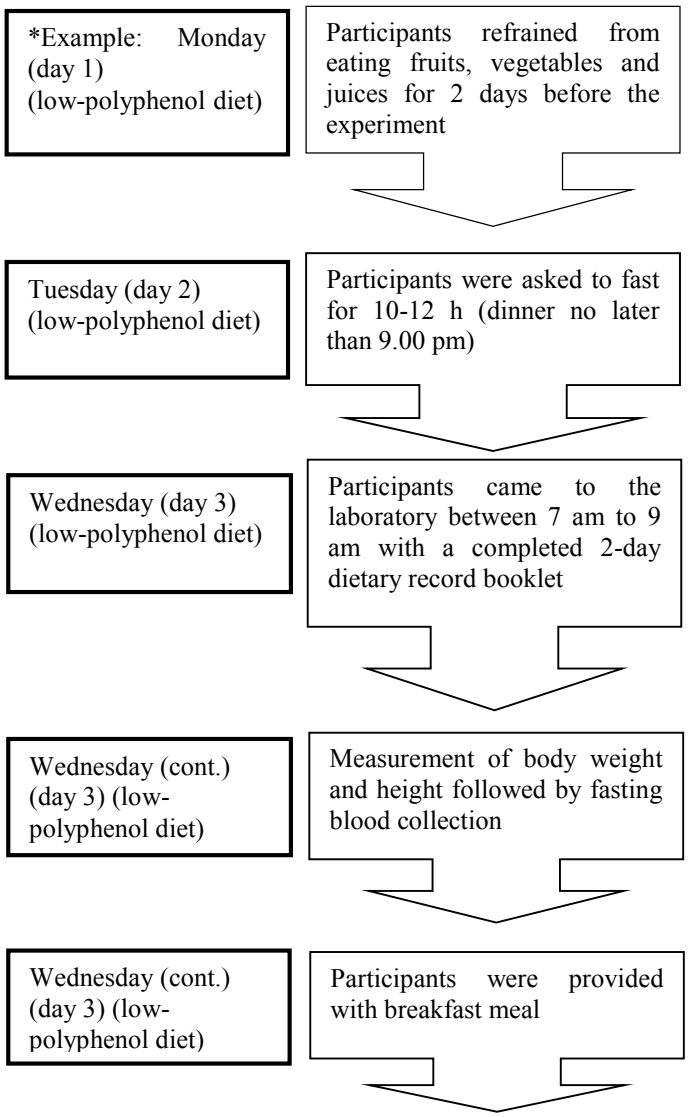


Figure 5-2. Details of procedures on the day of each visit.

On each study day, participants came to the laboratory early in the morning (between 7 and 9 am) after 10-12 h of fasting. Participants were advised to wear light clothing and shoes removed for height measurement using the Leicester Height Measure (maximum 210 cm) (SECA Ltd, Birmingham, UK) and body weight measurement using SECA 2562 weighing scale (maximum weight 200 kg, accuracy of 0.1 kg) (SECA Ltd, Germany).

Participants were provided with one of the following breads in a randomised order at each experimental trial:

i. White bread (WB)
ii. Black tea bread (BT)
iii. β-Glucan bread (βG)
iv. β-Glucan plus black tea bread (βGBT)

Breads were prepared according to the method described in **Section 2.1** and were given as breakfast (**Section 2.3.1**). Test meals containing 50 g of available carbohydrate were served with 15 g of butter spread (low fat), 25 g cheese and plain water (300 mL). Participants were asked to consume the entire serving within 10-15 min. During the postprandial period, volunteers were provided with 500 mL of water and no additional water was permitted.

Details on blood collection and biochemical analyses were described in **Section 2.4**.

5.2.6 Statistical analysis

Data was analysed using IBM Statistics SPSS (Version 22.0). Incremental area under curve ($iAUC_{0\text{-}180\ min}$) for glucose, insulin, CCK, PYY and GLP-1 were estimated using a trapezoidal rule (Vitaglione et al., 2009). Tests of normality for glucose, insulin, CCK, PYY and GLP-1 were performed using the Shapiro-Wilk test. Data distribution with p-value more than 0.05 was considered as normal. All data were normally distributed except insulin and PYY. Data for insulin and PYY were log-transformed to improve normality. This was a crossover study design and each subject served as their own control, therefore a General Linear Model (GLM) was performed to determine differences between breads (WB, BT, βG and βGBT) and time points (0, 30, 60, 120 and 180 min). BMI and baseline

value (for each parameters) were used as covariates and gender as between-subject factor. Using baseline as a covariate will increase the efficacy of the analysis rather than subtraction from baseline alone. Furthermore, the use of baseline subtraction can introduce artefactual effects due to random differences at baseline (i.e. whether these differences are statistically significant or not) (Blundell et al., 2010).

The data for GLM test meet the following assumptions:

i. The dependent variable is measured at continuous level (eg. glucose levels measured as mmol/L, which is continuous data).
ii. The independent variable should consist of at least two categorical, "related groups" or "matched pairs" (This study is a cross over study hence subjects are present in both groups).

Values with $p < 0.05$ were considered significant.

5.3 Results

A total number of 16 (8 males and 8 females) subjects were recruited and 15 subjects (8 males and 7 females) completed this four-arm crossover trial. One female subject did not complete the last two trials and was subsequently excluded from the analysis. Subjects' characteristics are shown in **Table 5-1**.

Table 5-1. Participant characteristics (n = 15)

Characteristics	**Mean ± SD**
Age (years)	24.0 ± 7.7
Weight (kg)	70.8 ± 16.8
Height (cm)	171.2 ± 11.2
Body Mass Index (BMI, kg/m^2)	23.8 ± 3.2
Systolic blood pressure (SBP, mmHg)	118.3 ± 10.4
Diastolic blood pressure (SBP, mmHg)	67.3 ± 6.4

Fasting plasma glucose levels were similar between treatments (**Figure 5-3**). Glucose levels gradually increased from 0 min to 15 min and reached peak levels at 30 min for all breads. Glucose levels gradually decreased from 30 min and returned to baseline at 180 min. There were no significant differences between breads at any time point. However, there were significant ($p < 0.05$) time interactions between baseline and 30 min for all breads except βG. βG showed the flattest glucose peak, i.e. the increment from baseline to peak concentration at 30 min did not change significantly. βGBT showed significantly ($p = 0.005$) higher (12.7%) incremental area under curve ($iAUC_{0\text{-}180\ min}$) when compared with βG (**Figure 5-4**). There were no significant differences in $iAUC_{0\text{-}180\ min}$ of βG when compared with WB.

Subjects showed similar baseline insulin levels (**Figure 5-5**). Insulin levels significantly ($p < 0.05$) increased after eating each bread at 30 min in the range of 42.8 ± 8.5 to 62.4 ± 17.9 mU/L when compared to baseline. However, insulin levels did not change significantly between breads at 30 min. Insulin levels gradually decreased from 30 min to 60 min and significantly ($p < 0.05$) reduced at 120 min for all bread except for βG. The nadir insulin response was obtained at 180 min for all breads. There was a significant ($p = 0.018$) difference (3.2%) between BT and βGBT at 180 min (only at one time point). $iAUC_{0\text{-}180\ min}$ were similar between breads (**Figure 5-6**).

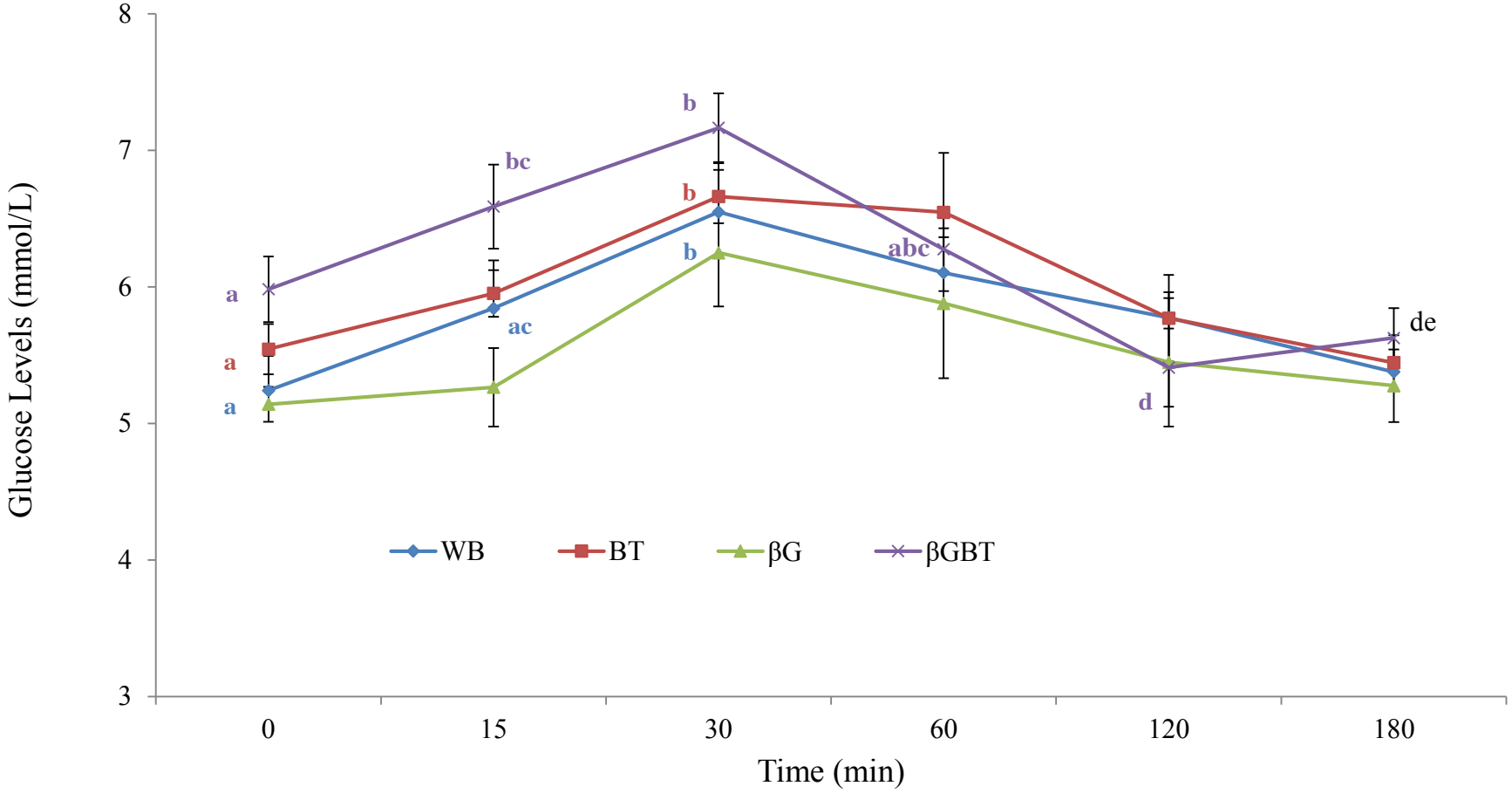


Figure 5-3. Glucose responses (mmol/L) after eating four different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. Values with different letters are significantly ($p < 0.05$) different between time points (General Linear Model, GLM).

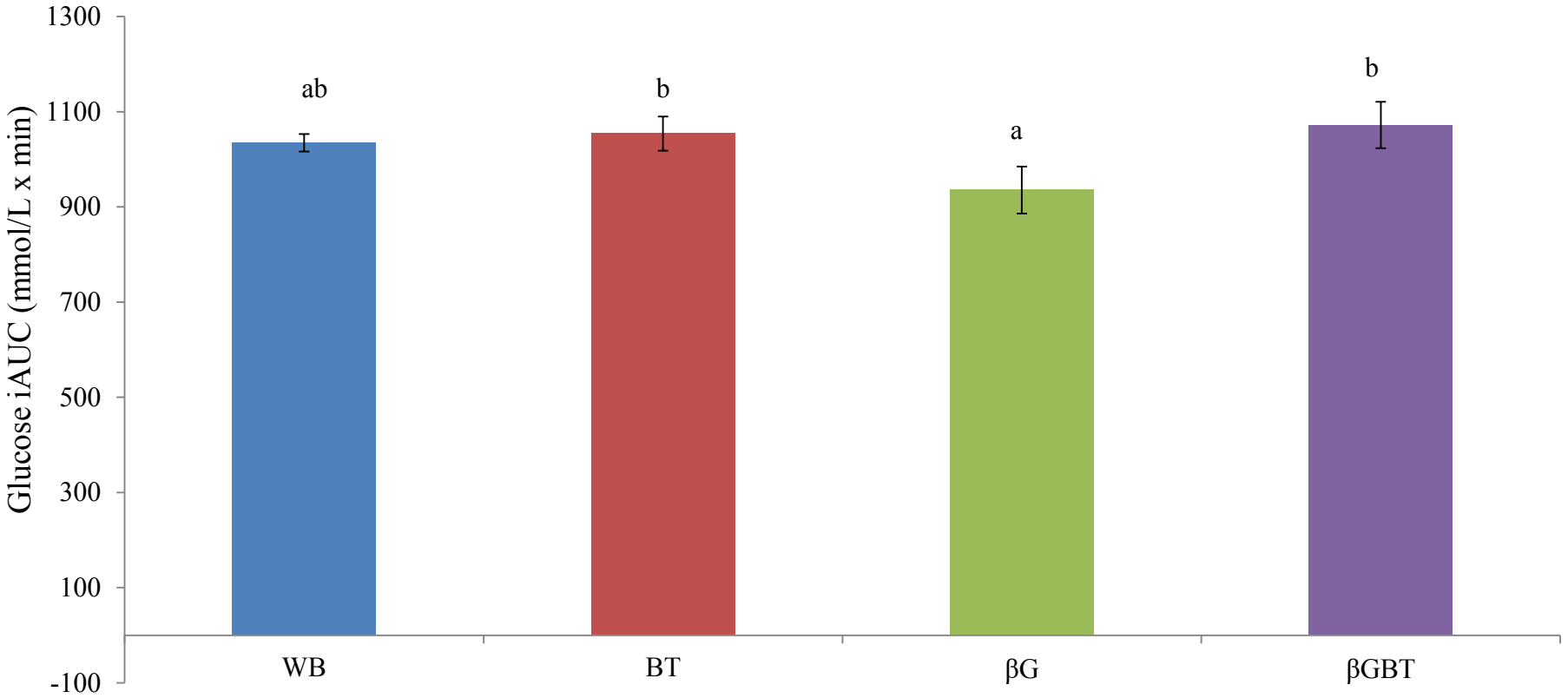


Figure 5-4. Glucose incremental area under the curve ($iAUC_{0\text{-}180\ min}$) (mmol/L x min) of different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. Values with different letters are significantly ($p < 0.05$) different between breads. βG *vs* BT ($p = 0.014$), βG *vs* βGBT ($p = 0.001$).

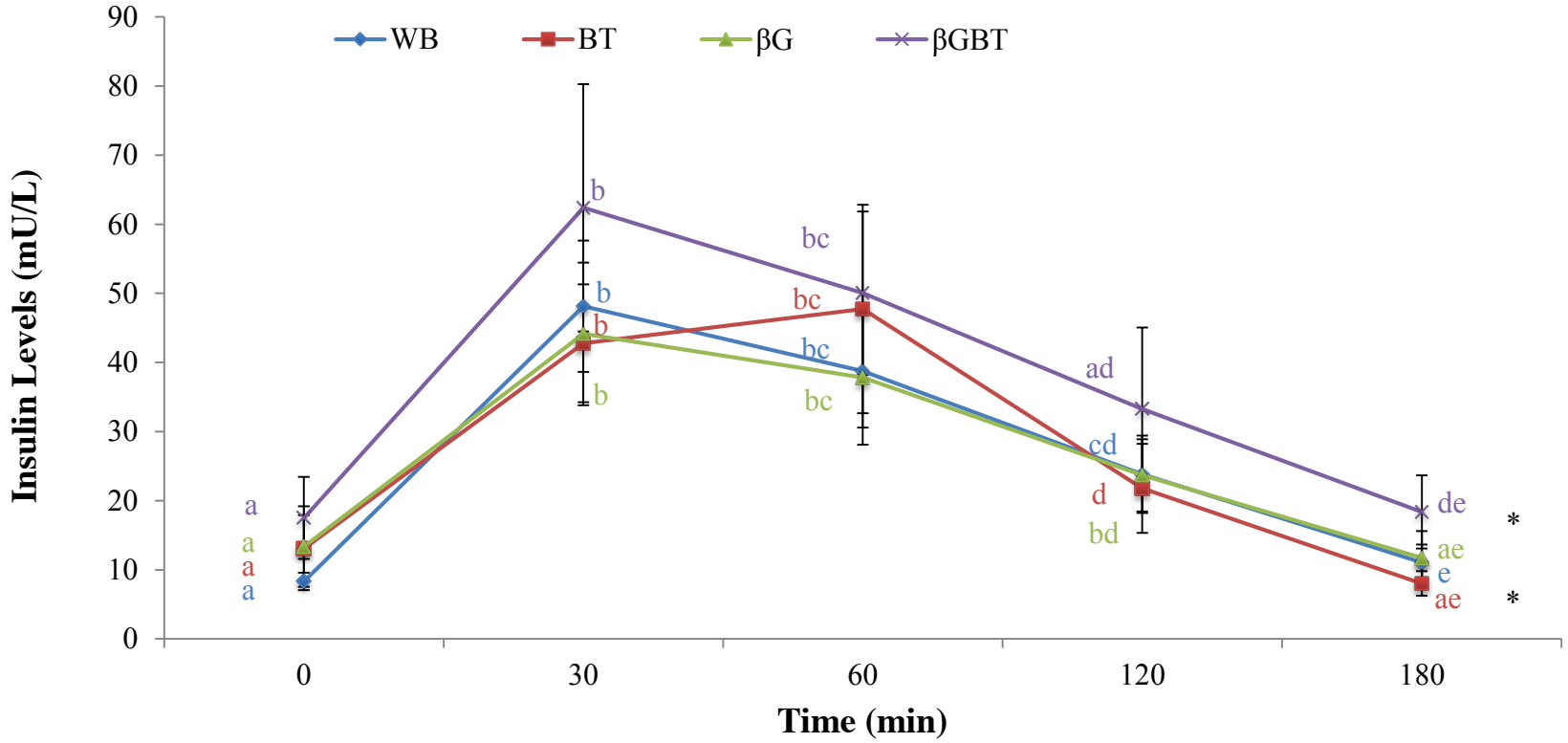


Figure 5-5. Insulin responses (mmol/L) after eating four different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. Values with different letters are significantly ($p < 0.05$) different between time points (General Linear Model, GLM). Asterisk (*) indicates significant ($p = 0.018$) values between BT and βGBT.

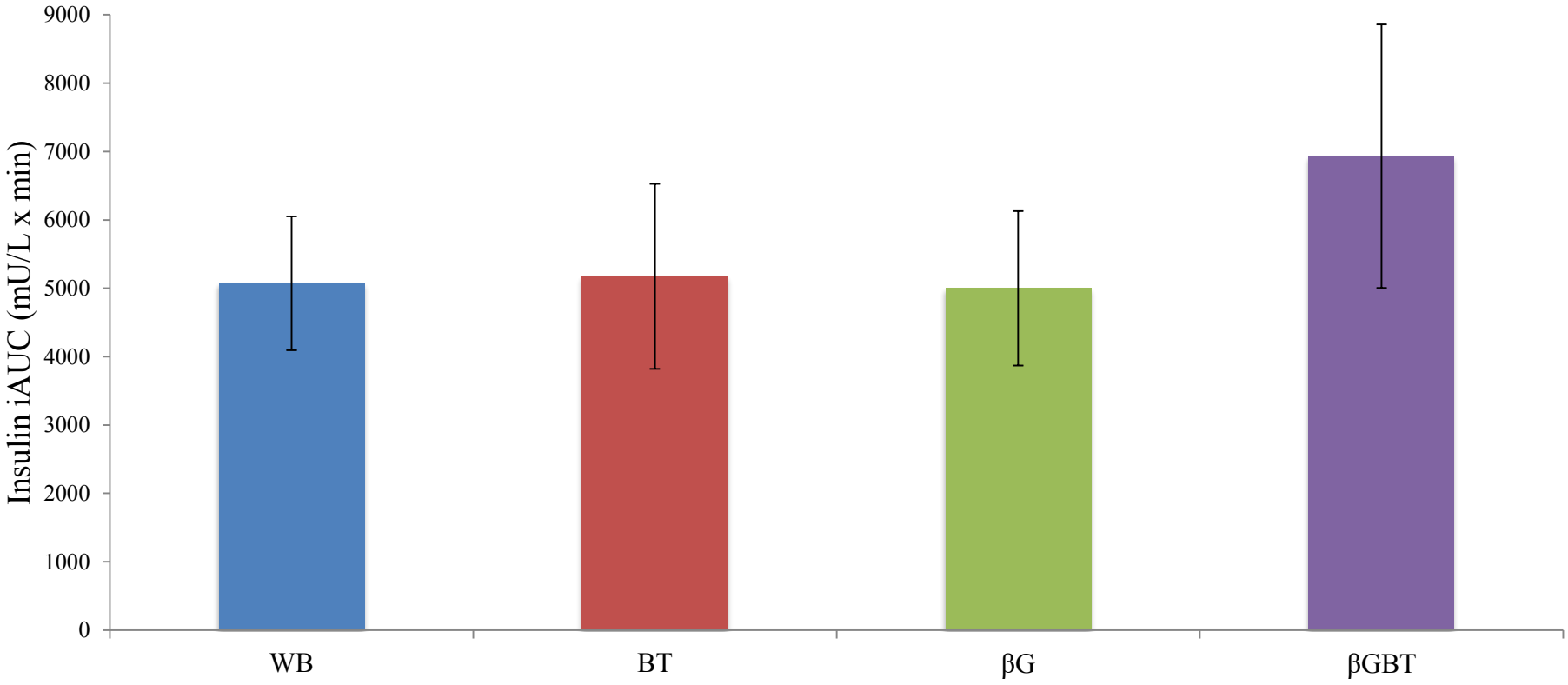


Figure 5-6. Insulin incremental area under the curve ($iAUC_{0\text{-}180\ min}$) (mU/L x min) of different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was no significant different between breads.

Fasting cholecystokinin (CCK) was similar between breads (**Figure 5-7**). Postprandial CCK at 30 min did not increase significantly when compared to baseline. CCK responses remained unchanged over the period of 60, 120 and 180 when compared with baseline and 30 min. Incremental $AUC_{0\text{-}180\ min}$ were similar between breads (**Figure 5-8**).

Fasting peptide YY (PYY) was similar between breads (**Figure 5-9**). Postprandial PYY increased from baseline to 30, 60, 120 and 180 min but did not change significantly between breads. There were no significant time interactions from baseline (0 min) to 30, 60, 120 and 180 min for WB, BT and βG breads. However, there were significant time interactions for βGBT at 30 and 180 min ($p = 0.008$), and a trend towards significant between 60 and 180 min ($p = 0.056$) (**Figure 5-9**). βGBT showed -11.9% lower ($p = 0.045$) incremental $AUC_{0\text{-}180\ min}$ when compared with BT (**Figure 5-10**). There was a trend towards significant ($p = 0.054$) for total AUC between βG and BT.

Fasting glucagon-like peptide 1 (GLP-1) was similar between breads (**Figure 5-11**). There were small increments in postprandial GLP-1 at 30 min but did not change significantly when compared with baseline. There was non-significant decreased in GLP-1 levels between breads from 30 min to 60, 120 and at 180 min when compared to baseline. There were no significant time interactions between baseline, 30, 60, 120 and 180 min among four breads. Incremental $AUC_{0\text{-}180\ min}$ of GLP-1 were similar between breads (**Figure 5-12**).

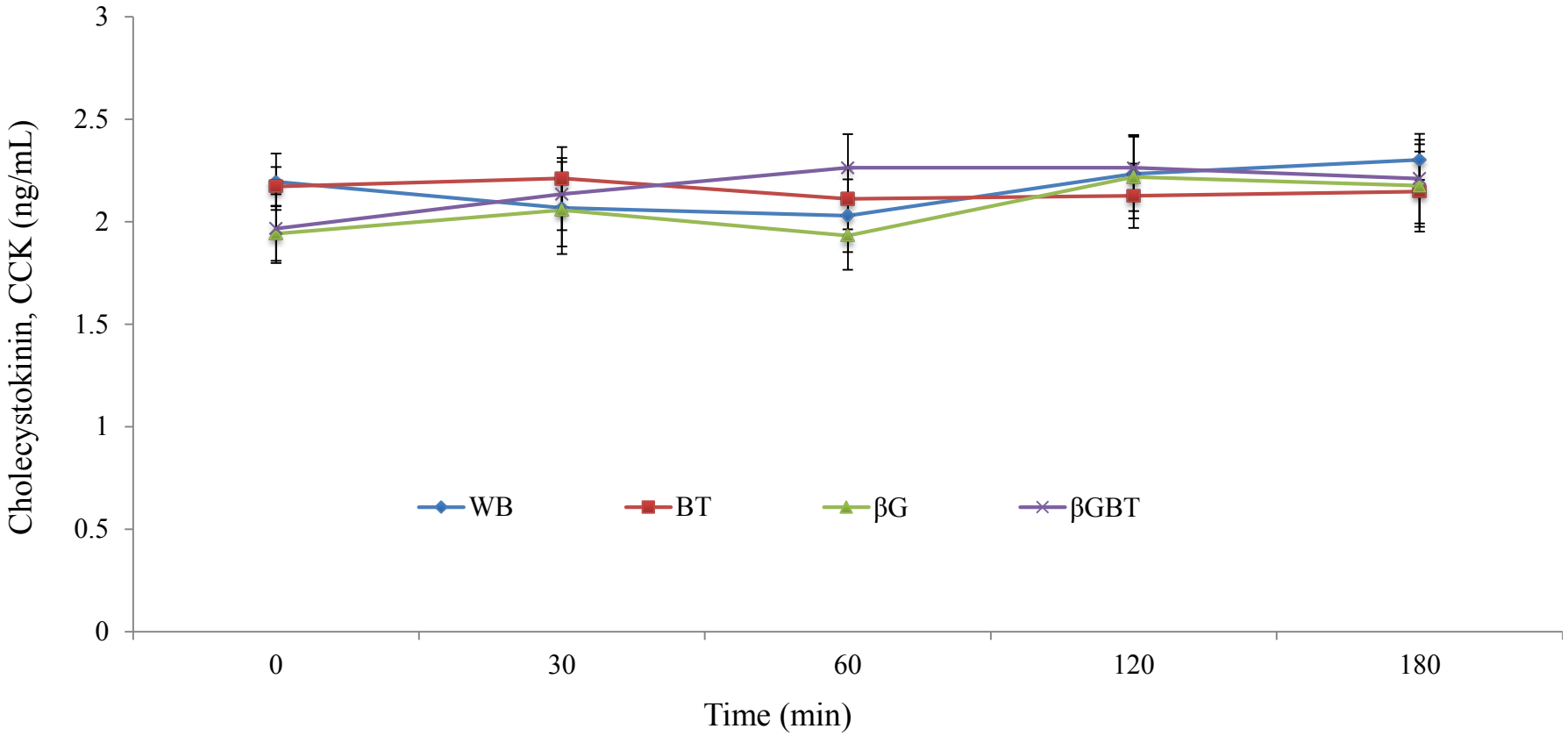


Figure 5-7. Cholecystokinin (CCK) responses (ng/mL) after eating four different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was no significant difference between breads. Normal fasting range: 1 – 2 pM (French et al., 1993).

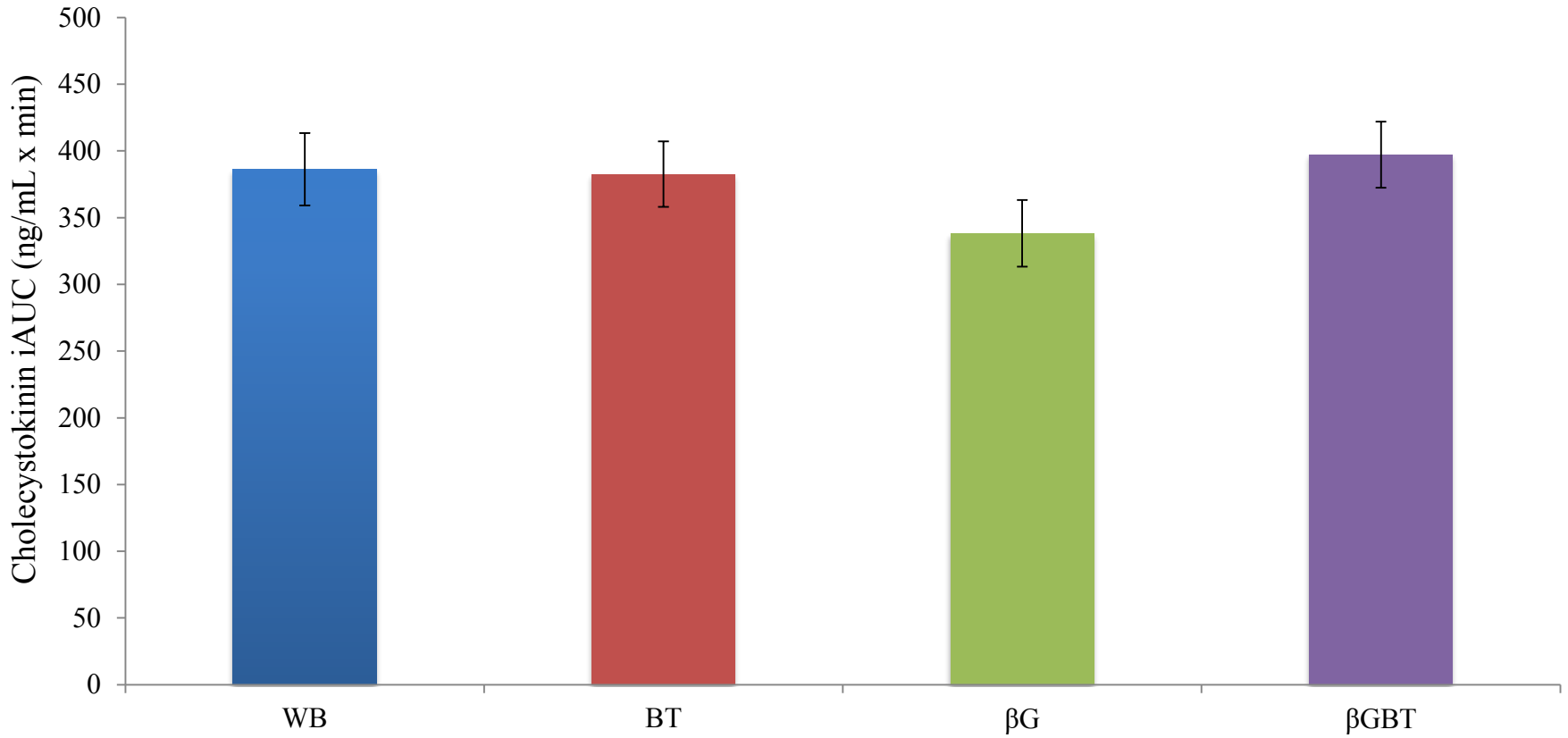


Figure 5-8. Cholecystokinin (CCK) incremental area under the curve ($iAUC_{0\text{-}180\ min}$) (ng/L x min) of different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was no significant difference between breads.

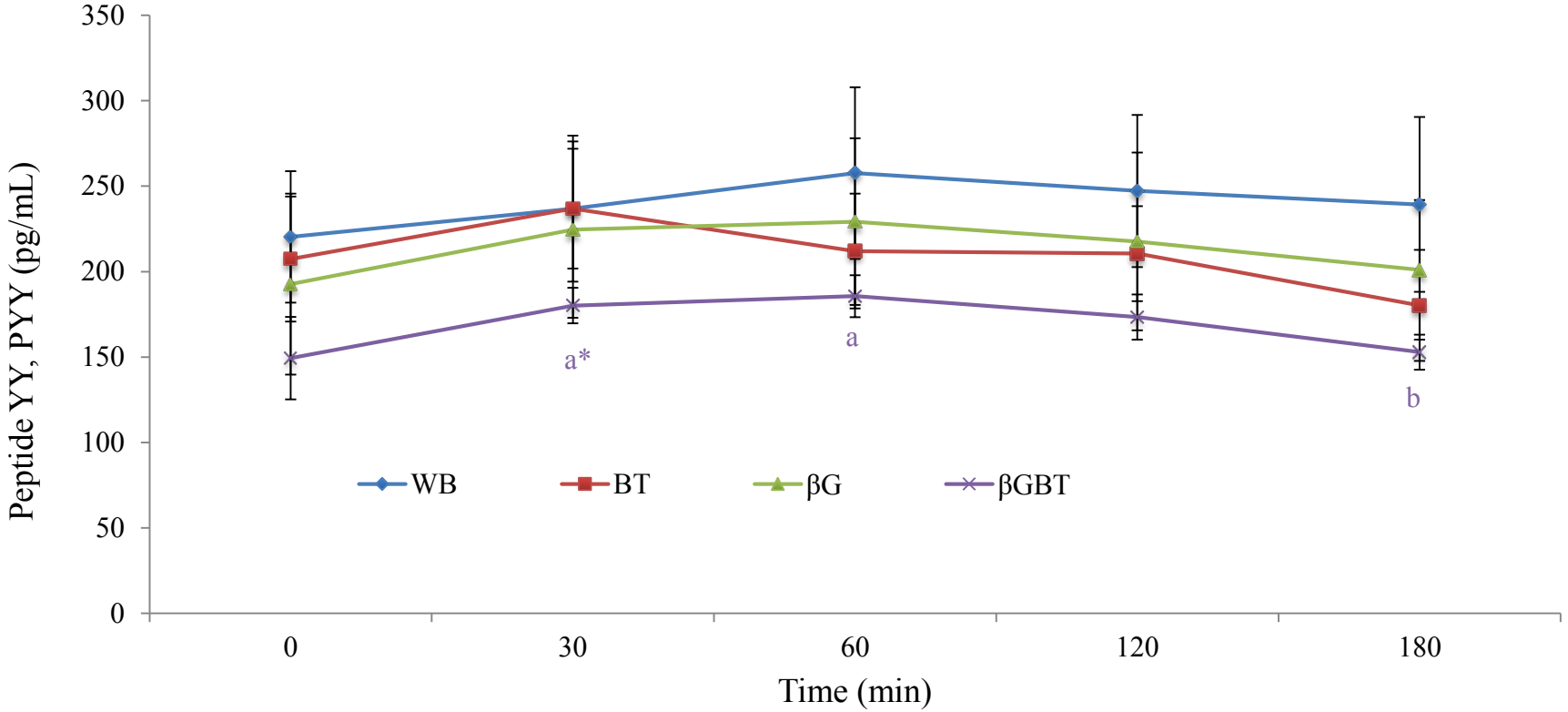


Figure 5-9. Peptide YY (PYY) responses (pg/mL) after eating four different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was a significant ($p = 0.008$) difference for βGBT at 60 *vs* 180 min. Asterisk (*) indicates a trend towards significant ($p = 0.056$) for βGBT at 30 *vs* 180 min. Normal fasting range: 84 – 168 pg/ mL (Essah et al., 2007).

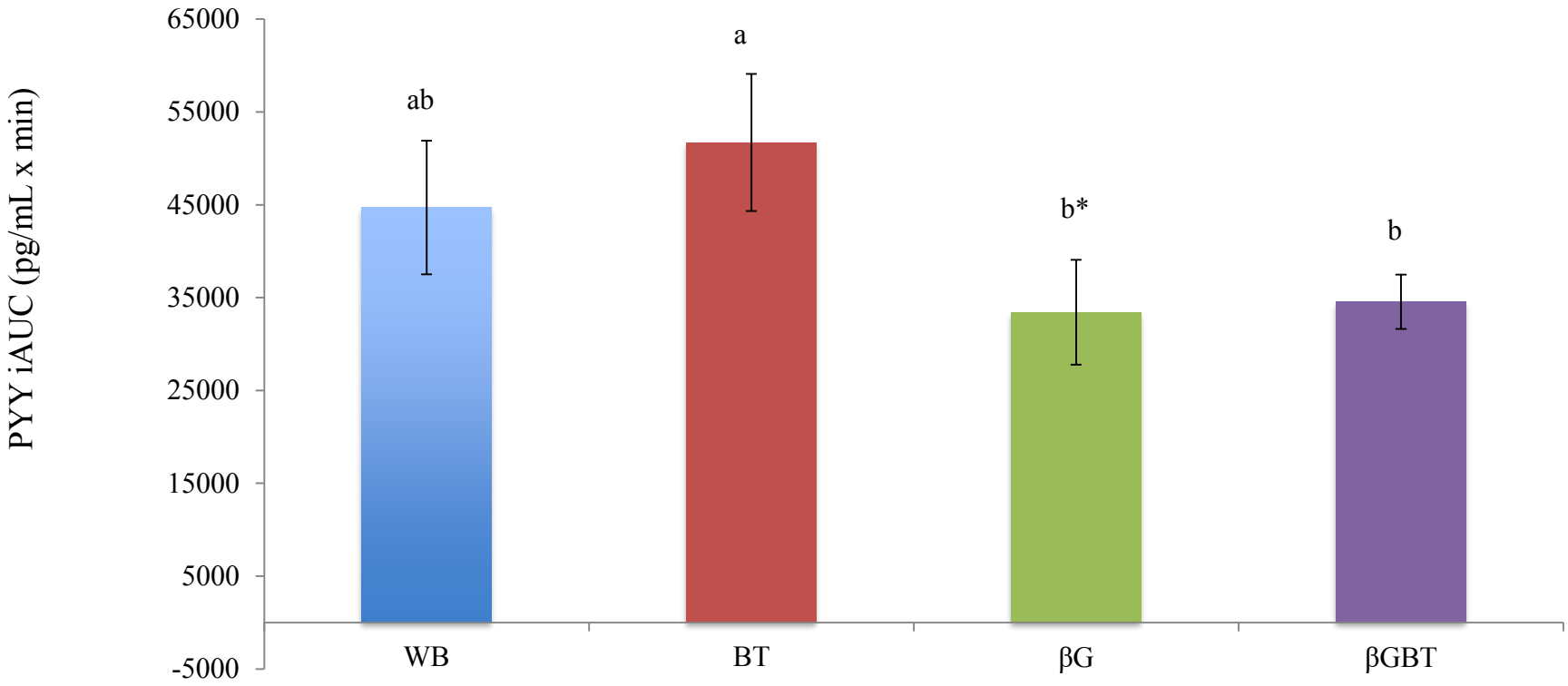


Figure 5-10. Peptide YY (PYY) incremental area under the curve ($iAUC_{0\text{-}180\ min}$) (pg/mL x min) of different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was a significant ($p = 0.045$) difference βGBT and BT. Asterisk (*) indicates a trend towards significant ($p = 0.054$) between βG and BT.

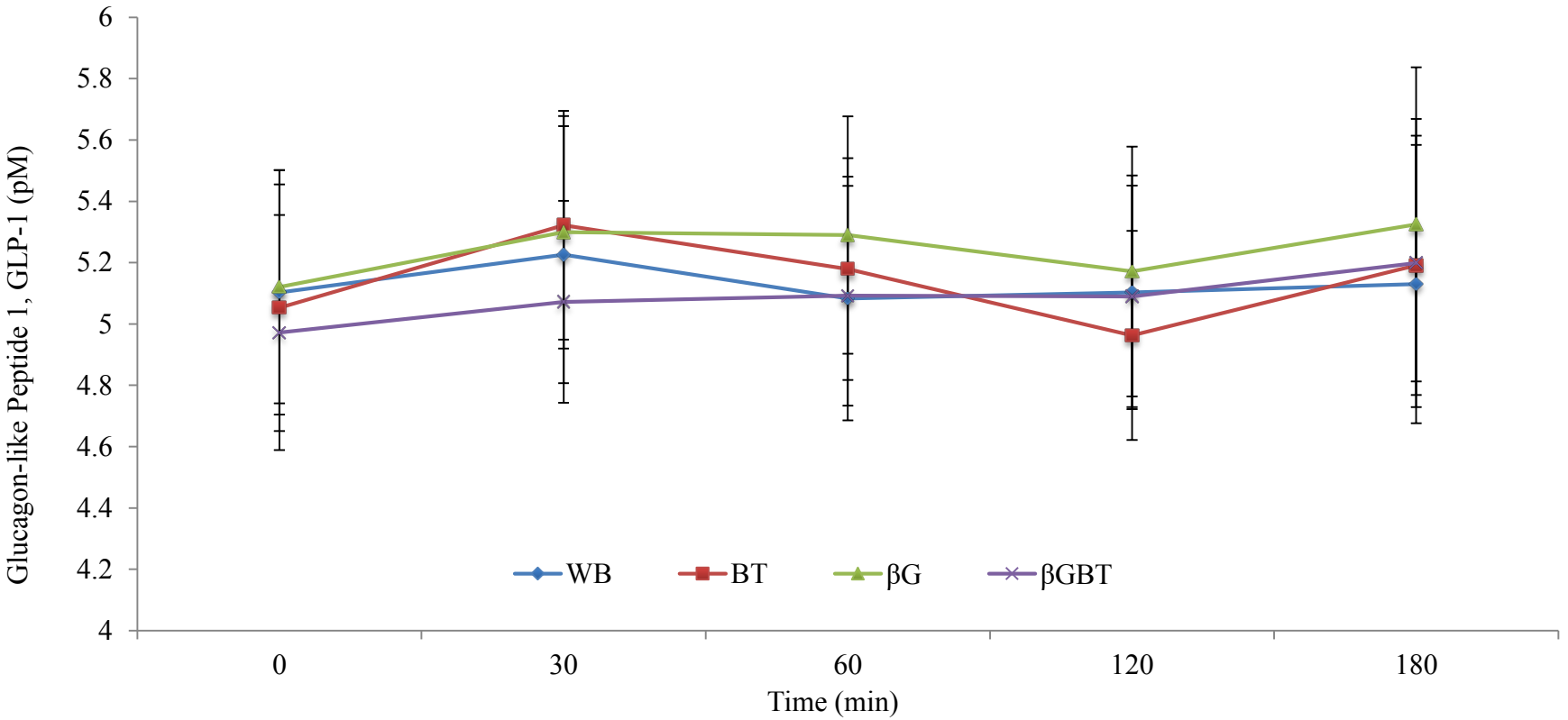


Figure 5-11. Glucagon-like peptide 1 (GLP-1) responses (pM) after eating four different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was no significant difference between breads. Normal fasting range: ~ 10 pM (Flint et al., 1998)

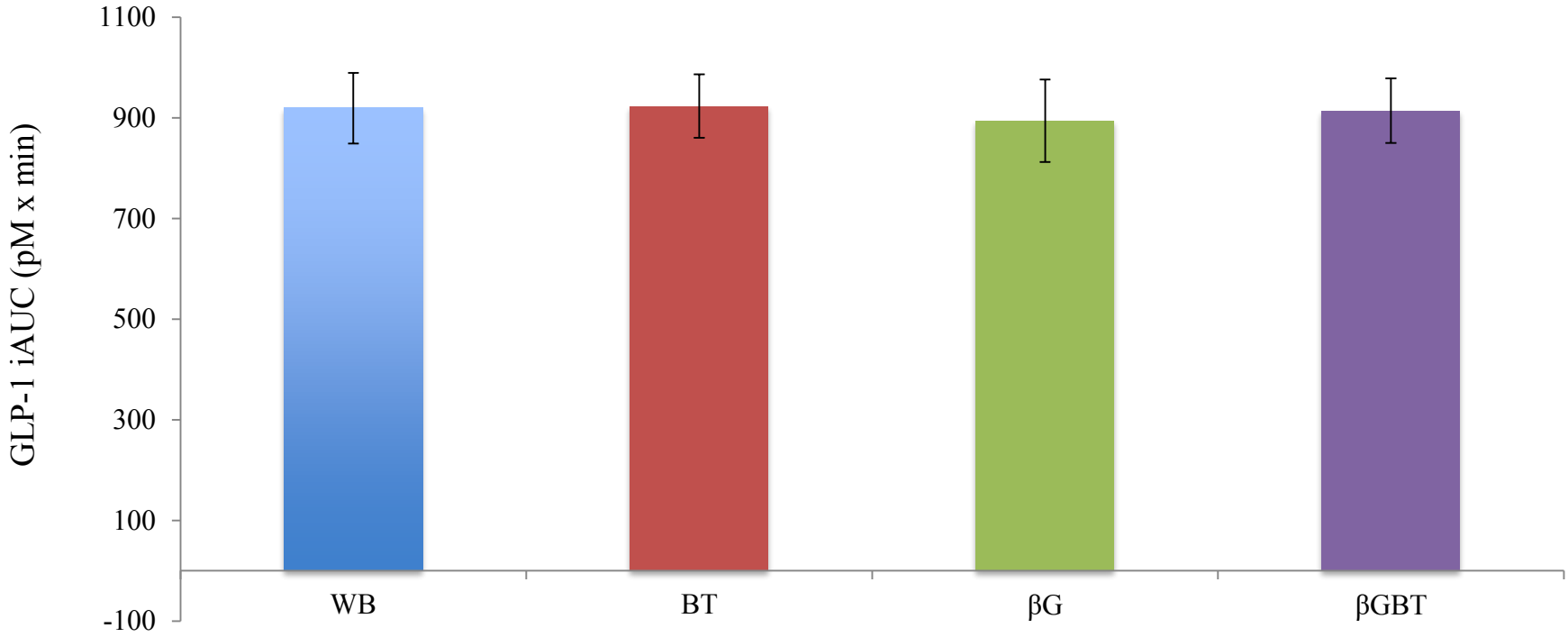


Figure 5-12. GLP-1 incremental area under the curve ($iAUC_{0\text{-}180\ min}$) (pM x min) of different breads. Values are expressed as mean ± S.E.M. WB, white bread; BT, black tea bread; βG, β-Glucan bread, βGBT, β-Glucan plus black tea bread. There was no significant difference between breads.

5.4 Discussion

The aim of this study was to determine whether the combination of two functional ingredients (β-glucan and black tea) in bread could reduce postprandial glucose and insulin responses. In the **Chapter 4**, we demonstrated that combining β-glucan and black tea in bread increased perceived satiety, fullness and reduced prospective food intake among healthy subjects. However, eating behavior is a complex process that involves metabolic processes that drive hunger, and satiety and sensory characteristics of food that drive food choice (Blundell et al., 2010). Fatima et al. showed that perceived satiety and fullness correlated with the appetite hormone (peptide YY) (Fatima et al., 2015). Hence, the second aim of this study was to determine the effects of these breads on appetite hormones.

According to the European Food Safety Authority (EFSA), β-glucan intake of 4 g per 30 g available carbohydrate reduces postprandial glycaemic responses without disproportionally increased postprandial insulinaemic responses (Agostoni et al., 2011). Our study showed the addition of 7 g β-glucan in bread (βG) containing 50 g available carbohydrate attenuated postprandial glucose responses (10 – 13%) without significant changes in insulin when compared to white black tea (BT) and β-glucan plus black tea bread (βGBT). To explain the possible mechanisms, we proposed that that the presence of β-glucan could preserve the elliptical structure of starch granules and this could form a 'barrier' to α-amylase-starch interactions, (**Chapter 3**) (Jalil et al., 2015). This action slows down the digestion of starch by α-amylase (amlylolysis) and hence may reduce postprandial glucose response. Similarly, Brennan et al. demonstrated that galactomannan from guar gum added during breadmaking became dispersed and mixed evenly with starch granules and protein matrix (Brennan et al., 1996). The overall appearance of the starch granules appeared to be 'glued' together by the galacatomannan. Vitaglione et al. demonstrated that the addition of 3 g of β-glucan per 55 g of available carbohydrate reduced (10%) postprandial glucose without altering the secretion of insulin levels when compared with white bread (Vitaglione et al., 2009).

We have previously shown that βGBT reduced short-term (10 min) *in vitro* starch hydrolysis when compared to WB, BT and βG (Jalil et al., 2015). However, in the study described in this chapter, we found no significant effects of βGBT in reducing postprandial

glucose when compared with other breads. This could be due to the following reasons: first, the size of starch molecules are different when being digested in the *in vitro* when compared with *in vivo* system (Hasjim et al., 2010). Preincubation of normal maize starch with artificial saliva and pepsin for 30 min formed large pores on the starch surface and also the formation of small starch segments. On the contrary, digesta recovered from pig small intestine showed similar morphology but more pores when compared with raw starch granule before digestion. Hence, this leads to the differences between *in vitro* starch hydrolysis and *in vivo* postprandial glucose responses. We employed simplistic *in vitro* digestion models to study starch digestion which may not totally reflect the dynamic digestion procedures *in vivo* (pigs or colectomized rats) (Hasjim et al., 2010; Marlett and Longacre, 1996). *In vivo* models are a better method in predicting postprandial glycaemic response. Brennan et al. found no correlation between *in vitro* glucose production and *in vivo* postprandial glycaemic responses and concluded the lack of correlation may be due to inherent subject-to-subject variation in postprandial glycaemic responses (Brennan et al., 2012). However, this is not the case in our study because we employed a crossover study design. Each subject served as their own control and this reduced intervariation between measurements. As discussed in **Chapter 3**, another possible explanation could be the modifications of food matrix by black tea. Black tea contains high molecular weight thearubigins and theaflavins. These (poly)phenols may have modified the food matrix of the bread by forming a cross-link between gluten and starch, which led to softer bread.

As discussed in **Chapter 1**, viscous guar gum inhibited convection of the luminal content in relation to mixing contractions and hence reduced glucose absorption through the epithelium. This may be a mechanism to explain how soluble β-glucan reduced postprandial glycaemic responses. Tosh et al. demonstrated reducing high MW β-glucan (from 2.2 to 0.410 x 10^6) significantly increased their solubility from 44 to 57% (Tosh et al., 2008). Different food processing techniques may also have impact on β-glucan solubility. The bread baking process decreased molecular weight and solubilised β-glucan in oat when compared when cooking oat as porridge (Johansson et al., 2007). Ellis et al. demonstrated that supplementation of high molecular weight guar gum in pigs significantly increased jejunal viscosity and dose-dependently reduced glucose absorption and insulin secretion (Ellis et al., 1995). This study suggests an important role was played by the

enteroinsular axis in attenuating the glycaemic response after a meal containing high molecular weight guar gum. Different viscous fibres may have different physico-chemical properties. Hence, the physiological effects of different types of viscous fibre are not comparable to each other. These soluble fibres have different chemical structures (eg. different types of sugar backbone, molecular weight and solubility) and may have different effects on viscosity when added in foods and also in the gastrointestinal tract. Edwards et al. showed that the ingestion of both xanthan and locust gum reduced gastric emptying but had no effects on blood glucose (Edwards et al., 1987). A further test indicated that acidification (mimic gastric condition) and re-neutralisation (mimic duodenal secretions) reduced the viscosity of the mixture (dilutional effects) (as discussed in **Chapter 1**).

Gastric satiety is based on mechanical action in the stomach while intestinal-derived satiety is nutrient-dependent whereby the satiety signals are released when nutrients interacted with the intestinal wall (Kristensen and Jensen, 2011). There is interplay between gastric emptying and gut hormones. Cholecystokinin (CCK) is a major gut hormone released from duodenum, jejunum, the proximal small intestine, in response to the presence of nutrients (protein and fat) (Holt et al., 1992; Liddle et al., 1985). CCK stimulates secretion of pancreatic enzymes, causes contraction of gallbladder, slows gastric emptying and increases motility in the large intestine (Beyer et al., 2008). CCK receptors (CCK-A and CCK-B) are both present in the brain and stomach and play a pivotal role in the regulation of food intake. In this study, we hypothesised that the blunted effect of βG on postprandial glucose responses may be due to the effects of CCK. However, in this study, postprandial CCK responses were similar between breads. CCK increases in response to fat and protein in the small intestine (Liddle et al., 1985). All breads tested here had similar protein and fat content, and hence had a little effect on CCK response.

In **Chapter 4**, we demonstrated βGBT and βG decreased perceived hunger, increased perceived satiety and reduced perceived prospective food intake up to 3 h after breakfast. Putting the results of these two studies together, it may be suggested that the effects may not be through stimulation of CCK. This is consistent with one study by French et al., which demonstrated CCK was not related to hunger and satiety and also with the rate of gastric emptying in humans (French et al., 1993). Gibbons et al. demonstrated that high-fat

diet stimulated postprandial CCK responses but was not related to suppression of hunger (Gibbons et al., 2015). These authors suggest that CCK measurement at the end of meal intake (postprandial) was not related to postprandial appetite control. Instead, CCK should be measured during a meal to see the action of CCK on satiation through neural mechanism activated through vagal nerve. Using a beverage as a food matrix, Juvonen et al. showed low viscosity (< 250 mPa.s measured at shear rate 50 s^{-1}) β-glucan beverage significantly increased CCK ($AUC_{0\text{-}180\ min}$) by 80% compared with high viscosity (> 3000 mPa.s measured at shear rate 50 s^{-1}) β-glucan beverage (Juvonen et al., 2009). This may be related to the viscosity of the products. The high viscosity beverage decreased mixing in the small intestine and prevented close interaction between nutrient and GI mucosa for stimulation of enteroendocrine cells to release peptide CCK (Juvonen et al., 2009).

Similar to CCK, another gastrointestinal hormone peptide tyrosine tyrosine (PYY) acts through colonic L-cells and the arcuate nucleus within the hypothalamus (Chambers et al., 2014). This interconnection allows for the stimulation of appetite (orexigenic) and appetite suppression (anorexigenic), in response to SCFA through activation of G protein-coupled receptor 41 (GPR41) and GPR43. Both receptors presence on the colonic endocrine L-cells, at which the same cells that secretes PYY and glucagon-like peptide 1 (GLP-1) (Murphy et al., 2006). Both GPR41 and GPR43 are physiologically regulated by acetate, propionate and butyrate with stronger preference of GPR41 for acetate and propionate and GPR43 for propionate and butyrate (Brown et al., 2003; Le Poul et al., 2003; Burger et al., 2012; Nilsson et al., 2003). In **Chapter 3**, fermentations with β-glucan increased propionate production but did not change significantly when compared with white bread. The (poly)phenol-β-glucan could be a substrate for colonic fermentation by gut microbiota. However, the present study was continued for up 3 h postprandially and a longer period study (6-9 h) is needed to determine the colonic effects of (poly)phenols and β-glucan on satiety.

Vitalione et al. demonstrated bread containing 5.5% β-glucan per available carbohydrate significantly increased $AUC_{60\text{-}180\ min}$ PYY compared with control bread (Vitaglione et al., 2009). In our study, bread containing 14% β-glucan per g available carbohydrate was used, and hence, it could be argued that that the dose difference could have a stronger effect in

stimulating PYY. We found that bread prepared with β-glucan resulted in a 12% reduction in the $iAUC_{0\text{-}180min}$ of PYY when compared with BT bread. In our study, GLP-1 did not change significantly between breads. Kristensen et al. showed supplementation of soluble low- and high-mucilage flaxseed (12 and 15 g) in buns did not change postprandial PYY and GLP-1 over 7 h (Kristensen et al., 2013). Comparisons for the effect of soluble fibre on appetite hormones are difficult as they have different physical and chemical properties and also depend on how they are prepared in food or a beverage.

A liquid, low viscosity beverage prepared with β-glucan evoked higher PYY and GLP-1 response ($AUC_{0\text{-}180min}$) with 420% and 155%, respectively, when compared with high viscosity β-glucan beverage (Juvonen et al., 2009). This suggests that the presence of β-glucan decreased mixing in the small intestine and prevented the interaction between nutrient and the stimulation of PYY release. However, the addition of oat bran in pudding (semisolid) containing 5 g of β-glucan had no effect on PYY (Juvonen et al., 2011). This may be due to reduced viscosity in the stomach and small intestine after a semisolid meal compared to liquid meal. However, these are acute (one day) and short (3 h postprandial) feeding trials. It should be noted that short-term effects may be different to ingesting fibre for longer periods of time.

Supplementation of 16 g of soluble oligofructose in a beverage for 2 weeks increased $AUC_{0\text{-}230\ min}$ GLP-1 by 94% when compared with control (Verhoef et al., 2011). A lower dose (10 g) increased $AUC_{0\text{-}420\ min}$ GLP-1 by 9% compared with control. In a dose-escalation study, Pederson et al. showed increasing oligofructose dose from 15 to 55 g as beverage in a time frame of 5 weeks dose-dependently increased PYY in the range of 2 to 51% when compared with the control group (Pedersen et al., 2013). The authors suggested that the delay in gastrointestinal motility was due to increased osmotic pressure in the gut. Regardless of the food matrix, an intake of 8 g oligofructose twice a day (as powder form) for 2 weeks significantly increased PYY at 10 min compared with baseline (Cani et al., 2009). Supplementation of highly viscous PolyGlycoPlex (containing soluble glucomannan, sodium alginate and xanthan gum) for 2 weeks (5 g in week one and 10 g in week two) with cereal and plain yogurt significantly reduced PYY response by 33% when compared with control (Reimer et al., 2010). It is possible that long-term fibre

supplementation might change gut microbiota composition and confer health benefits through the production of SCFA.

We have discussed the physical and chemical characteristics of soluble fibres affecting postprandial glycaemia, insulinaemic and appetite hormones. The bread food matrix itself may also play an important role in affecting those responses. Choosing bread as a food matrix to deliver two functional ingredients is feasible as breadmaking is relatively inexpensive and easy. However, breadmaking involves the complex formation of an elastic gluten network (Belton, 1999). At a low hydration level, there is an increase in water-protein hydrogen bonds, but the hydrogen inter-chain is strong and will not break easily. There is a balance between residues involved in the inter-chain hydrogen bonds and those that are hydrated. This leads to the formation of 'trains' and 'loops' regions. The 'trains' represent groups of polymer-surface interactions while 'loops' represent groups of polymer-solvent interactions. In this study, the addition of β-glucan competed with gluten for water; therefore, more water was needed to compensate for water uptake by β-glucan (Jacobs et al., 2008). As the hydration proceeds, the presence of (poly)phenols from tea may form an interaction between gluten network in the dough. Gluten subunits are rich in glutamine, glycine and proline (Tatham et al., 1990). Protein-(poly)phenols is formed through hydrophobic interactions between proline hydropohic sites with ring structure of (poly)phenols (Bordenave et al., 2014).The addition of soluble pectin and (poly)phenols during dough development and baking formed a cross-linking with gluten network which lead to more water holding and softer bread (Sivam et al., 2011). At this point, the addition of (poly)phenols and fibre reduces the mobility of hydrated segments because of the competition among protein, (poly)phenols and fibre for water (Sivam et al., 2013).

Apart from gluten, there is also an interaction between tea (poly)phenols (TPL) and starch granules. There was evidence of interactions between TPL and high amylose corn starch (HACS) (Chai et al., 2013; Liu et al., 2011). HACS is naturally high in amylose and considered as low glycaemic response (Englyst et al., 1996). Surprisingly, the presence of 10% (w/v) TPL in high amylose corn increased postprandial glycaemic responses. The presence of TPL disrupted the normal process of starch retrogradation and specifically altering the normal process of amylose forming an ordered crystalline structure (Chai et al.,

2013). The addition of 10% (w/v) TPL with HACS significantly increased postprandial glucose response when compared with control without HACS (Liu et al., 2011). Increased postprandial glycaemia was achieved through accelerated digestion of HACS by α-amylase enzyme activity.

We postulate that the interactions between black tea and/or in combination with β-glucan lead to the following outcomes: 1) (poly)phenols in black tea form an interactions with gluten network, increased in elasticity, soften the final bread, increased the surface area and increased starch digestibility 2) β-Glucan 'coated' the starch granule, preserving their elliptical structure; reduced gluten elasticity, leads to a more solid bread, reduced starch digestibility and hence reduced glycaemic response 3) the combination of black tea (poly)phenols and β-glucan leads to the formation of (poly)phenols-gluten-β-glucan-starch complex, increased elasticity and (poly)phenols interaction with gluten network. This however leads to increased starch digestibility due to high surface area, which subsequently leads to a higher glycaemic response. Moreover, the sample size of this study was based on the EFSA's health claims on β-glucan in reducing glucose and insulin responses. Hence, this study was based on one outcome (glucose response) and may be underpowered for the effects on other parameters such appetite hormones.

In conclusion, this study suggests that consuming 7 g of β-glucan in bread containing 50 g available carbohydrate reduced postprandial plasma glucose without adversely increased insulin response when compared with black tea and black tea plus β-glucan. There were no additional effects of adding black tea in bread with β-glucan on glucose and insulin responses. The addition of β-glucan attenuated the PYY response. Black tea and/or β-glucan had no effects on CCK and GLP-1 responses.

Further study is needed to determine acute (6-9 h) and long-term (eg. 2 weeks and more) effects of consuming these breads on postprandial glycaemic responses and appetite hormones. Long-term consumption of these breads may change the gut microbiota composition, produce more SCFA and increase satiety.

Chapter 6: General Discussion

The major chronic diseases - cardiovascular diseases (CVD), cancer, chronic obstructive pulmonary disease and type 2 diabetes - are all associated with preventable biological and behavioural risk factors (WHO, 2010). Well-known biological factors associated with these diseases are: high blood pressure, high plasma cholesterol and being overweight, as discussed in **Chapter 1** of this study. Behavioral risk factors are modifiable within individuals and include: an unhealthy diet, physical inactivity and tobacco use. For example, a recent prospective study investigating the effect of unhealthy diets found an association for increased body weight (≥ 3 kg) over a period of 3 years, in those eating out at fast-food shops and eating while watching TV (Leon-Munoz et al., 2016). However, curbing these diseases through reducing the risk factors associated with them is not an easy task. It involves a population-based multi-sectoral, multidisciplinary and culturally relevant approach (WHO, 2016a). The World Health Organization (WHO) has devised a plan to tackle these problems through eating a healthy diet, and these include: 1) limiting the intake of sugar and salt 2) limiting total fat intake and choose unsaturated fat instead of saturated fat 3) increasing the consumption of fibre through the intake of fruit, vegetables, pulses, wholegrain and nuts (WHO, 2016b).

There are several barriers which have been associated with increasing fruit and vegetable intake, such as socio-demographic factors, individual food preferences, lack of awareness/knowledge and cultural issues (Mak et al., 2013; Dumbrell and Mathai, 2008; Bryant et al., 2015; WHO, 2016d). Based on an age-stratified focus group discussion, young men (18 – 25 years) indicated that fruit and vegetables were not part of a young men's culture and they did not eat a lot of fruits and vegetables (Dumbrell and Mathai, 2008). Higher frequency of eating out and eating takeaways is associated with lower consumption of both fruits and vegetables (Mak et al., 2013).

A review of randomised trials found no consistent effect on dietary fibre intake and body weight or energy intake (SACN, 2015). However, there is enough evidence to suggest that dietary fibre intake is beneficial for reducing risk of cardiovascular disease and reduced

incidence of haemorraghic stroke (SACN, 2015). In Western countries, however, dietary fibre intake is generally less than adequate for the prevention of major non-communicable diseases (NCD) (Marlett et al., 2002). In the UK, a minimum of 30 g of dietary fibre a day is recommended for the adult population (British Nutrition Foundation, 2006a; SACN, 2005). However, the current intake of dietary fibre in the UK is almost half of this recommendation with 14 g/day. In the US, the recommended fibre intake is about 25 g/day and 38 g/day for women and men, respectively (McRorie, 2015). However, research suggests that 90% of the US population do not meet this minimum recommendation.

Apart from these community-based interventions, food scientists can also play a major role in helping to achieve the minimum dietary fibre intake recomendation in the population. This could be achieved through the development of functional foods. Functional foods are foods that have additional nutritional benefits beyond their basic nutritional value (British Nutrition Foundation, 2016c). However, it is unclear what type of dietary fibres should be used and how much needs to be added in order to achieve health benefits. The type of food (vehicle) that is the most suitable as a functional food development also needs to be explored.

In this thesis, the selection of the most suitable dietary fibre was based on European Food Safety Authority (EFSA) recommendations. Based on scientific evidence, EFSA has approved a health claim for 4 g of β-glucan isolates per 30 g available carbohydrate to maintain desirable postprandial glucose and insulin reponses (Agostoni et al., 2011). There is also strong evidence from RCT that a higher intake of isolated β-glucans and oat bran leads to lower LDL-cholesterol, triacylglycerol and total cholesterol concentrations and lower blood pressure (SACN, 2015).

Starchy foods such as bread, pasta and *chapatti* have been used for the development of functional foods with added fibre (Cleary et al., 2007; Wang and Zhou, 2004; Bourdon, 1999). Bread is considered to be the most popular starchy food in the Western world (Pearn, 1998). Previous studies showed that bread prepared with soluble fibres were feasible and reduced postprandial glucose responses (Vitaglione et al., 2009; Juntunen et al., 2002). The traditional staple uses of bread has now changed to that of functional foods

that confer additional health benefits (Pearn, 1998). Moreover, the health benefits for β-glucan based on EFSA recommendation could be met with one serving of bread.

Developing a bread prepared with soluble fibre such as β-glucan showed promising health benefits. However, the major obstacle in developing bread with the addition of soluble fibre is their negative impact on product palatability and acceptability (Ellis et al., 1981; Ellis et al., 1991). There may be a food matrix interaction between gluten (protein), starch, and β-glucan in bread that could modify the final product's texture becoming unfavourable. It is important to keep a balance between a product's acceptability and the amount of fibre needed to have any additional health benefits (Hall et al., 2010).

Black tea is rich in (poly)phenol monomers and polymers (Roowi et al., 2010; Del Rio et al., 2004). These (poly)phenols inhibited α-glucosidase activity in the small intestine and potentially reduced plasma glucose responses in humans (Bryans et al., 2007; Tsuneki et al., 2004; Park et al., 2009; Satoh et al., 2015). Tea (poly)phenols were stable in functional foods and the final products (i.e. bread and biscuit) had similar sensory characteristics to the control (Sharma and Zhou, 2011; Pasrija et al., 2015; Lu et al., 2010). The presence of (poly)phenols in these products may also contain strong antioxidant, anti-inflammatory and other beneficial properties. With this in mind, would the addition of black tea improve the palatability of β-glucan bread, and would these two ingredients act synergistically in reducing the postprandial plasma glucose response? The development of such a bread was explored in **Chapter 3** and its effects on postprandial responses studied in **Chapter 5**.

Previous studies have shown the importance of bread acceptability in nutrition studies to increase the feasibility for general consumption (Ellis et al., 1981; Jenkins et al., 2002). Based on these early developments, it is important to determine the relative palatabilty of high fibre breads when compared to white bread (control). In **Chapter 4**, the relative palatabality and acceptability in healthy volunteers were also compared.

6.1 Summary of results

The results of this thesis have been divided into three sections:

The results of the first study (**Chapter 3**) showed bread prepared with β-glucan and black

tea (βGBT) did not significantly reduced overall but early (10-min) *in vitro* starch hydrolysis when compared with white and black tea breads (**Figure 6-1**). The predigestion of bread with α-amylase increased the total (poly)phenols released from black tea bread (BT). However, digestion with pepsin and pancreatin had no effect on the total (poly)phenols released from breads. Fermentations with β-glucan increased propionate production but did not change significantly. Black tea (positive control), or when added in bread alone or in combination with β-glucan, had no impact on total SCFA production.

The second study (**Chapter 4**) showed β-glucan (βG) bread reduced perceived hunger, and decreased the perceived desire to eat and prospective food intake (**Figure 6-2**). βGBT increased fullness, decreased perceived satiety and prospective food intake when compared with white bread (WB). Having βG and βGBT at breakfast had no effects on energy intake at lunch. However, BT bread intake reduced energy intake at lunch when compared with white bread. WB and BT breads showed similar palatability characteristics. βG and βGBT showed good overall acceptability. However, taste, texture and palatability of βG and βGBT were lower than with WB and BT. Female subjects showed a lower preferences for taste, texture and palatability of βG and βGBT when compared with white bread.

Results from **Chapter 5** showed that βG reduced glucose incremental $AUC_{0\text{-}180\ min}$ when compared with BT and βGBT breads (**Figure 6-3**). There was no additional effect of adding black tea with β-glucan (βGBT) on blood glucose and insulin responses. βG attenuated peptide YY (PYY) responses when compared with other breads. There were no additional effects of adding black tea or black tea with β-glucan on PYY, cholescystokinin (CCK) and glucagon like peptide-1 (GLP-1).

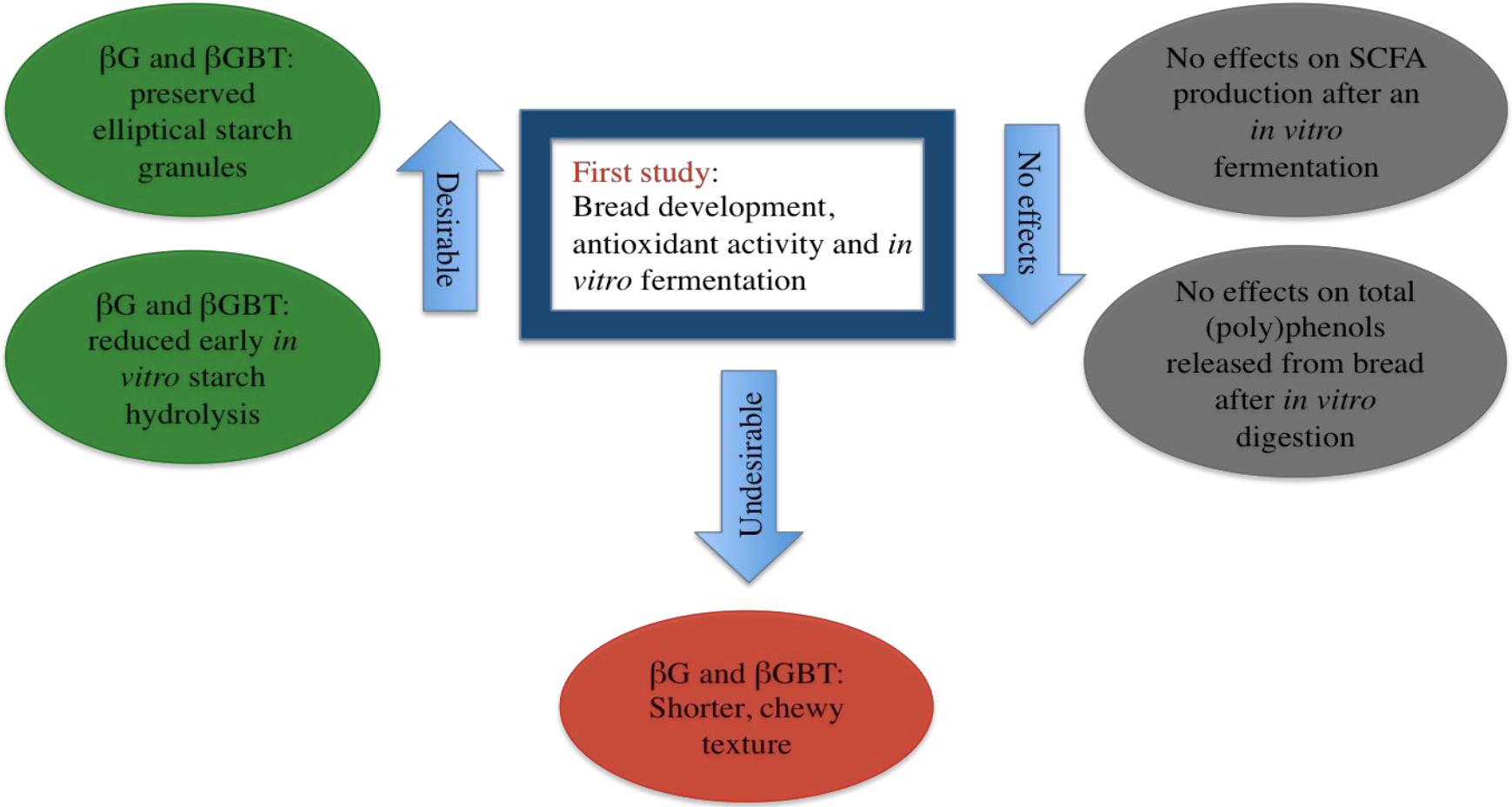


Figure 6-1. Results summary for Study 1 (bread development, antioxidant activity and *in vitro* fermentation)

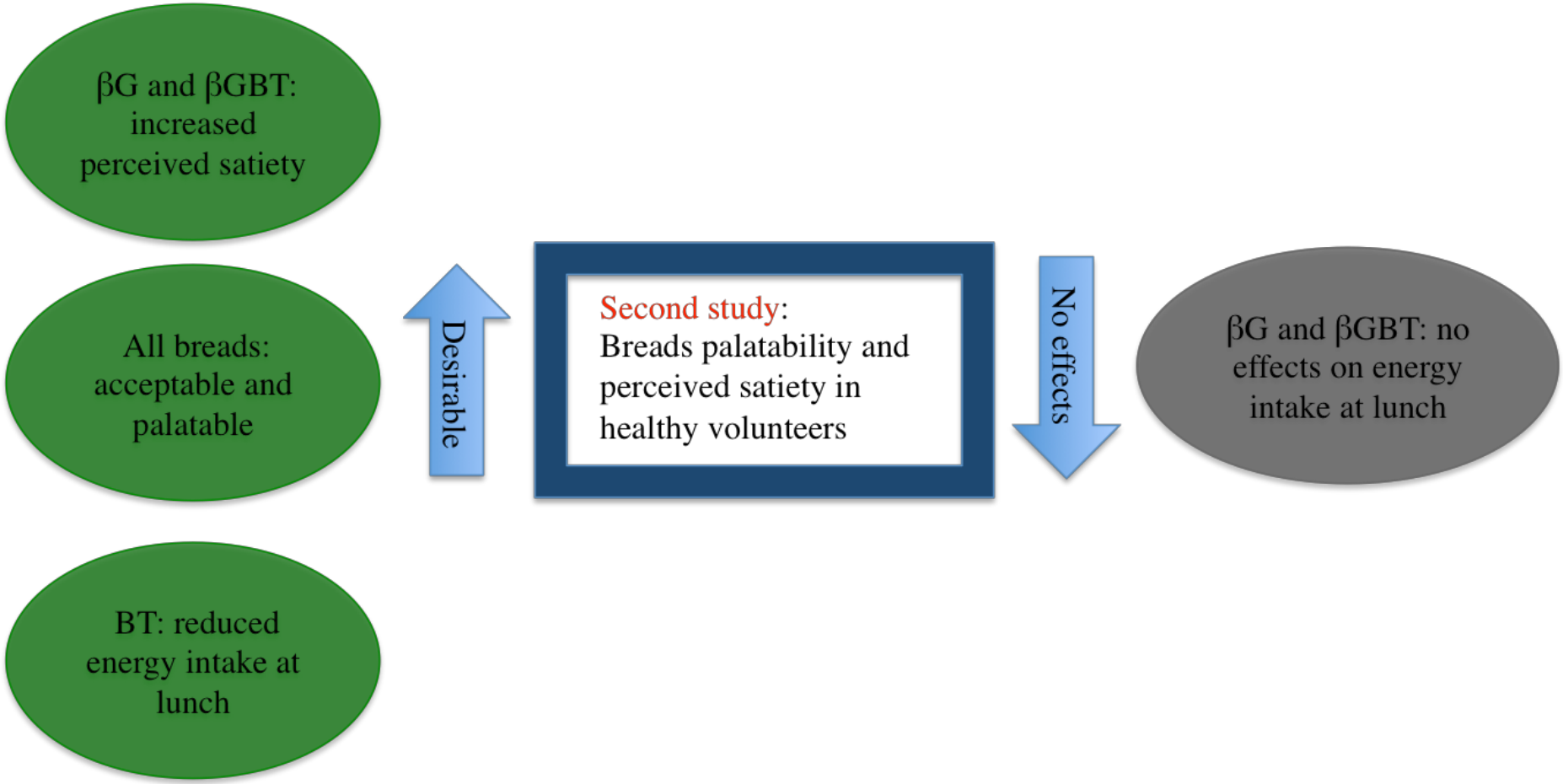


Figure 6-2. Results summary for Study 2 (breads palatability and satiety)

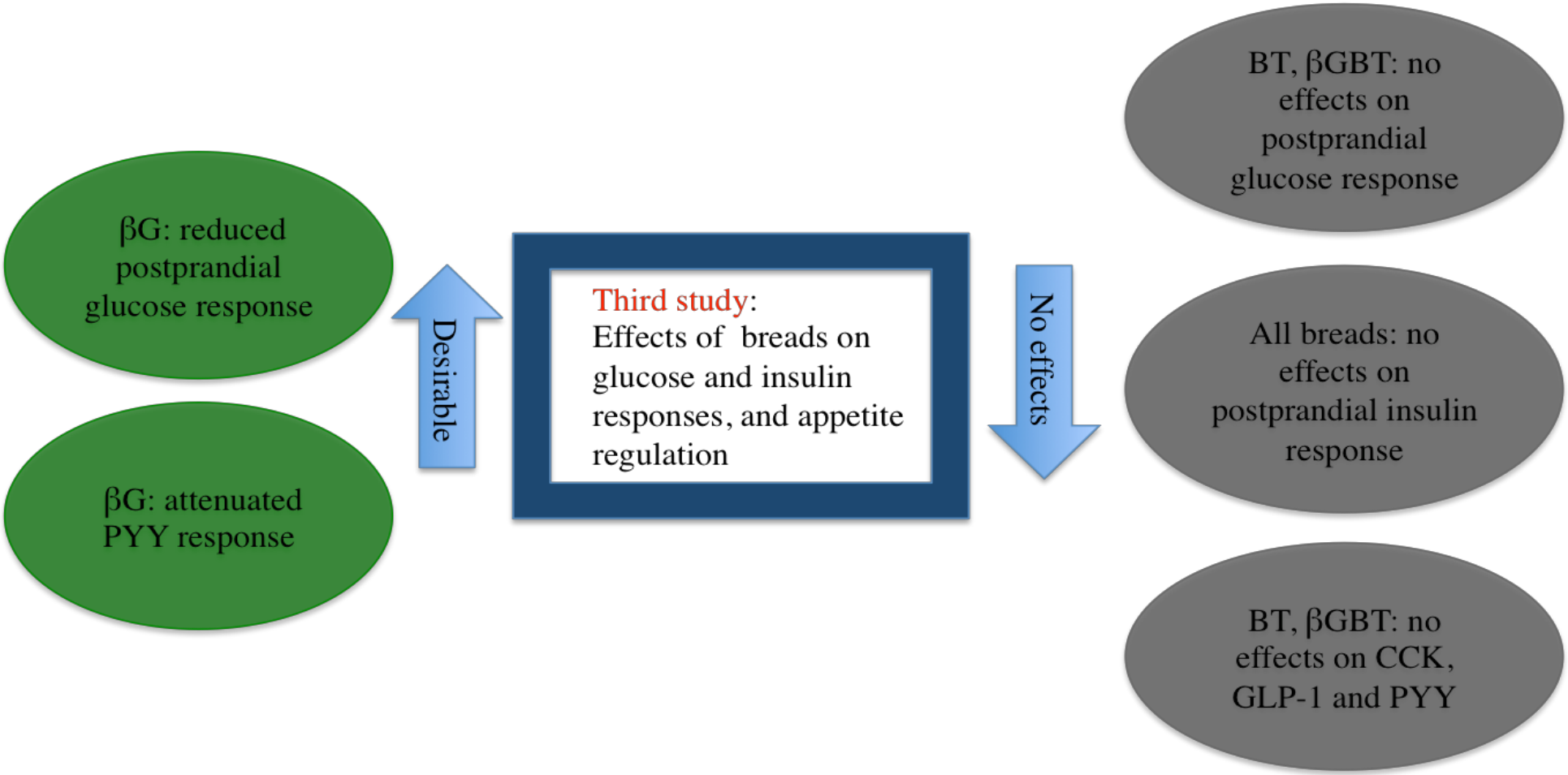


Figure 6-3. Results summary for Study 3 (effects of breads on glucose and insulin responses, and appetite hormones)

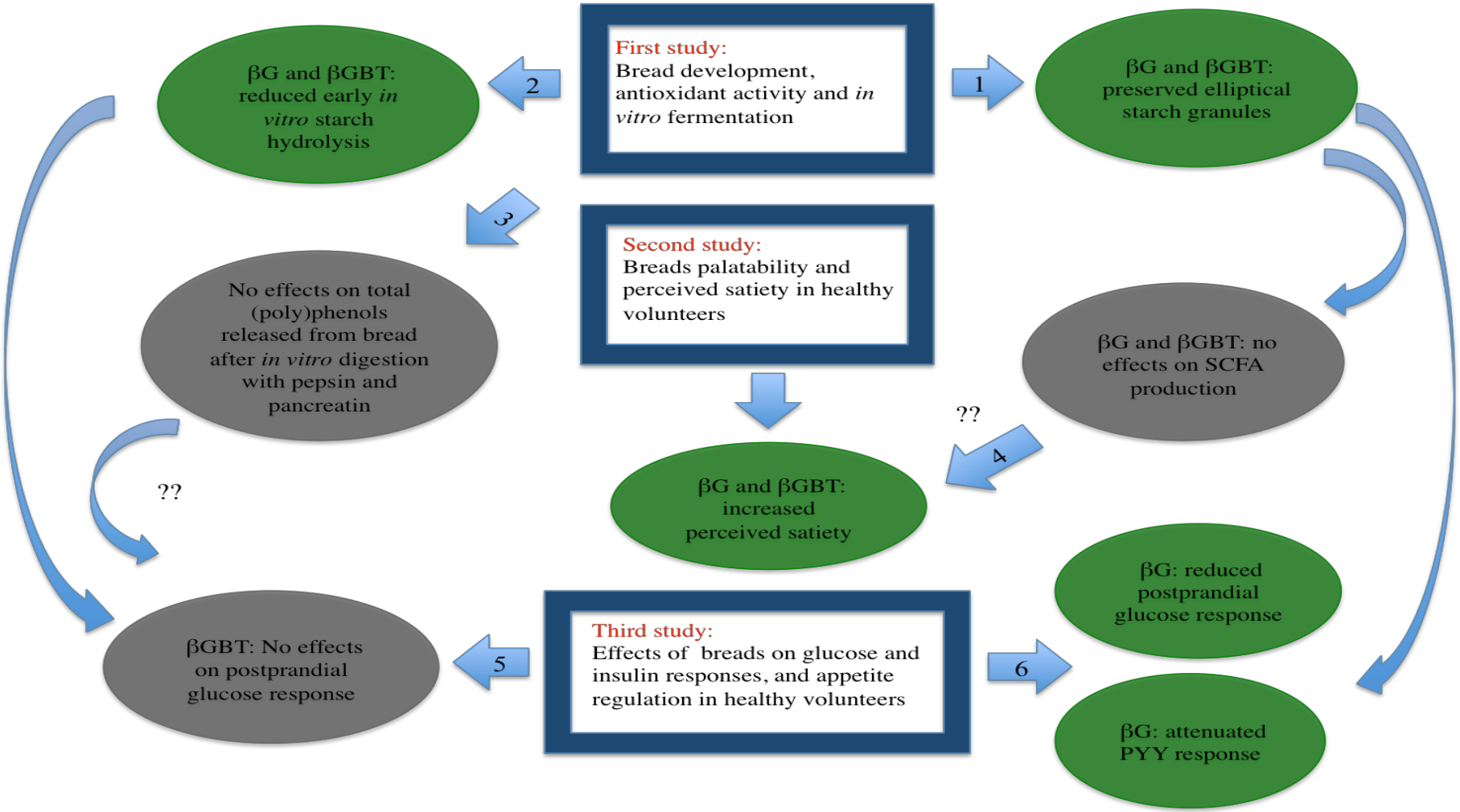


Figure 6-4. Relationship between *in vitro* and *in vivo* study and possible mechanistic explanations. Each number in the blue arrows is used for in-text explanation in **Section 6.3**. Green oval, significant effect; Grey oval, no significant effect.

6.2 Relationship between *in vitro* and *in vivo* studies, and possible explanations

The studies described in this thesis showed no relationship between *in vitro* starch hydrolysis and *in vivo* glucose response for βGBT bread in the human studies. Why is this?

In study 1, two functional ingredients (β-glucan and black tea) were selected based on their established health benefits. Breads prepared with β-glucan and/or black tea preserved the elliptical structure of some starch granules (**arrow 1**) (**Figure 6-4**). Digestion with α-amylase increased (poly)phenols released from black tea bread (**arrow 2**). These two factors might reduce *in vitro* starch hydrolysis and had the potential to have beneficial effects *in vivo*.

However, there was no effect of adding these two functional ingredients regarding the postprandial glucose response in humans (**arrow 5**). This could be explained by the following factors: firstly, the presence of (poly)phenols in black tea forms cross-links between the gluten network, and this led to a softer bread (Sivam et al, 2011). This softer bread may be more susceptible to the enzymic activity of α-glucosidase in the small intestine and resulted in increased postprandial glycaemia (Satoh et al., 2015). As discussed in **Section 1.8.3**, Koh et al. (2010) showed that black tea reduced *in vitro* α-glucosidase activity using a physiologically relevant dose of 0.56 mg/mL. Secondly, tea (poly)phenols reached their maximum plasma concentration between 0.8 to 2.3 h after intake of 300 - 500 mL green tea (Stalmach et al., 2009; Stalmach et al., 2010). This may have beneficial effects on plasma glucose, as the peak postprandial glucose response occurred at 30 min after an oral glucose tolerance test (Aldughpassi et al., 2008; Louie et al., 2008). However, as discussed in **Section 1.8.3** (**Table 1-12**), only one out of four studies showed that black tea reduced postprandial glucose response. The amount of black tea used in the present study was relatively small, with 30% (78 mg total polyphenols per bread portion) of that consumed from a standard cup of black tea (261 mg total polyphenols/250 mL) (Rothwell et al., 2012). Thirdly, the *in vitro* starch hydrolysis model is simplistic and does not completely mimic the physiological environment of the small intestine. Thus, the *in vitro* starch hydrolysis may overestimate the amount of starch hydrolysed during the incubation period.

As discussed above, the addition of black tea in bread led to a softer bread and may be more susceptible for enzymic activity in the small intestine. However, the addition of black

tea together with β-glucan in bread may have different physiological effects. Black tea contains a higher amount of high molecular weight theaflavins and thearubigins when compared with green tea. These (poly)phenols may not be absorbed in the small intestine and become a subject for colonic fermentation to phenolic acids. Although black tea is widely consumed in the UK, they are less extensively studied when compared with green tea. An intake of 700 mg of theaflavins (equivalents to 30 cups of black tea) showed a plasma concentration of 1 ng/mL (at 2 h) (Mulder et al., 2001). A low concentration of thearubigins in plasma may indicate that it may be a subject for fermentation in the colon, but this needs further investigation. Regarding humans, Roowi et al. showed that flavan-3-ol (monomer) from green tea being absorbed from the small intestine to the colon and degraded into phenolic acids by colonic microbiota (Roowi et al., 2010). An intake of two cups of green tea (500 mL) showed higher plasma concentration of flavan-3-ols in the range of 46 – 268 ng/mL (Lee et al., 1995).

Although little is known about the fate of black tea (poly)phenols, we hypothesised that the (poly)phenols-β-glucan complex may reach the colon, and would become a substrate for bacterial fermentation for the production of SCFA (**arrow 4**). A high propionate ratio is characteristic of β-glucan fermentation (Hughes et al., 2008). Propionate has been implicated as a signalling molecule for reduced food intake through stimulation of hepatic vagal afferents (as discussed in **Chapter 1**, **Section 1.6.3**), by activation of G protein-coupled receptors located in the colonic epithelium. Physiological concentrations of SCFA activate these receptors, although different receptors have different preference for each SCFA (Brown et al., 29003; Le Poul et al., 2003). These receptors are located in the colonic endocrine L-cells, and secrete the anorexigenic hormones peptide tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1). Hence, SCFA, particularly propionate may be involved in appetite regulation.

Alhabeeb et al. showed that 10 g/day inulin propionate ester (which releases large amounts on propionate in the colon) increased perceived satiety (increased satiety and fullness, decreased desire to eat), when compared with an inulin control (Alhabeeb et al., 2014). However, no change was observed for gastric emptying when determined using ^{13}C in expired breath, and this study suggests the effect of propionate on satiety might be centrally mediated by the action of PYY and GLP-1. Chambers et al. showed the same novel propionate ester (400 mmol/L) increased PYY and GLP-1 in primary cultured

human colonic cells (Chambers et al., 2015). Further investigation regarding humans (acute study) showed that 10 g/day of inulin-propionate ester reduced energy intake (14%) when compared with control (inulin). It was demonstrated that propionate entered the colon at 240 min, as determined by ^{13}C enrichment in expired breath air. PYY and GLP-1 significantly increased at 240 – 420 min in the treatment group when compared with control.

In our study, *in vitro* propionate production did not increase significantly after fermentation of βG and βGBT at 6 and 24 h for when compared with WB (**arrow 4**). A higher intervariation in the gut composition between donors could be one possible explanation. Carlson et al. showed 2-fold differences in SCFA production among individuals (Carlson et al., 2016). The addition of β-glucan decreased rather than increasing PYY levels (**arrow 6**). Comparisons between studies are difficult as each study used different types of dietary fibre, varying dosages and associated food matrix (as discussed in **Chapter 1**, **Table 1-9**). There were differences between food-matrix (solid *vs* liquid) on the observed outcomes. β-Glucan increased product viscosity when added in liquid food, but not when added to solid food such as bread and biscuits (Johansson et al., 2007; Åman et al., 2004). Verbeke et al. showed more propionate was produced from barley porridge and not from barley kernel (Verbeke et al., 2010). This could be due to the higher water holding capacity of oat porridge when compared with barley kernel. Oat porridge decreased intestinal transit time and allowed more time for bacterial fermentation and propionate production.

6.3 Reflections on methodology: strengths and limitations

First study: Effects of functional breads on *in vitro* starch hydrolysis, antioxidant activity and *in vitro* fermentation (**Chapter 3**).

Strengths:

Two well-known functional ingredients (β-glucan and black tea) were used for the development of functional bread. The *in vitro* study (**Chapter 3**) provided useful evidence for planning studies in **Chapter 4** and **5**. The reduction in early starch hydrolysis and the microscopy study suggested a food-matrix interaction when β-glucan and black tea were added in bread.

Bread development using a home bread maker was rapid and feasible for this study. The home bread maker allowed for the preparation of fresh bread, which in turn avoided a freeze-thaw cycle to be used for *in vivo* study. The freeze-thaw cycle affects starch retrogradation and this could have an effect on the measured outcome, particularly blood glucose response (Lan-Pidhainy et al., 2007).

The *in vitro* digestion model, mimicking human digestion (mouth, stomach and small intestine), allows for the removal of starch and protein. However, this procedure does not take into account the degree of absorption of metabolites in the intestine. The retentates produced after the dialysis (after removal of monosaccharides, disaccharides and low molecular weight polyphenols) were used for the determination of antioxidant activity and also substrate for the batch *in vitro* fermentation. The substrate used for this study mimics what would enter the colon in terms of β-glucan, (poly)phenols, undigested protein and resistant starch. Day-to-day variation of this *in vitro* digestion method was determined by measuring glucose released and total starch in the digestion residue (Aura et al., 1999).

Studying bacterial fermentation in humans is difficult because the proximal human colon where most fermentation occurs is not easily accessible. Hence, the *in vitro* batch fermentation technique offers a fast, rapid and reliable method for estimating bacterial fermentation in the colon. This method was validated in 8 laboratories, using 40 healthy volunteers from different geographical regions and with similar experiment environments (Edwards et al., 1996). It mimics the physiological aspects of the human colon as follows: 1) Fermentation bottle is placed in a shaking water bath at 50 strokes per minute to mimic peristalsis 2) Phosphate buffer is used to prepare faecal slurry and eliminating the requirement of the sample to be bubbled continuously with CO_2 3) A higher concentration of faecal slurry (16% w/v) is used which reduces the need for excess fermentation medium. However, this method is not without its limitations, which shall be discussed next. There is high inter-personal variation in the gut microbiota composition.

Limitations:

The miscoscopy study of the bread structures (starch and gluten) was based on staining method and visualised under light microscope and hence could not ascertain the changes at supramolecular level. Other technique such as scanning electron microscope could possibly show changes at supramolecular level. The *in vitro* digestion might lead to the

loss of (poly)phenols at higher intestinal pH, but this loss could be prevented if the (poly)phenols are absorbed in the small intestine. There were high inter-individual variations in the *in vitro* batch fermentation that could reduce the power of the study. A post-hoc calculation was done using G*Power software. Based on sample size of 4 volunteers (faecal donors) and propionate production from an *in vitro* fermentation as an outcome, the present study achieved a power of 54%. To achieve a power of 80%, 4 more volunteers are required to detect any differences.

The enzymes used during the digestion procedures could give a false positive result to the total (poly)phenol content, as macromolecules (e.g. protein, fat) are known as interference factors for the Folin-Ciocalteu reaction during total (poly)phenol analysis (Saura-Calixto and Diaz-Rubio, 2007). The *in vitro* batch fermentation is a validated method mimicking most physiological conditions. However, this method does not mimic the rate of SCFA absorption in the colon and hence may overestimate the concentration of SCFA in the fermentation bottle. The freeze-drying technique used prior to *in vitro* fermentation may have increase or decrease the retrograded starch in the retentate and this should be considered in future study.

Second study: Effects of functional breads on palatability and satiety (**Chapter 4**).

Strengths:

This chapter provided useful data on the bread specific characteristics (texture, aroma, palatability, smell), and these breads were relatively acceptable compared with white bread. The satiety study was conducted without venous cannulation and hence more similar to the actual eating environment. The *ad libitum* lunch administered allowed for freedom of food choice when compared to a monotonous lunch (single food item), and could also avoid the boredom of eating the same food for every study trial.

Limitations:

The breads used in this study differed in texture, colour and aroma. This was blinded during the trial, but subjects could still make a sensible guess of which bread is which and hence potentially reduce the power of this study. The types of foods presented during the *ad libitum* lunch were not appetising for some subjects and they might not eat well during the session. Some subjects might overeat during the lunch session due to the large amount

of food available on the table. However, the foods were cut into smaller portions in order to eliminate portion related cues (Fatima et al., 2015). The eating environment (in the food testing lab) was artificial and may not represent the normal eating environment for subjects; this may provide different cues for the eating behaviour of subjects. However, the lab was equipped with a dining table and chairs which were placed facing the window in order to avoid feelings of boredom. Subjects were allowed to use their laptop, read or do their own work, but were advised not to search online or read about anything relating to food. In addition, subjects were advised to avoid strenuous physical activity before the trial day, as this would deplete their glycogen (liver and muscle) content. Glycogen has a storage capacity of 12 – 48 h and this will switch to oxidative metabolism of fats and ketones (Lean, 2015).

The adaptive visual analogue scale (AVAS) scale used in this study has a limitation. AVAS scoring does not always correlated with objective measure of energy intake later during a second meal. However, for pragmatic reason, AVAS has been recommended as a standard set of basic scales for self-assessment of perceived satiety measurement in healthy adults (Marsh-Richard et al., 2009).

Third study: Effects of functional breads glucose and insulin responses, and appetite hormones (**Chapter 5**).

Strengths:

This study had a similar study design as that in **Chapter 4** but was conducted on different subjects. It was a cross-over study trial and hence could be more powerful in reducing intervariation between individuals. This study was powered (n = 16 based on plasma glucose levels as the main outcome) and should be able to detect changes in blood glucose levels (Vitaglione et al., 2009).

Previous studies investigated satiety and biochemical aspects together but in this study the two were separated for the following reasons:

1) Eating while being cannulated is not a normal eating condition.
2) Subjects might feel hungrier and eat less due to feeling uncomfortable with the cannulation.

Subjects were also advised to keep their diet low in (poly)phenols for two days before the study visit. This meant avoiding fruits and vegetables, tea or coffee which can be difficult for many to achieve, and may have affected their eating behaviour during the trial. This should eliminate any (poly)phenols metabolites in the circulation that would interfere with the studied outcomes. No side effects or abdominal discomforts were reported during the trial. This functional food is generally safe and feasible for general consumption.

Limitations:

Body mass index of the subjects were in the range of 17 to 30 kg/m^2, which may be a contributing factor for higher intervariations between subjects. The postprandial was measured for up to 3 h, and this time was too short for any colonic effects (SCFA production). Measurements of plasma insulin and gut hormones (CCK, PYY and GLP-1) are based on ELISA principle and were sensitive to storage and analysis. However, the samples were stored in -80°C immediately after being separated from the plasma before analysis. The samples were analysed batch-wise within 3 months to reduce the variations. The method used for glucose analysis (Reflotron® analyser) is not the most reliable and accurate for the determination of glucose concentration. This could lead to false positive or false negative results. A post-hoc calculation was done using G*Power software. Based on the sample size of 15 volunteers and $iAUC_{0\text{-}180\ min}$ glucose as an outcome, the present study achieved a power of 76%. To achieve a power of 80%, two more volunteers are required in order to detect any differences.

6.4 Implications for food industry

As there is growing consumer interest in the relationship between food and health, the commercialisation of these breads is an area of interest within and outside of academia. However, it is easy to take advantage of the broad definition of functional food and launch products that do not actually have any health benefits but are marketed based on insufficient scientific background or empty promises. There are different types of breads available in the market prepared with whole fibres such as flaxseed, cracked wheat, poppy seeds, linseed, kibbled buckwheat and buckwheat kernels. These products might have additional health benefits but none of them were approved by EFSA. Hence, this poses a huge challenge for both consumers and the food industries to promote healthy eating. An independent market survey by Euromonitor International showed that customers in the UK

are not ready yet for the healthy options. Consumers are more prepared for bread such as Hovis' 'best of both' that is white bread with added wholemeal (Euromonitor, 2004). This bread is appealing to both children (it tastes like white bread) and parents (it contains more fibre).

The breads were produced with a home bread maker, which is suitable for small-scale use. The cost of producing this bread is similar to that of other instant pre-mix bread flour available in the market, such as ASDA's white bread and whole meal bread mix flour and Tesco's white, multiseed and wholemeal bread mix flour. The price range of these products is from 0.80 to 1.50£ per packet (500 g flour mix for one bread). Based on the labels, these breads do not contain any preservatives and are suitable for traditional hand baking or using a bread maker. The use of fresh bread means the avoidance of preservatives, such as calcium propionate as antifungal agent (Belz et al., 2012). Scaling-up the baking technique by the food industry means that they must add calcium propionate in order to increase the shelf life for storage and transportation before they reach customers. In a large-scale production, calcium propionate increases an average shelf-life between 10-12 days. However, the addition of preservative might increase the production cost of the bread. Moreover, it is still unknown whether the addition of calcium propionate might have an impact on the taste of these breads. However, these bread mix recipes might contain flour treatment agents such as α-amylase, amyloglucosidase, glucose oxidase and hemicellulase. α-Amylase breaks down amylose into smaller sugar unit maltose, maltotriose and α-limit dextrans and also debranching some of the amylopectin chains. This process allows additional sugars for yeast fermentation (Bakerpedia, 2016a). Glucose oxidase functions by oxidizing glucose to gluconic acid and hydrogen peroxide. Hydrogen peroxide strengthens the disulphide and non-disulphide cross-links in the gluten network and hence improving dough development (Bakerpedia, 2016b). In regards to large-scale production, the food industry needs to focus on preserving the functionality and at the same time maintaining the palatability of this bread.

6.5 Implications for healthy eating at the population level

Bread is the main source of starchy food in the UK (SACN, 2015). These breads contain 419 – 423 kcal and correspond to 21% of the total daily energy requirement for adults. Hence, this bread could be considered as one of the options for healthy food choice. Acute intake of these breads might be useful in maintaining desirable blood glucose levels

without increasing insulin responses. This study confirms the positive health benefits of 4 g β-glucan per 30 g of available carbohydrate for maintaining desirable blood glucose, as claimed by EFSA. This was an acute effect, and long-term study (chronic consumption) is needed to determine whether there exist any developmental changes in the gut microbiota. Chronic consumption of this bread will change the gut microbiota composition and produce higher SCFA, particularly propionate. Moreover, consuming this bread along with the increased intake of fruits and vegetables could help consumers achieve the minimum dietary fibre intake of 30 g/day (British Nutrition Foundation, 2016b).

Commercial food stores such as Tesco, ASDA and Sainsbury's have a range of sandwiches prepared with white, multiseeds or whole meal bread, priced between £1.50 to £2.50. These sandwiches are supplied on a daily basis and are usually placed on the cold shelf along with other 'food-on-the-go' items (e.g. ready-to-eat instant rice, pasta and chicken). This might specifically target those who have a limited time in which to prepare their own meals (e.g. university students, working people). Substitution of these 'traditional' breads with β-glucan bread could also be an alternative for the preparation of healthy sandwiches. However, the price of these sandwiches may not be affordable in deprived communities. Another alternative could be the development of lower cost products, such cereal with black tea and β-glucan.

6.6 Future research

There are several areas that can be further developed, based on the evidence from the present study as follows:

Food science:

1) Studying the rheological properties of bread dough as this will provide useful information on the food-matrix interaction in bread dough and the final product.
2) How to study (poly)phenol stability under simulated *in vitro* digestion. This could be achieved, for example, through microencapsulation of tea (poly)phenols with dextrin before being added in bread.
3) Some consumers like the bread to be toasted and this process might have an impact of the starch retrogradation. Simplistically, starch retrogradation is the formation of ordered amylose and amylopectin chains structure after cooling of heated starch (in a presence of water) (Wang et al., 2016). Higher starch retrogradation was

associated with lower glucose response in healthy volunteers (Burton et al., 2008). Moreover, storage conditions such as freezing (between 2 to 7 days) and thawing (overnight at room temperature) might increase starch retrogradation.

4) Breads can be developed using a combination of other soluble fibres, such as guar gum (highly viscous) and gum Arabic (non-viscous) with black or green tea.
   Determine the food-matrix interactions between β-glucan and black tea with starch and protein (gluten) in bread.

Nutrition research:

1) Study the physico-chemical properties after an *in vitro* digestion, such as solubility, degree/rate of fermentation and viscosity. This could provide an insight of how these breads behave in the gastrointestinal tract.
2) Measuring the energy intake (satiety study) in the afternoon in a free-living setting for up to 8 h after breakfast, which will reflect more natural eating behaviour.
3) Long-term study is needed to confirm whether there is an effect of consuming this bread on a daily basis on blood glucose, insulin, lipid profiles, and appetite hormones.
4) Long-term study is needed to confirm that these breads are resistant in the upper gastrointestinal tract, being fermented in the colon and stimulate the growth and/or activity of beneficial bacteria (eg. *lactobacillus* and *bifidobacteria*) (prebiotic effects).
5) Determine the effects of eating β-glucan bread with a cup of black tea on glucose, insulin, gut hormones and energy intake.
6) Study the (poly)phenols released from the breads in plasma and urine, particularly thearubigins and theaflavins.
7) Study the health benefits of interactions between (poly)phenols metabolites produced from black tea and SCFA produced from β-glucan.

6.7 Conclusions

There were structural differences in bread with and without β-glucan. Bread with β-glucan could preserve some of the 'native' starch granules, and formed more continuous gluten matrix when compared with white bread. Adding β-glucan alone beneficially reduced *in*

vitro starch hydrolysis and postprandial glucose response, without negatively affecting insulin response. These results provided further evidence for the health benefits of β-glucan claimed by EFSA.

However, adding black tea with β-glucan reduced early *in vitro* starch hydrolysis but this effect disappeared when tested in humans. The absence of 'absorption' capacity and also the simplicity of 'complex nutrients-enzyme' reaction in *in vitro* starch hydrolysis models may have affected this. Hence, further study is needed to consider the results obtained from *in vitro* study carefully before embarking on *in vivo* study.

Predigestion with α-amylase released (poly)phenols from BT and βGBT breads. However, digestion with pepsin and α-pancreatic amylase had no effects on antioxidant activity. βG and βGBT showed higher acetate, propionate and total SCFA (acetate, propionate and butyrate) at 24 h, but did not change significantly when compared with WB. These results suggest there is no additional effect of black tea *per se* or in combination with β-glucan on antioxidant activity and SCFA production.

βG and βGBT breads showed lower taste, texture and palatability when compared with WB and BT. However, the breads showed good overall palatability when compared with white bread. WB and BT were similar in terms of palatability characteristics. Eating BT and not βG or βGBT as a breakfast meal reduced energy intake at lunch when compared with white bread.

Overall, each of the studied breads possess both strengths and weaknesses, as follows:

1. White bread (WB)

Strong point: Good overall palatability.

Weak point: Increased *in vitro* starch hydrolysis, increased perceived satiety.

2. Black tea bread (BT)

Strong point: Good overall palatability, reduced energy intake at lunch.

Weak point: Increased *in vitro* starch hydrolysis.

3. β-Glucan bread (βG)

Strong point: Good overall palatability, reduced *in vitro* starch hydrolysis, reduced postprandial glucose response, reduced PYY.

Weak point: Reduced taste, texture and palatability.

4. β-Glucan plus black tea bread (βGBT)

Strong point: Good overall palatability, reduced *in vitro* starch hydrolysis.

Weak point: Reduced taste, texture and palatability.

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