Understanding the intrinsic and extrinsic properties of a *starch branching enzyme II* wheat mutant and their impact on glucose response

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Abstract

Wheat is a major source of dietary carbohydrate but the starch in many wheat-based products is rapidly digested, causing a rapid increase in blood glucose levels after consumption. Previous research has developed a novel type of wheat carrying mutations in *starch branching enzyme II a* and *b* (*sbeII*) genes, characterised by altered starch structure, thought to be less susceptible to amylolysis. The *sbeII* wheat is known to have a higher amylose content than conventional wheat, but its processing performance, starch digestibility and glycaemic power have not yet been studied in detail.

The aims of this PhD project were to examine the intrinsic and extrinsic properties of starch from a *sbeII* wheat, to investigate its susceptibility to starch amylolysis *in vitro*, and explore its potential use to lower glycaemic response to wheat-based foods *in vivo.*

A series of *in vitro* studies were undertaken to characterise the starch and non-starch polysaccharide components of *sbeII* wheat and to determine the effects of hydrothermal processing on *sbeII* starch susceptibility to hydrolysis. These studies demonstrated that starch retrogradation is a key mechanism limiting starch amylolysis in foods made with *sbeII* wheat.

Based on this knowledge, two test foods (semolina pudding and bread) were then developed using *sbeII* wheat to measure the glycaemic impact of *sbeII* foods *in vivo*. Their effect on postprandial glycaemic response was determined in two double-blind cross-over intervention studies. Evidence from these *in vivo* studies suggested that *sbeII* bread can elicit a lower glycaemic response compared to the control bread, however, due to the relatively small size of the study cohort, the extent of the decrease in glycaemic response may need to be further investigated. Overall, these studies have advanced current understanding of *sbeII* wheat starch and its potential to improve glycaemic properties of wheat-based foods.

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"Digestibility of processed durum wheat with increased resistant starch"

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"Starch resistance to digestion in sbeIIa/b wheat bread: estimating the effect of high-amylose bread on glycaemic response"

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Oral presentations

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Publications

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Abbreviations

AACC	American Association of Cereals and Grains
ADP	Adenosine Diphosphate
AGP	Arabinogalactan
AGPase	ADP-glucose pyrophosphorylase
AMG	Amyloglucosidase
AOAC	Association of Official Agricultural Chemists
AX	Arabinoxylans
AXOS	Arabinoxylan Oligosaccharides
BBSRC	Biotechnology and Biological Sciences Research Council
BMI	Body Mass Index
CCAT	Cereals and cereal applications testing
CGM	Continuous Glucose Monitoring
CI	Confidence Interval
CIELAB	Device-independent colour space L* a* b*
CONSORT	Consolidated Standards of Reporting Trials
CRF	Clinical Research Facility
CV	Coefficient of Variation
DBE	Debranching Enzyme
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl sulfoxide
DP	Degree of Polymerisation
DSC	Differential Scanning Calorimetry
DWB	Dry Weight Basis
EFSA	European Food Safety Authority
EGC	Eluent Generator Cartridge
EHT	Electron High Tension
EMS	Ethyl Methanesulfonate
FDA	Food and Drug Administration
FP	Finger-prick
GBSSI	Granule-bound Starch Synthase I
GI	Glycaemic Index
GIPIRS	Glycaemic Index Pudding with Increased Resistant Starch
GLUT2	Glucose Transporter 2

GOD	Glucose Oxidase
GOPOD	Glucose Oxidase/Peroxidase
GOS	Mixed-linked β-glucan oligosaccharides
HI	Hydrolysis Index
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
iAUC	incremental Area Under the Curve
ICC	International Association for Cereal Science and Technology
IF	Interstitial Fluid
IRAS	Integrated Research Application System
ISA1	Isoamylase I
ISA2	Isoamylase II
IU	International Units
JAR	Just-About-Right
LOS	Logarithm of Slope
LS	Laser Diffraction
MGAM	Maltase-glucoamylase
MLG	Mixed-linkage β-glucan
NHS	National Health System
NIHR	National Institute for Health Research
NNUH,	Norfolk and Norwich University Hospital
NSP	Non-starch Polysaccharide
PAHBAH	p-hydroxybenzoic acid hydrazide
PIDS	Polarization Intensity Differential Scattering (PIDS),
PVP	Polyvinylpyrrolidone
QIB	Quadram Institute Bioscience
REC	Research Ethics Committee
REST	Resistant Starch study
RS	Resistant starch
RS4	Resistant Starch type 4
SBE	Starch Branching Enzyme
SBEI	Starch Branching Enzyme I
SBEII	Starch Branching Enzyme II
SCFA	Short Chain Fatty Acids
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate

SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SEM	Standard Error of the Mean
SGLT1	Sodium-glucose cotransporter 1
SI	Sucrase-isomaltase
SIV	Starch Synthase IV
SS	Starch Synthase
SSI	Starch Synthase I
SSII	Starch Synthase II
SSIII	Starch Synthase III
TA-XT2	Texture Analyser
TES	Techniques used/ Equipment/ Standard specifications
TFA	Trifluoracetic acid
TGW	Thousand Grain Weight
TILLING	Targeting Induced Local Lesions in Genomes
TOT	Total
TPA	Texture Profile Analysis
TS	Total Starch
UKAS	UK's National Accreditation Body
UKRI	UK Research and Innovation
VAS	Visual Analogue Scale
WE	Water-extractable
WT	Wild-type

Symbols

C_∞	Product concentration or amount of starch digested at end of digestion
C_t	Product concentration or amount of starch digested at time, t.
D	Dimensions
G0, G1, ... Gn	Blood glucose concentrations (subscript numbers indicate the time interval)
n	Number
R^2	Coefficient of determination
t	Time
W	Kendall concordance coefficient
Y_0	Endogenous sugar present at the start of the reaction
λ	Wavelength

Chapter 1 Introduction and literature review

1.1 Introduction and project overview

Large intakes of available carbohydrates, starch in particular, can represent a metabolic challenge for the body as these are readily digested to release large amounts of glucose into the systemic circulation. It is generally accepted that reducing the daily intake of available carbohydrates (carbohydrates that are easily digested) or favouring fibre-rich foods can have beneficial effects on health. Yet, most of the wheat-based foods consumed are made from finely milled wheat grains containing mostly available starch.

Wheat is one of the main staple crops and a major source of carbohydrates worldwide. In 2019, the United Kingdom produced approximately 16 million tonnes of wheat;[1] about half of the total production is used to make white flour (45%).[2] About 70% of the dry weight of the mature wheat grain is made of starch, proteins, and cell wall polysaccharides, encapsulated within the bran, germ and endosperm.[3] Like other staples, wheat retains most of these structural components of the intact grain when minimally processed, however, wheat is usually consumed after several processing steps. The processing, which includes milling and cooking processes, aims at improving palatability and nutrient availability. When milling to white flour, the starchy endosperm is the main component being recovered, of which about 80% is starch, 10% protein with low amounts of cell wall components, minerals, and phytochemicals.[4]

Consuming wheat-based foods made from refined white flour is usually associated with larger glycaemic responses, as large intakes of available starch can be readily digested in the small intestine. Postprandial glycaemia plays an important role in the aetiology of common chronic diseases such as non-insulin dependent (type 2) diabetes and cardiovascular diseases. Over time, high glycaemic loads and a poorly controlled postprandial glycaemia may lead to the dysregulation of metabolic processes, increasing the risk of developing type 2 diabetes.[5] Postprandial hyperglycaemia is also a direct risk factor for the development of cardiovascular disease; prevention and management of CVD in diabetes is often focused on attenuating postprandial glycaemia.[6]

The current intake of carbohydrates for the UK adult population (19-64 years of age, men and women) is approximately 45% of the daily energy intake.[7] According to the National

Diet and Nutrition survey, between 2016 and 2019 white bread consumption contributed to approximately 10% of the daily intake of carbohydrate of which, most of the dry weight is starch.

The UK Dietary Reference Values reports a recommended intake of carbohydrates of 50% of the total energy intake, with no more than 5% from free sugars.[8, 9] Here it is important to distinguish between available carbohydrate that are easily digested in the upper gastrointestinal tract and non-starch polysaccharides that are fermentable in the larger intestine (fibre). The physiological impact of foods on human health is usually estimated based on their chemical composition. This approach does not take into account the structure of foods and the physico-chemical properties of complex macromolecules such as carbohydrates, that play a major role in nutrient digestion, microbial fermentation, metabolisable energy and postprandial metabolism.[10] Foods with similar nutritional value can elicit very different metabolic responses and different effects on health. This is particularly important for starch-based foods; it is well known that foods containing the same amount of starch can produce variable postprandial glycaemic responses, determined by the rate and extent of starch digestion.[5] Therefore it is not sufficient to quantify only the starch content of foods to predict their physiological effect.

One important factor, which is the main topic of this PhD project, is that starch structure and characteristics determine the extent of starch digestibility and sugars released during digestion. For instance, increasing the amylose content of starch (one of the two forms of starch) is thought to result in starch with lower susceptibility to enzymatic hydrolysis. In cereals, this has been extensively investigated using both chemical and biological approaches to obtain high-amylose crops. Among others, the use of a non-transgenic technology to induce genetic mutations, known as TILLING, allowed the generation of wheat carrying mutations in enzymes involved in starch synthesis to manipulate starch structure. The *starch-branching enzyme II* (*sbeII*) wheat mutants generated by TILLING were characterised by starch with increased amylose content and increased resistance to enzymatic digestion, compared to conventional wheat starch.[11, 12] However, while structural changes can be measured in raw starch, it is not fully understood how such changes translate when *sbeII* starch is processed into foods and whether altering the starch susceptibility to hydrolysis is sufficient to modulate glycaemia. Therefore, this project was designed to gain understanding of the role of *sbeII* starch characteristics on its susceptibility

to hydrolysis and their potential to reduce postprandial glycaemia. As starch-based foods are consumed after processing, the processing performance of *sbeII* wheat was evaluated. To this aim, the structure, properties, and susceptibility to hydrolysis of starch were measured *in vitro* on foods made from *sbeII* durum and bread wheats. Different types of processing were tested to investigate their influence on starch characteristics. Foods with potential to attenuate glycaemia were used in *in vivo* studies: initially, a pilot study was used to determine glycaemic index of *sbeII* pudding and study the feasibility of pudding as test food in larger intervention studies. Then, a larger intervention study was used to determine the effect of *sbeII* starch in bread on post-prandial glycaemia. The *sbeII* wheat carbohydrate characteristics were compared to those of a wild-type control representing conventional wheat and the properties of *sbeII* wheat contributing to the effects observed are discussed.

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1.1.1 Thesis outline

Chapter 1 introduces the research context. Current knowledge and research gaps are highlighted through a review of the relevant literature. These are reflected in the project aims and objectives, stated at the end of this Chapter.

Chapter 2 provides an overview of the origin and preparation of the wheat materials and foods, and the methodologies used in this project.

Chapter 3 reports the intrinsic characteristics of *sbeII* wheat that underpin the results reported in the following chapters.

Chapter 4 reports the extrinsic characteristics of *sbeII* starch as a function of processing. These studies were designed to determine *sbeII* starch performance through processing and to explore the role of processing on starch susceptibility to hydrolysis.

Chapter 5 reports the results of an *in vivo* feasibility study to measure glycaemic response to *sbeII* foods. This chapter includes measurements of the excursions in peripheral venous blood glucose concentrations in response to a *sbeII* food (pudding) and a WT control, expressed relative to a suitable control such as glucose (glycaemic index).

Chapter 6 reports the results of an *in vivo* glycaemic response study carried out to investigate the role of *sbeII* starch in bread in modulating post-prandial glycaemia. This is one of the first studies to measure post-prandial glycaemic responses in interstitial glucose using a continuous glucose monitoring system.

Chapter 7 reports the general conclusions from the various studies conducted as part of this PhD project providing scope for future work.

1.2 Literature review

1.2.1 The nutritional role of wheat starch

Starch is an important dietary carbohydrate with a complex structure and a major component of cereal-based staple foods. It is mostly consumed in a cooked form to enhance its palatability or used to add structure and texture to foods. With cooking, starch complex structure is altered becoming more accessible to digestive enzymes. Once cooked, large intakes of available starch can have a great impact on postprandial glycaemia, as the large quantities of glucose released during starch digestion alter the metabolic steady state (section 1.2.7.1). Starch impact on postprandial glucose homeostasis and the related metabolic responses depend on the rate of starch digestion and absorption in the small intestine (section 1.2.7.2). Starchy foods, including many cereal products such as wheat bread, are known to elicit large glycaemic responses that are frequently associated with an increased risk of developing type 2 diabetes and other related cardiovascular complications.[13] However, a small fraction of the starch identified as resistant starch (RS) can escape digestion in the small intestine to reach the colon where it can be fermented by the microbiota, similarly to dietary fibre components. RS content is variable in commonly consumed foods depending on the starch botanical origin and the processing used. Starch's resistance to digestion depends on a number of factors; it is not always possible to predict a starch-based food metabolic effect solely from the starch chemical composition (section 1.2.11). Most research categorises carbohydrates, including starch, according to their chemical composition and expected properties, however, the chemical composition alone may not reflect fully their properties and functionality, especially when found within a food matrix.

This thesis is focussed on the factors influencing starch resistance to digestion, including its structural and physico-chemical characteristics when raw and isolated and when processed within a food matrix. These characteristics are of great nutritional importance when evaluating post-prandial glycaemic response to starch-based foods.

1.2.2 Starch

1.2.2.1 Structural hierarchy of wheat starch

Starch is a ubiquitous storage polysaccharide in wheat and the main source of available carbohydrates from wheat-based foods. It is synthesised and stored in the form of granules

within the plastids of cells in the leaves, seeds and vegetative storage organs of plants.[14] The focus of this review is starch from wheat grains, its synthesis, its composition and the physico-chemical properties that determine starch behaviour during processing and its digestibility within a food matrix.

In wheat, sucrose produced by photosynthesis in the leaves is transported to the developing grain where it is converted to starch, to serve as energy reserve for the seed germination. Wheat grains consist of an embryo (germ), rich in lipids, minerals and antioxidants, which is surrounded by the endosperm tissue, where starch is stored. This is coated by several outer layers of fibrous material (Figure 1.1). These three components have a distinct nutritional composition and role within the grain. The endosperm is predominantly made of starch granules stored in packed cells of variable size and shape, depending on their location; starch represents around 83–84% of the dry tissue weight at maturity and it is the main component of refined flour obtained by milling wheat grains.[2] The endosperm tissue contains also protein and dietary fibre (cell walls) that provide mechanical strength and maintain the structural integrity of the tissue. Depending on the botanical origin, starch characteristics can be very diverse in terms of granule size (0.1–100 µm in diameter), shape (round, lenticular, polygonal), size distribution (uni- or bi-modal) and composition (amylose, amylopectin, branching frequency and length); granules can be found as individual items (simple) or associated into clusters (compound).[15, 16] In the endosperm of wheat grains, starch granules have a bimodal distribution; lenticular A-type granules are typically between 15–35 µm in diameter whereas smaller, spherically shaped, B-type granules are between 2-10 µm. The two main granule populations are also thought to have different chemical composition and properties, with B granules containing a lower proportion of linear glucan chains (amylose) than A granules. The different size and composition of starch granules is of interest as these could directly affect the starch susceptibility to hydrolysis, discussed later in section 1.2.8.

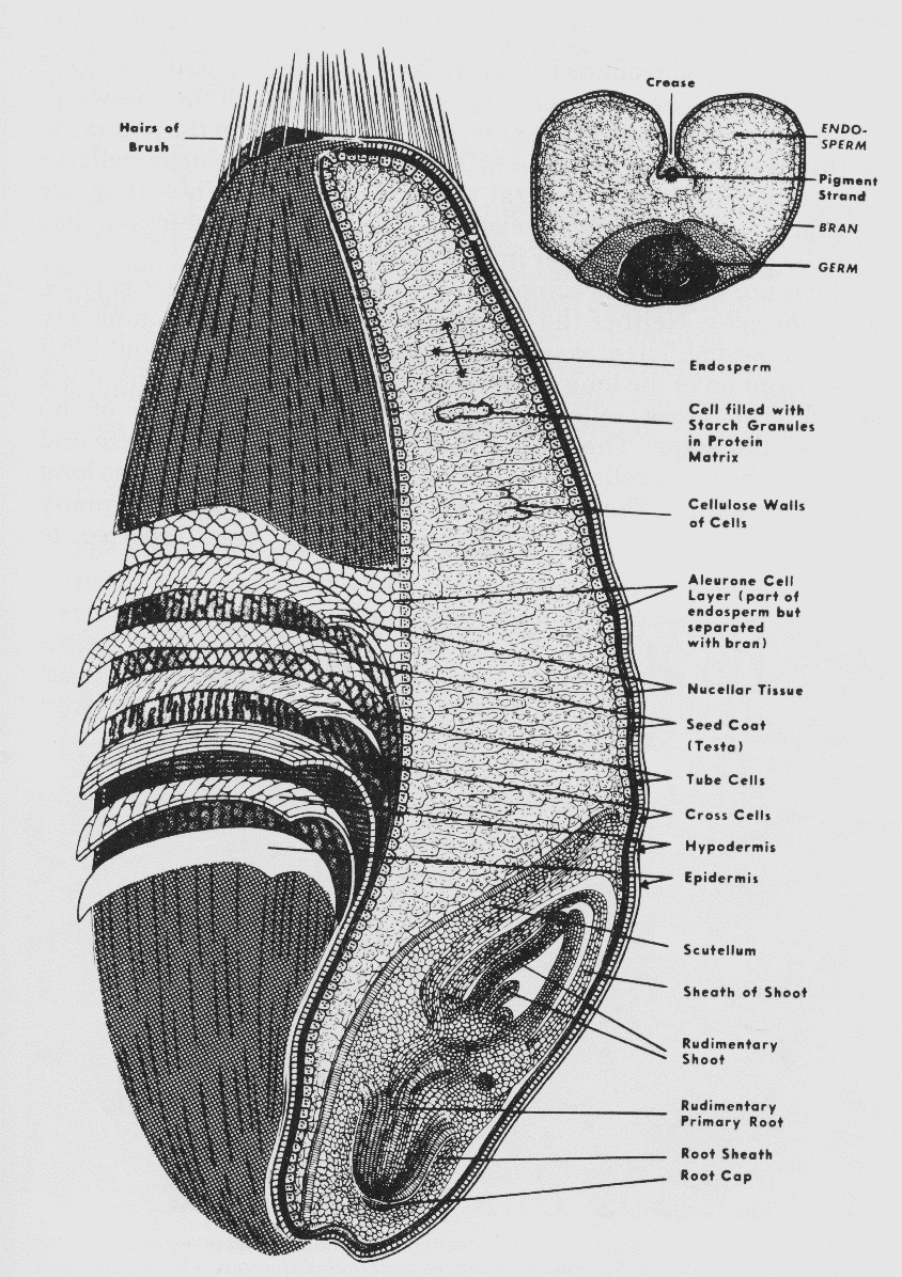


***Figure 1.1 Wheat grain illustration from Hoseney 1992[17]**, showing the bran, the germ and endosperm in longitudinal and transversal sections.*

1.2.2.2 Starch composition and structure

Starch is an insoluble glucan composed of two polymers of glucose, amylopectin and amylose. Its molecular structure determines the physico-chemical properties of starch when processed into a food, its accessibility to digestive enzymes and consequently, the rate and extent to which sugars are release from starch glucan chains and absorbed into the blood stream in the body.

The physico-chemical properties of wheat starch vary depending on the hydrothermal processing applied. The following description applies to native (raw) starch from wheat,

unless otherwise specified. The changes that can occur to starch during processing are described in section 1.2.5.

Amylopectin is the main component of starch, accounting for approximately 70%-80% of the starch weight. It is a large, branched molecule made of α-1,4-linked glucose chains between 6 and >100 glucosyl residues long. About 6% of the glucosyl residues carry α-1,6-bonds that form branch points distributed at regular intervals along the axis of the molecule.[18] Adjacent chains of about 12-15 glucosyl residues can pack together in ordered arrays or clusters to form crystalline lamellae. These alternate with amorphous lamellae, containing the branch point, with a periodicity of 7-10 nm to form semi-crystalline granules (Figure 1.2a).[19] Starches with frequent longer branches such as potato starch (>22 residues) are usually characterised by an open structure, which is less crystalline and has a higher capacity to absorb water.

Amylose is a minor component of starch, ranging between 20% and 30% depending on the starch botanical origin. Its chemical composition also varies depending on the plant species. In wheat, amylose consists of long chains of approximately 1000 α-1,4-linked glucose residues, mostly linear with very few (< 1%) α-1,6 branching points (Figure 1.2b).[20] The location of amylose within the starch granules is not well defined; because of its structural characteristics and low crystallinity, it is thought to reside mostly in the amorphous regions of the granule. Nonetheless, some amylose may also be present within the semi-crystalline areas at the periphery of the starch granule, interspersed amongst the amylopectin molecules.

Amylose and amylopectin make up the majority of the granule matrix, with small amount of proteins (0.4-0.6%) and lipids (0.8%), present on the granule surface originating from the intracellular matrix.[21, 22] Starch structural characteristics and branching pattern are the main determinants of starch granule morphology (Figure 1.2c). In wild-type starches, amylopectin molecules are organised radially with the non-reducing end of chains pointing towards the periphery of the granule. This organisation confers a characteristic crystallinity to the starch that can be observed by microscopy as a birefringence pattern. Amylose does not appear to contribute to the granule crystallinity; normal and amylose-free (waxy) starches can display a strong birefringence indicating similarities in the degree of starch crystallinity. On the

other hand, the granule crystallinity of high amylose starches is disrupted, and the birefringence pattern is less visible.

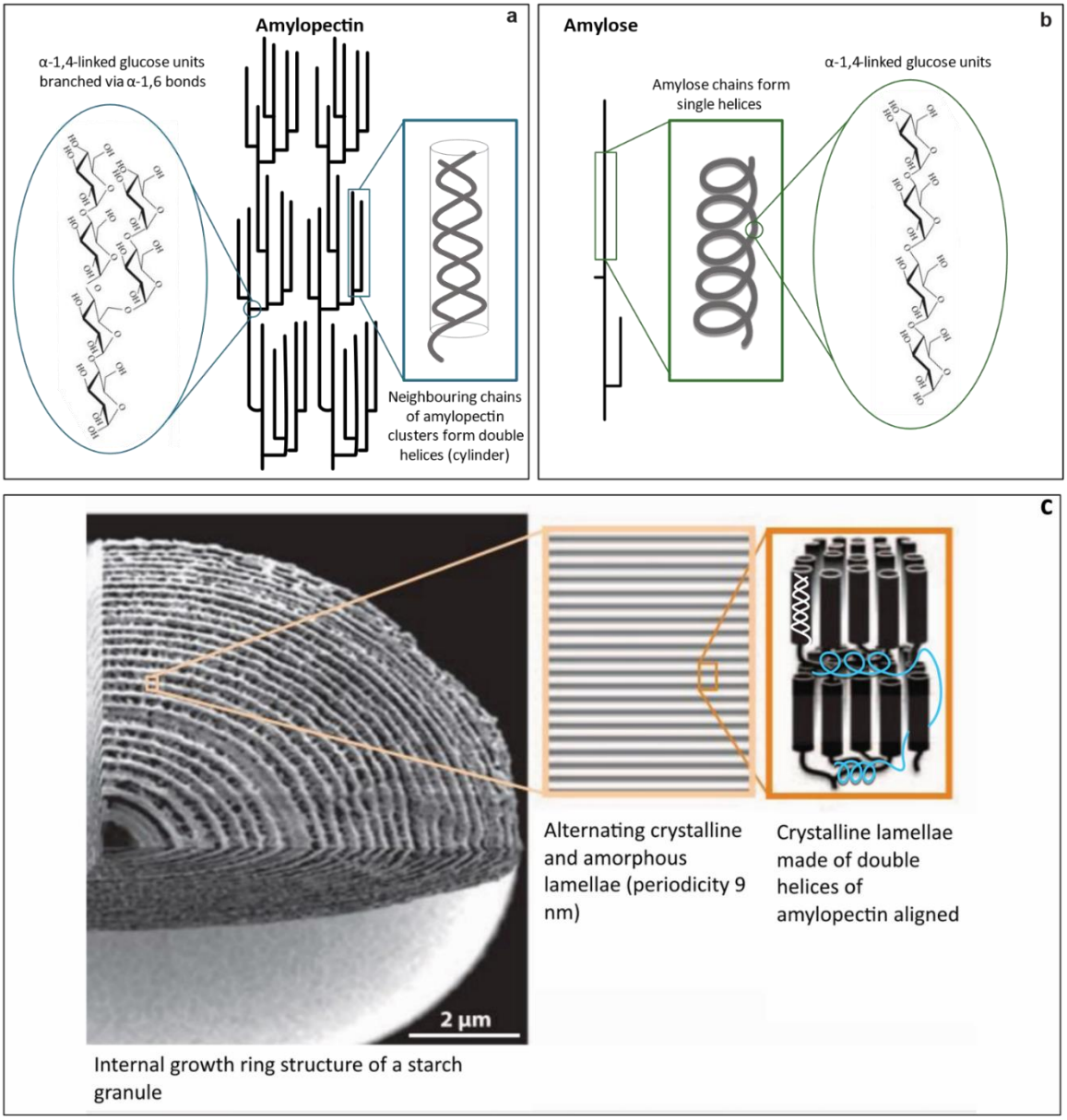


Figure 1.2 A schematic representation of amylopectin (a) and amylose (b), and the structures adopted by the constituent chains. *Figure adapted from Zeeman et al. (2010) and Pfister et al. (2016).*[23, 24] *Double helices packed together in organized arrays (cylinders), form crystalline layers (lamellae) within the granule. These alternate with amorphous lamellae formed by the regions containing the branch points, with a 9- to 10-nm periodicity.* ***c.*** *On the right, the crystalline lamellae containing the linear part of amylopectin chains aligned into double helices (white), interspersed with amylose chains (light blue). Alternating semi-crystalline and amorphous regions form 'growth rings' within the granule (left) with a thickness of approximately 200–400 nm.*

The vast literature available regarding the structural and molecular characteristics of starch components highlights how the ratio of amylose and amylopectin fractions determine the structure of starch granules and confer specific physico-chemical properties to the starch. It

is possible for starch granules to be made entirely of amylopectin or to have a high content of amylose. For instance, starches can have an amylose content as low as 1% (waxy starch)[25] or as high as 85% in wheat[26] and close to 100% in barley.[27]

Starch digestibility is strongly influenced by the ratio and structures of amylopectin and amylose because these factors determine the ordered architecture of the starch granule.

1.2.2.3 Starch biosynthesis in the grain

Starch fine structure is defined during starch biosynthesis by the action of a series of enzymes. The following description applies to starch synthesis within endosperm cells, during the development of wheat grains. The substrate for starch synthesis in wheat is adenosine diphosphate (ADP)-glucose, generated by ADP-glucose pyrophosphorylase (AGPase) in the cytosol from a hexose-phosphate precursor that is synthesised from sucrose imported from photosynthetic tissues. ADP-glucose is then transported into the plastid where it is used as substrate to form glucan chains. Within the plastid, there are 3 families of enzymes that work synergistically towards the formation of starch polymers: starch synthases (SSs), starch-branching enzymes (SBEs), and debranching enzymes (DBEs) (Figure 1.3a). SSs join glucose residues from ADP-glucose to the non-reducing end of a glucan on the surface of the granule. The SBEs create branches by cleaving α-1,4 bonds to transfer the released reducing ends to a C6 hydroxyls linked by α-1,6-bonds; these can be on the same glucan chain (intrachain transfer) or to an adjacent chain (interchain transfer), (section 1.2.2.3.1). DBEs remove some of the branches created making the structure highly ordered to form a starch granule (Figure 1.3b). Each enzyme family contains several classes and isoforms, some of which are tissue-specific and have distinct substrate specificities. For instance, there are five classes of SSs: SSI, SSII, SSIII, SIV and granule-bound SSI (GBSSI). GBSSI is primarily involved in synthesis of long linear chains that constitute amylose. There are two classes of SBEs: SBEI and SBEII, consisting of two distinct isoforms, SBEIIa and SBEIIb. The precise role of each isoform during starch synthesis remains difficult to define and it can vary depending on the botanical species. This is of interest because the loss of one or more isoforms can have pleiotropic effects with changes in the distribution of chain lengths available as substrates for the remaining isoforms, alteration of the SS to SBE activity, and disruption of enzymatic complexes required for the formation of starch.

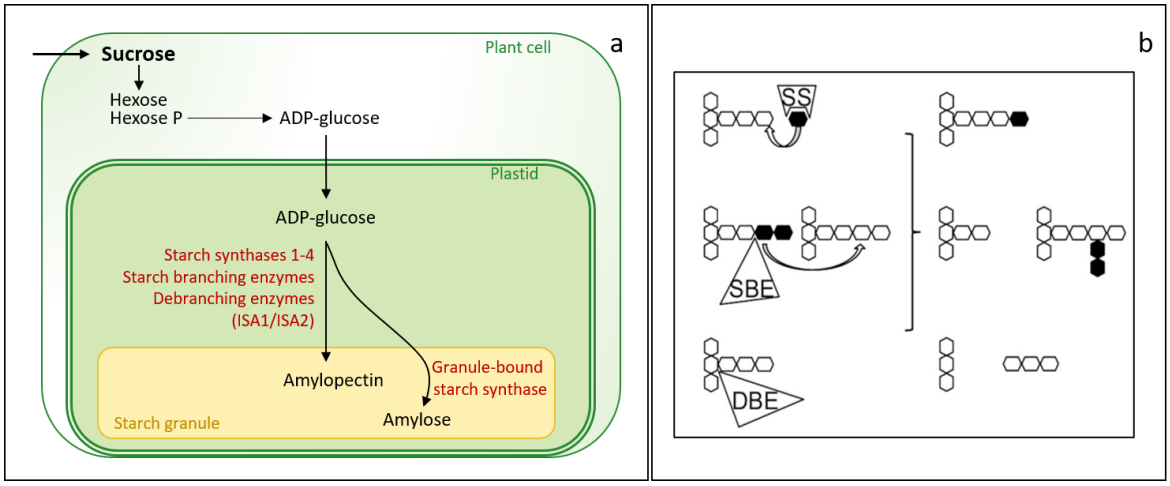


***Figure 1.3** a. **Pathway of starch synthesis within the endosperm tissue of cereals**; In non-photosynthetic cells, hexose phosphate derived from imported sucrose is converted into ADP-glucose in the cytosol and then imported into the amyloplast transported into the amyloplast. Glucose from ADP-glucose is transferred to starch polymers by starch synthases. Starch-branching enzymes and the debranching enzymes ISA1 and ISA2 form amylopectin branches. Granule-bound starch synthase forms linear chains of amylose. Adapted from Smith and Zeeman (2020). [28] b. The key enzymatic steps involved in starch synthesis and elongation, branching and debranching catalysed by starch synthase (SS), starch branching-enzyme (SBE) and debranching enzyme (DBE). Adapted from Li et al. (2015).*

1.2.2.3.1 The role of starch branching enzymes in starch biosynthesis

The various SBEs present in wheat have a great impact on the structural and functional properties of starch. For example, the activities of different SBE isoforms during starch biosynthesis can determine the branching frequency and length of starch polymers, thus governing starch digestibility and processing characteristics. The different isoforms of SBEs have different roles during synthesis and different substrate specificity. SBEI, which has a higher affinity for amylose as a substrate, requires glucan chains with a degree of polymerisation (DP) of at least 10–13 and up to DP 30; SBEII isoforms transfer shorter chains (DP 6–14) and prefer amylopectin as a substrate.[29] In cereals, SBEIIa and SBEIIb have different kinetic characteristics and tissue expression patterns. SBEIIb, in monocots, is found exclusively in non-photosynthetic tissues (like the endosperm) whereas SBEIIa is expressed ubiquitously and it is the predominant branching enzyme in wheat endosperm starch synthesis.[30]

In many plant species, the loss of SBEs activity leads to pronounced phenotypes; a well-known example of this is the loss of SBEI activity in pea (*Pisum sativum* L.) mutants resulting in a wrinkled phenotype (*rugosus, r*), studied by Mendel.[31] In the *r*-mutant peas lacking one isoform of starch-branching enzyme, starch biosynthesis was found to be 50% lower

compared with wild-type peas[32] and the amylose content was ~70% higher than the wild-type peas; the starch granule morphology was altered, showing internal fissures and altered gelatinisation behaviour (section 1.2.5);[33] and the starch susceptibility to amylolysis was lower than starch from wild-type pea.[34]

Similarly, in maize, the loss of SBEIIb activity (the predominant isoform in maize endosperm) in *ae*$^{-}$ mutant plants resulted in a 20% reduction in starch synthesis.[35] The starch accumulated was characterised by granules with severely altered morphology (fissures, irregular shape)[36] and it was composed of long internal amylopectin chains,[37] less frequently branched compared to normal starches, resulting in an dramatic increase in the proportion of amylose.[38]

In wheat, SBEIIa and SBEIIb are expressed in the endosperm, so to achieve a strong phenotype it is necessary to suppress expression of both genes. When this is done, levels of amylose of up to 70% can be generated as shown by studies using both TILLING and RNAi technologies.[11, 39, 40]

1.2.3 The botanical origin of wheat as a crop

Wheat is one of the main cereal crops used forhuman and animal consumption; about 95% of the wheat grown worldwide is hexaploid bread wheat and approximately 5% is tetraploid durum (pasta) wheat. While durum and bread wheat share similar ancestors, it is useful to consider their origin to understand challenges and opportunities of selecting wheat traits. Durum wheat (*Triticum turgidum ssp. durum*) and bread wheat (*Triticum aestivum* L. *ssp. aestivum*) are monocotyledonous plants of the Gramineae family, Triticeae tribe and *Triticum* genus. Wheat species were originally constituted of a set of seven chromosomes in diploid form. One of the first events that led to modern wheat species was the hybridisation of a diploid *Triticum urartu* (AA genome) with a diploid BB genome donor species related to *Aegilops speltoides,* now extinct, resulting in a fertile tetraploid wheat (AABB genome).[41] Because of the combined genetic background of different diploid species, tetraploid wheat was characterised by high yield and adaptability to different environments, leading to the domestication of the species as *Triticum turgidum,* the ancestor of durum wheat cultivars used for pasta production.

Bread wheat (*T. aestivum*) is the result of the further hybridisation of tetraploid wheat and a wild diploid species with a DD genome (*Aegilops tauschii*) resulting in a hexaploid wheat (AABBDD genome), approximately 7000 to 9500 years ago.

1.2.4 Wheat breeding and traits selection: challenges and opportunities

Selection of new traits in wheat can be challenging by conventional breeding because of the limited gene pool available, particularly genes regulating starch synthesis in the grain, and the high number of genes acting additively and synergistically. Furthermore, wheat can tolerate a much higher number of mutations compared to diploid species. Because of the relatively recent polyploidization of wheat species, tetraploid and hexaploid wheat have multiple functional copies of most genes making wheat species adaptable and robust to changes. For instance, the loss-of-function of one gene can be masked by the other copies of the same gene (homoeologues).[41] This characteristic allows wheat plants to tolerate changes naturally occurring in a genome and adapt well to new environments. Therefore, traits selection in wheat often requires the targeting of several or all gene copies.

While this can represent a challenge when selecting wheat traits by conventional breeding, it was found to be an advantage when using ethyl methanesulfonate (EMS) mutant wheat by TILLING. 'Targeting Induced Local Lesions in Genomes' (TILLING) was first described by McCallum *et al.* in 2000[42] as a new method to screen mutant populations for 'lesions' (i.e. mutations) in a gene of interest. The organisms produced by this method do not differ from those obtained by traditional mutation breeding, so genetically modified organism issues do not arise. EMS mutations have been successfully applied in crop improvement since the late 1990s, they have a long safety record and are therefore not subjected to the obligations stated by the GMO Directive.[43-46]

The TILLING approach allows the identification of lesions within a sequence, regardless of its phenotypic effect, which are then categorised based on their potential to disrupt protein functionality. Because of the high mutation density tolerated by wheat plants, EMS is used to generate a large number of lesions that results in a high probability of identifying loss-of-function mutations. The combination of partial and or complete loss of function mutations can be used to create a spectrum of phenotypes.

In order to generate new phenotypic traits in crops, mutations in gene(s) of interest can be identified using *in silico* techniques from the TILLING research database (http://www.wheat-

tilling.com/). Several null alleles need to be then combined by crossing to achieve the desired phenotype. For instance, there are several enzymes involved in starch synthesis and formation of the branch points within the wheat grain (section 1.2.2.3). The TILLING approach was used in wheat to identify and combine null mutations in each of the three homoeologues genes encoding for one of the enzymes responsible for the branched structure of amylopectin (*sbeII*). Slade *et al.* (2012)[39] showed that plants containing null alleles in two homoeologous copies of *sbeIIa* showed no change in starch composition compared to the wild-type control. This suggested that the remaining copy of *sbeIIa* had compensated for the two missing copies. Combining null alleles of all three homoeologues instead resulted in a clear phenotype; starch from mutant plants was characterised by a significant change in starch branching structure, altered granule morphology and a significant increase in the proportion of amylose in the grain, compared to wildtype grains. Thus, the phenotypic traits can vary depending on the number of homoeologues targeted and the type of mutations involved (complete or partial loss-of-function), which is advantageous when attempting to modulate starch structure based on specific end-use characteristics. The use of TILLING enabled the generation of cultivars producing starch with a range of desired properties, including starch with increased amylose content (*sbeII*), without the need for post-extraction processing.

1.2.5 Processing starch: swelling, gelatinisation and retrogradation

The ordered granular structure of starch undergoes changes when mechanical forces or heat are applied.

Starch structure is considerably altered when the processing involves shear forces, water and heat. When isolated starch is heated in water, starch undergoes an irreversible transition named 'gelatinisation'. This transition begins with starch swelling; as water enters the granules, the amorphous regions expand destabilising the crystalline regions and allowing increasing amounts of water to enter the granule. As the granule hydrates, the proportion of amorphous material increases within the granule because of the loss of organised structure. With heat, hydrated granules undergo a melting transition where the hydrated double helices unravel; the granule structure is lost as the amorphous material forms a paste or a gel (Figure 1.4). Increasing proportions of amorphous material result in greater accessibility of the substrate to amylolytic enzymes, which facilitates starch digestion. Amylolytic enzymes attack the amorphous regions of starch during the initial stages of hydrolysis; a higher proportion and availability of amorphous areas determine the

rate and extent of amylase action. Therefore, a high degree of starch damage caused by milling or aggressive processing causing starch to gelatinise can results in high susceptibility to enzymatic digestion.

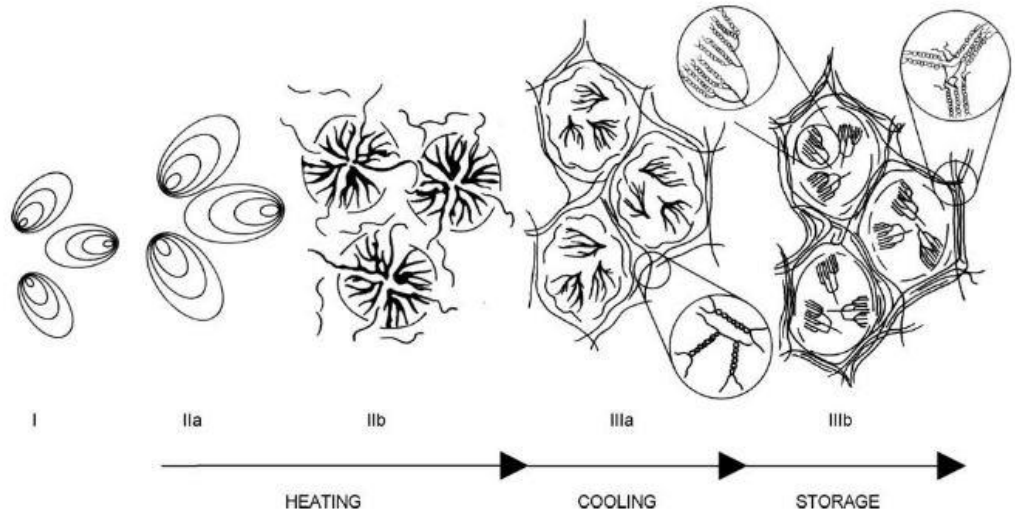


Figure 1.4 Starch in water behaviour during heating, cooling and storage from Goesaert et al. (2005)[47] *(I) Raw starch granules; (IIa) granules swelling due to hydration, (IIb) amylose leaching and partial granule disruption during gelatinisation starting to form a starch paste; (III) retrogradation: gelatinised amylose forms a network with cooling [a] followed by amylopectin forming new ordered or crystalline structure during storage [b].*

The rate and extent of this process greatly depend on the starch composition and on the processing characteristics, specifically the amount of heat and water. It is generally accepted that gelatinization is rapid when starch is heated between 50 and 100 °C in excess of water (moisture >70 %); changes in starch composition (amylose to amylopectin ratio, branching frequency and length) determine starch pasting properties (the ability to form a paste by interacting with water) and behaviour during processing. High amylose starches (> 50%) are generally more thermally stable than wild-type starch, requiring higher temperatures to gelatinise (> 120 °C) and to produce highly viscous pastes.[48, 49] Therefore, high-amylose starches in conventional cooking may not present the same structural properties (i.e. viscosity) as normal starches, which could affect the textural characteristics of foods.

Once gelatinized, the relative proportions of amylose/amylopectin in starch have no direct effect on starch digestibility. An increase in the amylose proportion, and consequent increase in gelatinisation temperature, can however cause the starch to retain a partially crystalline structure or to incompletely gelatinise under conventional cooking conditions, resulting in cooked starch that is more resistant to hydrolytic enzymes.[50, 51] Thus, the

processing applied can have a great influence on the starch characteristics. It should be considered that within a food matrix starch behaviour may differ, regardless of the starch composition. The presence of cell wall or protein structures can restrict starch hydration, swelling and therefore gelatinization. These are important considerations as changes in the starch physico-chemical properties during processing play a role in the digestibility of starchy foods.

Starch gelatinisation is an irreversible process as the starch α-glucan chains in the amorphous state cannot return to their native structure, but they can form new ordered or crystalline structures upon cooling, in a process called 'retrogradation'. As for the gelatinisation process, starch retrogradation depends on starch chemical composition and cooling conditions. A low rate of cooling favours the formation of more organised structure as amylose and amylopectin chains re-associate into double helices, forming starch crystallites with low susceptibility to amylolysis. This phenomenon occurs at a faster rate for amylose chains compared to amylopectin. This is thought to be due to the length of glucan chains: longer linear chains are more likely to align and form stable double-helices favouring amylose above amylopectin in the retrogradation process (Figure 1.4). Upon minutes from the start of the cooling process, amylose retrogradation begins, resulting in strong gels, while amylopectin retrogradation is a slower process commonly observed over days of storage. The rate of starch retrogradation is also dependent on the water content and cooling temperature, whereby low temperatures (+4 °C or lower) favour a faster retrogradation. In processed foods, the amylose retrogradation is thought to contribute to properties relating to texture and digestibility, whereas amylopectin retrogradation is an important factor in staling of baked products, like bread and biscuits.

1.2.6 Processing wheat: from harvest to foods

The first step of the processing chain of wheat is milling, where mechanical forces are used to separate the fibrous outer layers of the grain (dehulling) from the starchy endosperm, which is then fractured producing a wide range of particles sizes, depending on the type of mill, the type of wheat and the end-use quality required. To produce refined white flour, the outer layers of the grain and the germ, rich in fibres, lipids and micronutrients, are removed prior to milling; the starchy endosperm tissue is then fractured and reduced to an increasingly small particle size by a set of rollers or stones to expose and release the starch granules from the cellular matrix. The milled fractions are then separated by sieving based

on their particle size, to give refined flour. The resilience of these tissues to processing may vary resulting in flours of coarse or fine particle size. Depending on the severity of the milling process and the hardness of the wheat grain, starch intact structure can be damaged resulting in loss of crystallinity. For instance, hard grains like durum wheat grains are characterised by strong protein-starch bonds resulting in coarse semolina when milled, rather than refined flour. The reduction in particle size is associated with a reduction in the number of intact cells, and an increase in damaged starch granules present in the milled material that are more accessible to digestive enzymes (section 1.2.8). Damaged starch has greater water absorption capacity compared to raw intact starch and is more prone to gelatinisation because of exposed amorphous regions; these are also easily accessible to digestive enzymes with implications for end-use quality of milled wheat and digestibility of wheat-based products.[52, 53]

The processing performance of wheats with novel starch properties, such as the *sbeII* mutant wheat, has yet to be explored fully. Changes in the starch structure usually alter the end-use quality of flour and products quality that can determine consumer acceptance. Furthermore, changes in texture and viscosity of starch-based foods can influence the extent of the metabolic response to foods and ultimately, glycaemic control.

The resistant forms of starch present in foods are often classified as a type of fibre because they are known to resist digestion in the small intestine and to reach the colon where they can be fermented by the microbiota.[54] The increased proportion of amylose in flour is usually associated with an increase in RS, therefore, high amylose flour is expected to exhibit end-use quality characteristics similar to fibre-rich flour. Nonetheless, the definition of “dietary fibre” is very broad and includes nutrients characterised by different physico-chemical composition such RS and non-starch polysaccharide (NSPs). Depending on the type and quantity of the dietary fibre used, different outcomes can be obtained for process parameters and quality characteristics of the product. A study by Almeida *et al.*[55] on different fibre sources and their effects on bread quality parameters found that increasing quantities of RS (0 – 20 g per 100 g of flour, high-amylose maize starch) in bread doughs altered dough viscosity. Consequently, the required mixing time was increased for the dough to reach a similar consistency as the control dough. Overall, they described RS as an “inert” fibre when considering the quality parameters because adding RS to the formulation did not affect the quality of the product nor the acceptance of crust colour and appearance,

aroma and taste evaluated in their sensory test. In contrast, adding increasing quantities of wheat bran to the bread formulation reduced specific volume and crumb luminosity and increased high-speed mixing time, crumb chroma (colour purity) and crumb moisture content. The addition of fibre to flour for bread making usually results in increased water absorption and dough moisture, decreased mixing and fermentation tolerance, lower bread volume and changes in crumb structure compared to conventional white bread.[56] High-fibre wheat bread is usually firmer than conventional white wheat bread, particularly after a long storage period.[57] Breads enriched with RS (chemically modified pea starch) are known to have a lower volume and increased density due to the higher water binding capacity of RS, which leaves less available water for the development of the gluten-starch network.[58] The increased bread hardness could be linked to several factors including: a lower resistance of the dough to mixing and extension and therefore, a weaker gluten network,[59] or to changes in the starch properties (pasting and gelatinisation) due to the limited water available or the altered viscoelastic properties of the dough.[60] Therefore, the processing of fibre-rich flour into foods such as bread requires a method optimised for the characteristics of the flour and dough, which may differ compared to a regular bread dough, particularly for mixing and proofing conditions.[61]

In this PhD project, the starch and non-starch intrinsic characteristics were examined on isolated starch from wheat grains, intact or milled. Studies on the starch susceptibility to amylolysis and starch resistance to digestion were performed on milled wheat and/or food products. The processing performance of *sbeII* wheat was evaluated to produce *sbeII* products of similar characteristics to the WT controls and ultimately, to evaluate the effect of altered starch properties on glycaemic response.

1.2.7 Digestion, metabolism and physiological responses

1.2.7.1 Starch digestion in humans

The digestion of starchy foods begins in the oral cavity where the food material is mixed with saliva containing digestive enzymes secreted by the salivary glands. Here, the salivary α-amylase initiates starch digestion resulting in a steady increase of maltose and proteins released from the food matrix because of mechanic and enzymatic breakdown.[62, 63] Food mastication and exposure to salivary α-amylase can be relatively brief (seconds), therefore the extent of the starch breakdown by salivary amylase is often debated, particularly when

compared to the action of other digestive enzymes present in the upper gastrointestinal tract.[64] The mastication also plays the important role of reducing the particle size of the food ingested, breaking down the food matrix and increasing nutrient (i.e. starch) exposure to digestive enzymes.[65]

Depending on the structural characteristics of foods, masticated and lubricated foods can form a *bolus* in the oral cavity. Upon entry in the stomach, the activity of the salivary α-continuous until the increasing acidity of the gastric juices inactivates it (pH between pH 3.0 and 3.8).[66, 67] Here the bolus is mixed with gastric acids and digestive enzymes (mostly proteases and lipases) forming the *chyme*. The peristaltic movements in the lower part of the stomach further disrupt the food particles present in the chyme, which then moves into the small intestine. Here, the acidity of the gastric juices is neutralised by bicarbonate, mixed with mucins (secreted from the Brunner's glands) and bile, released from the gallbladder, to approximately pH 6 to then gradually increase to pH 7.4 in the terminal ileum.[68] In the upper part of the small intestine, the duodenum, the chyme is exposed to digestive enzymes produced by the pancreas, including pancreatic α-amylase, initiating starch digestion by producing maltose, isomaltose, maltotriose, and α-limit dextrins (section 1.2.7.2). These oligosaccharides are further digested by 'brush-border enzymes' secreted by endothelial cells on the intestinal mucosa, specifically maltase-glucoamylase (MGAM), which hydrolyses α-1,4-linkages in maltose and dextrins, and sucrase-isomaltase (SI), which hydrolyses the α-1,6-linkages in isomaltose and α-limit dextrins.[69] The glucose produced by the synergistic action of amylolytic enzymes is then absorbed from the intestinal mucosa into the systemic circulation (Figure 1.5). The absorption of glucose from the lumen depends on the action of transport proteins located in the brush-border and basolateral membranes and on luminal glucose concentration; when the glucose concentration is low (i.e., during the fasting state), active transport mediated by the sodium-glucose cotransporter 1 (SGLT-1) is used to move glucose across the brush-border. When luminal glucose concentration is high (i.e. following a meal), the glucose transporter 2 (GLUT2) transporter moves to the brush-border, providing bulk absorption of glucose by facilitated diffusion.[70] These transporters bring sugars (mostly glucose) across the brush-border into the enterocyte cells of the mucosa. Most of these sugars then exit the cells through the basolateral membrane via facilitated transport by GLUT2 and drain into the hepatic portal vein to be transported to the liver.[71] Thus, absorbed sugars elicit a measurable rise in plasma glucose concentration.

Starch reaching the end of the small intestine (terminal ileum) undigested passes into the colon with the other undigested component of the meal, including non-starch polysaccharides (NSP) and other dietary fibres. These are fermented by the microflora resident in the colon that can break down carbohydrates not digested and absorbed in the upper gut, to produce short chain fatty acids (SCFA), which are rapidly absorbed. SCFA are utilised by the human host in several metabolic pathways, they represent a primary energy source for the colonic epithelium. The rate of SCFA production and absorption is dependent on fermentation substrate, microbial composition and colonic transit time.[72] Fermentation of RS[73] and NSPs, particularly AX[74], results in an increased production of butyrate, thought to improve peripheral insulin sensitivity.[75, 76] An increased production and absorption of SCFA has been linked to improved hormonal regulation of a number of metabolic pathways including inhibition of lipolysis in the adipose tissue[77] and decreased secretion of the incretin hormones leading to improved insulin sensitivity and glucose homeostasis.[78] Gut microbial composition is largely determined by the habitual diet of individuals as shown by a very recent publication by Asnicar *et al.* (2021).[79] They showed that a healthy plant-based diet rich in fibre is predominantly associated with butyrate producers, such as *Roseburia hominis* and *Faecalibacterium prausnitzii;* these also positively correlate with markers of metabolic health.[80] Other species that correlated negatively with health markers were *Clostridium* species and the *Ruminococcus gnavus* and *F. plautii*, normally associated to poor overall health. With regard to the postprandial glucose response to meals, they identified *Prevotella copri* and/or *Blastocystis* presence with a favourable postprandial response. Furthermore, studies focusing on colonic fermentation of foods often rely on stool samples collected by participants. These samples may contain microbial material from other parts of the digestive systems; as shown by Asnicar and colleagues who reported the presence of *Streptococcus salivarius*, a component of the oral microbiota. While these associations are relevant when considering the individual responses to different diets, it should be considered that the rate limiting factor in postprandial glucose response is starch susceptibility to amylolysis in the small intestine as the amount of glucose entering the systemic circulation is directly proportion to the amount of starch digested in the duodenum.[81, 82]

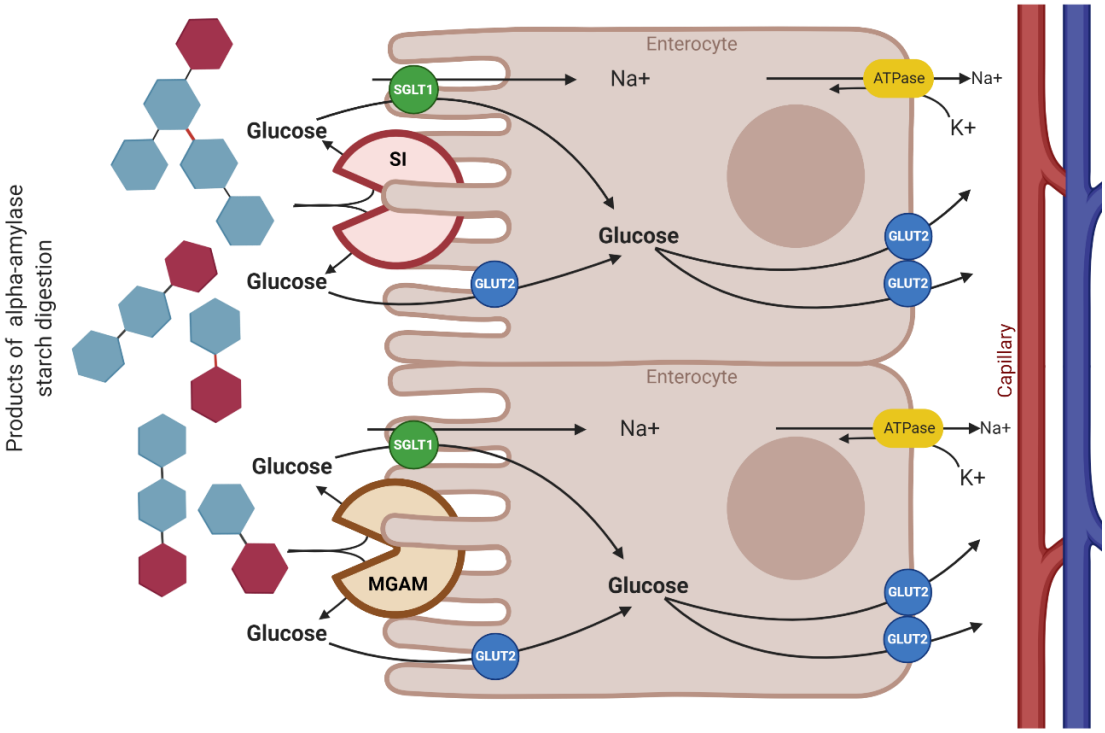


Figure 1.5 Digestion and absorption at the brush border membrane. *Starch is digested by α-amylase in the small intestinal lumen. The main products are maltose, isomaltose, maltotriose and maltodextrins: these are then broken down into glucose by brush-border enzymes (sodium-glucose linked transporter, SGLT1 and glucose transporter 2, GLUT2) embedded in the plasma membrane of the enterocyte: Sucrose-isomaltase (SI) cleaves α-(1,6) and some α-(1,4) bonds, maltase-glucoamylase (MGAM) cleaves α-(1,4) bonds only. The glucose produced is transported across the enterocyte to the blood through the basolateral membrane (GLUT2). Adapted from Sitrin 2014[83] with BioRender.com*

1.2.7.2 Enzymatic hydrolysis of starch

Starch digestion in the human gut involves several enzymatic degradation steps. Amylolytic enzymes are a group of enzymes operating on starch and derived oligo- or polysaccharides. The predominant class of hydrolases using starch as substrate are amylases. The α-amylases (salivary and pancreatic, α-1,4- glucan 4-glucanohydrolase; E.C. 3.2.1.1) are endo-glucosidases that initiate available starch breakdown by hydrolysing the internal α-1,4-glucoside bonds in large-chain polymers such as starch, glycogen and dextrins, producing linear or branched glucose oligomers (maltose, maltotriose, and other dextrins). The α-amylases are incapable of hydrolysing the α-1,6 branch points present in starch; the main product of digestion of amylopectin with α-amylases are limit-dextrins while amylose is hydrolysed to maltose and larger dextrins. The products of α-amylase activity cannot be directly absorbed into the bloodstream without further hydrolysis to smaller sugars by brush border' enzymes. As described above, the mucosal maltase-glucoamylase (E.C.

3.2.1.20 and 3.2.1.3, or amyloglucosidase) converts maltose to glucose and a sucrase-isomaltase (E.C. 3.2.1.48 and 3.2.1.10, or α-dextrinase) hydrolyse α-1,6 bonds of isomaltose and α-dextrins, continuing the digestive process and producing glucose. The amyloglucosidases, a class of exo- glucosidases, release single glucose molecules from the non-reducing end of α-1,4 oligo-or polysaccharides and hydrolyse α-1,6 branching points, converting oligosaccharides to glucose (Figure 1.6). As starch is progressively hydrolysed in the gut, the monosaccharides produced by amylolysis are absorbed from the intestinal mucosa and move into the systemic circulation through glucose transporters. Therefore, the concentration of blood glucose circulating is directly related to the rate and extent of starch hydrolysis. From a nutritional perspective, starch can have diverse effects on postprandial metabolism, depending on the rate and extent of hydrolysis in the gastrointestinal tract; this has important consequences for human health. A number of factors determine starch resistance to digestion (section 1.2.8) and it is well known that foods containing equal concentrations of starch can be metabolised to a different rate and extent. Starch with low susceptibility to amylolysis, such as starch with a low proportion of amorphous material, results in limited amounts of monosaccharides during digestion in the small intestine.[18] The resistant forms of starch that escape digestion in the upper gastrointestinal tract and reach the colon are known as resistant starch (RS); these are ultimately fermented by the microbiota in the large intestine. RS is a complex concept as starch resilience to digestion can be attributed to different factors (section 1.2.11).

Despite the complexities of starch digestion *in vivo,* starch amylolysis measurements obtained *in vitro* can be analysed using mathematical modelling to extrapolate kinetics parameters indicating rate and extent of amylolysis.[84, 85] These parameters were shown to correlate well with the increase in blood glucose concentration observed *in vivo* and can provide important insights in the susceptibility to amylolysis of novel starch-based foods and consequent blood glucose response. Starch amylolysis measured *in vitro* is based primarily on the action of α-amylase, the main amylolytic enzymes in humans. The enzymatic degradation of starch by amylase occurs by a pseudo first-order reaction, where the enzyme concentration remains constant over time and the rate of substrate degradation is inversely proportional to the starch concentration.[86]

For experimental purposes, porcine pancreatic α-amylase is often used as a model of human pancreatic α-amylase. Its amino acid sequence shows 83% identity with that of human

amylase;[87] its 3-D structure is similar to that of the other members of the family and has near-identical function to the human enzymes.[88] It is relatively inexpensive compared with human α-amylases and can be obtained with a high degree of purity.

The human α-amylase contains three structural domains, A, B and C (Figure 1.7). The A domain (residues 1–99 and 169–404) is the largest, forming a central eight-stranded parallel β-barrel; on one end it contains the active site residues Asp197, Glu233 and Asp300 and a bound chloride ion forming ligand interactions to Arg 195, Asn 298, and Arg 337, important for enzyme activation. The B domain is the smallest; its loop (100–168) forms a calcium binding site against the wall of the β-barrel of Domain A. The C domain (405–496) is made of an anti-parallel β-structure distantly associated with Domains A and B.[89] Contrary to other digestive enzymes, α-amylase is much smaller than its substrate however, this confers the enzyme the ability to access the starch glucan chains within intricate food matrices or within starch granules.

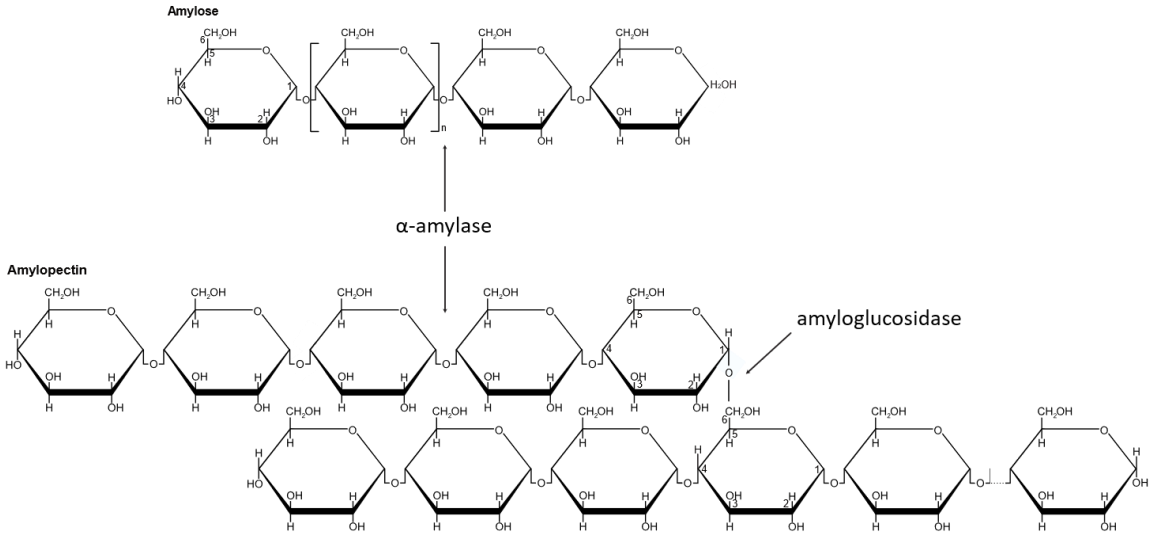


Figure 1.6 Enzymatic digestion of amylose and amylopectin. *α-amylase hydrolyses α-(1,4) linkages from linear regions of amylose and amylopectin producing linear or branched malto-oligosaccharides (typically maltose, maltotriose and limit dextrins). These products can be cleaved further by amyloglucosidase which acts on α-(1,6) branching points and on the release of single glucose molecules from the non-reducing end of a-(1-4) oligo- or polysaccharides, together with other disaccharidases. Adapted from Tester et al. (2004).*[90]

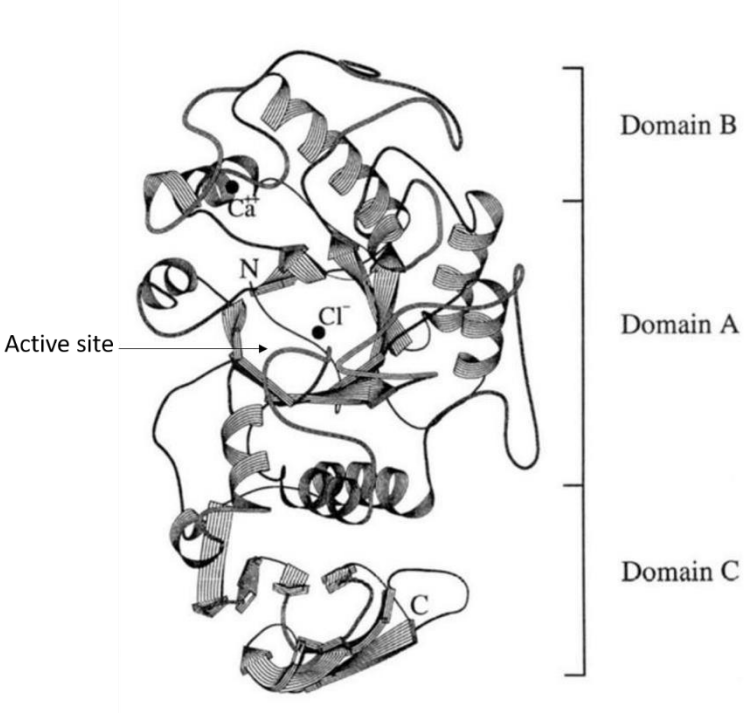


Figure 1.7 The structure of human pancreatic α-amylase determined to 1.8 A resolution using X-ray diffraction techniques by Brayer et al. (1995)[89] *The approximate position of the three structural domains present is indicated along with locations of the calcium and chloride binding sites and the N- and C-terminal ends of the polypeptide chain. The eight-stranded parallel β-barrel is thought to contain the active site region.*

1.2.8 Factors affecting starch susceptibility to hydrolysis by digestive enzymes

Starch hydrolysis by α-amylase can be described by three successive phases: enzyme diffusion towards its substrate; the enzyme adsorption on the substrate; and the catalytic event.[91]

Starch susceptibility to hydrolysis depends on a number of intrinsic and extrinsic factors. Intrinsic factors refer to structure and composition at a molecular level, where changes in granules size (section 1.2.2.1) or amylose-amylopectin ratio (section1.2.2.2) can greatly affect the starch physico-chemical properties and digestibility. Because of their highly ordered structure in the raw (native) state, starch granules are resistant to digestion. However, most starches are consumed after being processed, which increases nutrient availability and can alter starch properties (section 1.2.5). Processing is an extrinsic factor that can be used to manipulate starch characteristics. Milling is used to break cereal grain cells and expose the starch otherwise trapped within cells; the structural integrity of grain components (cell walls and starch granules) also influences nutrient availability (section 1.2.6). For instance, the rates of starch amylolysis and subsequent glycaemic responses of isoglucidic finely and coarsely milled wheat grains can be significantly different. Therefore,

the particle size of milled fractions can be used as predictor of starch accessibility to enzymes. The milled fractions are then usually hydrothermally processed to transform the ingredients into an acceptable food product such as bread. This processing also increases nutrient availability; the use of heat can cause the starch to gelatinise becoming an amorphous structure that favours starch susceptibility to hydrolysis (section 1.2.5). Thus, once gelatinised, the structural characteristics of native starches become less relevant.[49, 92] However, the degree of gelatinisation depends greatly on the structural characteristics of starch; high amylose starches are in fact more prone to gelatinisation than wildtype ones. If starch is subjected to cooling after cooking, amylose retrogradation can occur, which lowers starch digestibility as the glucan chains re-associate into semi-ordered structures (section1.2.5). Starch characteristics can change drastically with processing, therefore, studies investigating starch susceptibility to amylolysis should take into consideration the characteristics of raw and hydrothermally processed starch.

1.2.9 Glucose homeostasis and health implications

Dietary glucose entering in the systemic circulation is kept under tight control to ensure normal body functions are maintained. Several organs and tissues play a role in maintaining the glucose levels within the homeostatic range (4-6 mmol/L) through post-absorptive and post-prandial glucose fluctuations (Figure 1.8). The rate of glucose appearance and disappearance from the circulation defines the homeostatic range. This equilibrium known as glucose homeostasis is maintained by a tight feedback loop involving the liver, the pancreas and the glucoregulatory hormones they secrete, and the peripheral tissues (the brain, the muscles, and adipose tissue).[93] In normo-glycaemic individuals, the postprandial rise in glucose concentration triggers the release of insulin, a hormone secreted by pancreatic β-cells, which stimulates cellular glucose uptake and glucose disappearance such that peripheral blood glucose concentrations decrease, eventually reaching the fasting levels.[94] Glucose derived from the diet may be directly utilised for metabolic processes or stored in the muscles and in the liver in the form of glycogen (polymerised glucose). Increasing concentrations of circulating glucose also inhibit the endogenous glucose production by gluconeogenesis in the liver and kidneys, and the breakdown of stored glycogen in the liver (glycogenolysis) to minimize postprandial blood glucose elevations.[95] The glycogen stored in the liver is essential for the glucose homeostasis. During prolonged periods of fasting, available glycogen is gradually used to supply glucose into the circulation.

Since most tissues store very little glycogen, there is a constant glucose uptake from the blood that is transported to the tissues by facilitated diffusion. A low blood glucose concentration is not favourable to diffusion, therefore, when circulating glucose levels are low (in the postabsorptive state) a series of responses by nerves, hormones (i.e., glucagon, a hormone secreted from the α-cells of the pancreatic islets of Langerhans) are triggered to signal to the liver to promptly deliver glucose to the tissues in need.

Obesity and physical inactivity can result in metabolic alterations of the glucose homeostasis that, in time, can lead to abnormal glucose tolerance (when glucose fluctuates above the normal levels) and insulin sensitivity (when cells fail to respond normally to insulin released to counteract rising glucose concentrations) and progressively transition into hyperglycaemia.[96] It is estimated that most individuals in the pre-diabetic state (impaired glucose tolerance or fasting glucose) eventually develop type 2 diabetes (approximately 65%).[97] Impaired glucose tolerance increases the risk of developing type 2 diabetes because of the lack or decreased compensation for insulin resistance. While insulin secretion can be upregulated to maintain glucose tolerance, if β-cell function is inadequate, then hyperglycaemia develops.[98] A status of hyperglycaemia contributes to endothelial and vascular dysfunction due to the oxidative stress and inflammatory reactions that can progress to atherosclerosis and lead to cardiovascular events, a cause of major morbidity and mortality.[99, 100] Hyperglycaemia can also lead to other long-term conditions such as chronic kidney disease, particularly if combined with other risk factors such as hypertension, dyslipidaemia, non-alcoholic fatty liver disease and obesity;[101, 102] the combination of these metabolic alterations is termed metabolic syndrome and it is a strong determinant of diabetes and CVD.[103]

Controlling glycaemia can alter the natural progression of impaired glucose tolerance to diabetes; this can be achieved with lifestyle changes that include diet interventions.[104-106]

Dietary management of hyperglycaemia and prevention of type 2 diabetes target both quantity and quality of food consumed. Refined sugars intake (e.g. confectionaries, sugary drinks) should be limited or supressed in favour of complex carbohydrates.[107] Fat intake has little effect on postprandial glucose concentrations, but it appears to contribute to development of insulin resistance.[108] High-fat foods should be limited as high-fat diets can induce hyperglycaemia and obesity. Dietary proteins usually have an insulinotropic effect by promoting insulin secretion and blood glucose clearance. However, in the long-term, an

uncontrolled diet high in protein (consuming more than the recommended Population Reference Intake of 0.83 g protein/kg/day) can lead to hyperinsulinemia and, later, to insulin resistance.[109] Evidence from observational studies suggests that long-term high protein intake is associated with increased risk of developing metabolic syndrome or type 2 diabetes. [110-112]

The nutrients' source (plant or animal) and form (refined or complex), the composition of the diet and the presence of underlying conditions also play a role in the development of metabolic dysfunctions.

Large dietary intakes of carbohydrates can cause sharp increases in glycaemia which are hazardous for individuals that are unable to regulate their blood glucose levels; high-carbohydrate diets exacerbate the metabolic abnormalities of insulin resistance with deleterious effects. However, not all carbohydrates have the same physiological effect; the rate and extent of starch digestion in the small intestine plays an important role in regulating the rise in postprandial blood glucose and insulin concentrations.[5] The glycaemic response to foods is particularly important for individuals with abnormal blood glucose regulation, especially those with type 2 diabetes or metabolic syndrome. Foods eliciting low glycaemic responses (glycaemic index) can be used in the dietary management and prevention of obesity, type 2 diabetes, and cardiovascular disease.[113, 114] However, the Glycaemic Index (GI) calculation and methodology is often subject of controversies (discussed in chapter 5). The alternative approach adopted in this project was the combined use of an *in vitro* model, to measure starch-based foods rate and extent of digestion and to screen foods potential to elicit lower glycaemic responses *in vivo*.

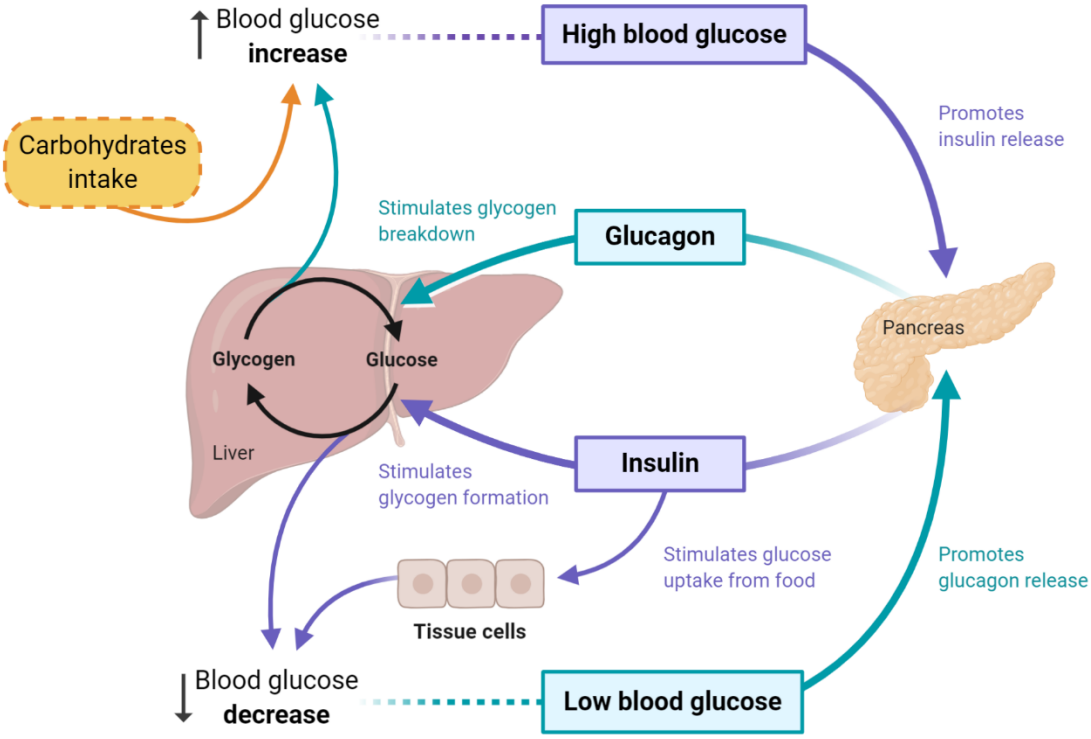


Figure 1.8 Glucose homeostasis regulation by insulin and glucagon. *When blood glucose is low, glucagon is secreted by the pancreas to stimulate glycogenolysis and increase the production of endogenous glucose. After a meal, high levels of exogenous glucose (from the diet) trigger the release of insulin to stimulate circulating glucose clearance. Muscle and adipose tissues uptake glucose and glycogenesis is triggered to form glycogen. Created with BioRender.com*

1.2.10 Plasma and interstitial glucose: a dynamic system for glucose control

Blood glucose levels are generally measured in the venous plasma. Arterial and venous blood glucose concentrations differ of approximately 3–5 mg/mL in the fasting state, with greater differences for greater glucose level in response to a meal.[115] Arterial blood glucose levels are generally higher than in venous blood: glucose diffuses from the arterial plasma to interstitial fluid (IF) as blood circulates through the capillary system, which is then cleared by the surrounding cells.[116]

The IF consists of interstitial water, its solutes and the structural molecules of the interstitial or the extracellular matrix that are found in the interstitial space (the microenvironment outside the blood, lymphatic vessels and parenchymal cells that is found within connective and supportive tissues).[117] The IF contains similar components to the plasma, except red blood cells and a small amount of proteins, allowing an efficient exchange of metabolites

and nutrients between them. It accounts for 15% of the bodyweight in adults, playing an important role in maintaining fluid and electrolyte balance in the body. [118]

From the blood, glucose transfuses into the IF by simple diffusion: the blood flow and the capillary permeability determine the amount of glucose delivered to IF.[119] The glucose concentrations in arterial blood and subcutaneous IF are highly correlated in healthy individuals. Thus, it is possible to continuously monitor subcutaneous IF glucose to reflect the kinetics for the increase and decline in glucose concentrations using a Continuous Glucose Monitoring system (CGM).[120, 121] However, any change in plasma glucose concentration metabolic rate or glucose uptake by cells, will affect the plasma-to-IF concentration difference by delaying the appearance of glucose in the IF; this is known as the physiological lag time between plasma and IF glucose. [122]

The glucose appearance in IF physiologically lags between 0 and 45 minutes behind blood glucose, depending on the efficiency of the glucose metabolism and the sampling site (arterial blood, venous blood or arteriovenous blood from the capillaries).[123] Several studies have assessed the lag time in individuals with diabetes reporting a range of 5 to 15 minutes and showing a large variability in glucose responses; this was partly due to the use of different techniques to measure it (e.g. microdialysis, real-time continuous glucose monitoring) and partly because of the complexity of the plasma-to-IF glucose relationship.[120, 124-126] When blood glucose changes rapidly, the equilibrium between plasma and IF glucose will be broken; reaching the equilibrium after a dramatic change takes a relatively long time, which in turns, amplifies the lagging time between them. However, there are some situations, such as during hypoglycaemia, when changes in blood glucose concentrations lag behind IF glucose concentrations. That is, when blood glucose becomes extremely low and the concentrations of glucose in other parts of the body may be even lower.[127]

Studies reporting the lag time are largely based on data from clinical trials involving patients with diabetes and may not describe glucose fluctuations in subject not affected by any metabolic conditions.[128] Thus, more studies are required to understand the reliability of CGM technology in normo-glycaemic subjects.

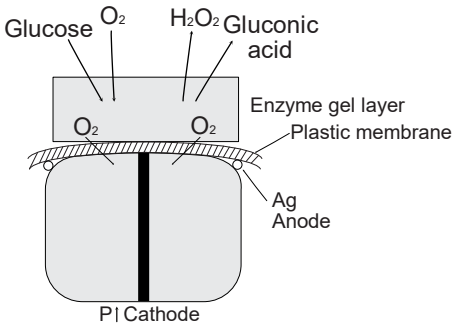
Glucose profiles obtained from CGM systems are a useful tool to understand naturally occurring and diet induced changes in blood glucose levels. This methodology could vastly

advance research focusing on the effect of carbohydrate foods on health, if suitable to detect glucose changes in healthy individuals.

Currently, more research is needed to determine the physiological factors affecting glucose measurements in continuous monitoring; these may include local tissue metabolism of glucose, local and regional differences in blood flow rates, arteriovenous shunting of blood, make-up of subcutaneous tissue and other unknown variables.[129]

1.2.10.1.1 Continuous Glucose Monitoring systems

Blood glucose level continuously fluctuates throughout the day and night due to natural metabolic changes that are tightly controlled to maintain homeostasis. Since traditional glucose markers such as glycated haemoglobin do not reflect accurately these dynamic fluctuations and plasma glucose measurements require invasive collection methods (cannulation or finger prick), research has focused on developing tools to monitor IF glucose level continuously. Glucose sensors were first introduced in the early 1960s by Clark and Lyons and consisted of a thin gel layer containing glucose oxidase enzyme (GOD), an oxygen electrode, an inner oxygen semipermeable membrane, and an outer dialysis membrane. Glucose concentration was calculated based on the change in the local oxygen concentration.[130] The detection method was improved by Updike and Hicks to capture glucose concentrations in biological fluids.[131] They used an 'enzyme electrode' to measure the oxygen diffusion flow through a plastic membrane. This principle is shown in Figure 1.9. In brief, when the enzyme electrode is in contact with a biological solution or tissue, glucose and oxygen diffuse into the membrane layer. Oxygen is reduced by glucose oxidase that is immobilised in a gel layer (acrylamide gel or similar) and gluconic acid is formed. The output of current generated can be measured after the diffusion process has reached the steady state (~3 minutes, depending on the thickness of the plastic and gel layers).



$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

Figure 1.9. Principle of enzyme electrode Glucose Oxidase (GOD) reaction adapted from Updike and Hicks, 1967 [131].

The first CGM system was approved for clinical use by the US Food and Drug Administration (FDA) in 1999. Since then, several CGM systems were launched on the market with different features regarding life span of the sensor, detection frequency, the ability to measure glucose retrospectively or in real time. In September 2016, the US FDA approved the *Abbott Libre Pro Professional* CGM system, a retrospective needle-type CGM, with a factory calibrated glucose sensor designed to last up to 14 days. Upon application, the sensor filament is guided into the dermis where IF glucose is measured (Figure 1.10). This type of retrospective CGMs rely on one-hour equilibration period to allow any local inflammatory response due to the needle insertion to decrease.

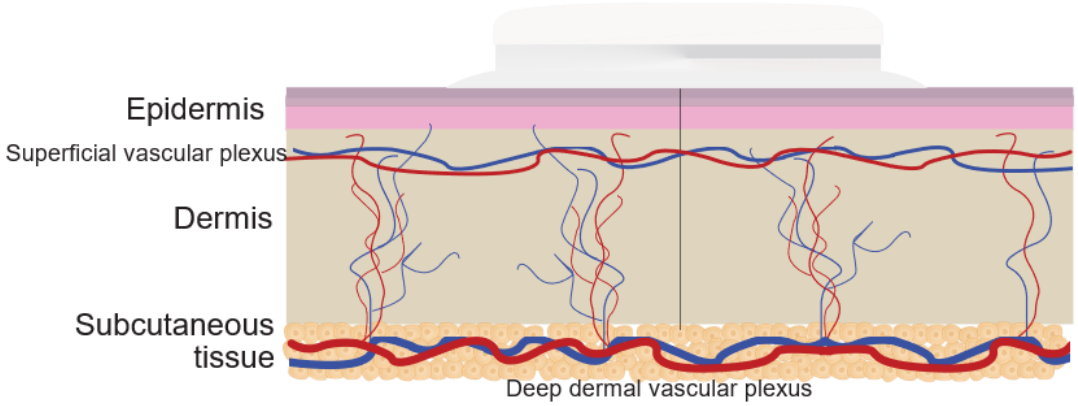


Figure 1.10. Skin layers and vascular system. *The CGM sensor filament reaches into the subcutaneous tissue to measure interstitial glucose before it is cleared by the surrounding cells, image adapted from Cengiz et al. 2009.*[116]

CGM technology has been validated and developed for use by individuals with diabetes individuals. The sensor optimization has allowed for monitoring of IF glucose levels to become less invasive and burdensome for participants. However, this system has not been validated to measure diet-induced changes in postprandial glucose levels of healthy individuals.

The use of a monitoring system with automatic and frequent determinations could revolutionise glucose metabolism research too, by providing glucose level readings over an extended period of time without requiring frequent sampling or finger pricks. The ability to detect and report high and low glucose values has been validated in overweight and individuals with diabetes.[120, 132] However, very few studies reported using such systems to measure the glycaemic response to foods of healthy volunteers.

1.2.11 Analytical and physiological studies of starch resistance to digestion

The release of oligosaccharides from starchy substrates has complicated kinetics, particularly when the substrate is in the form of inaccessible or intact starch granules, or retrograded starch, less susceptible to amylolysis. Using a mechanistic approach, the hydrolysis of starch by α-amylase is a function of the rate of enzyme binding to the substrate and/or the rate of substrate conversion to product.[133, 134]

This is a relatively modern concept since initially all starch was regarded as completely digestible in the small intestine once cooked. Studies in the early 80's attempting to measure NSP analytically, proposed a new concept of 'resistant starch' after observing that a portion of the starch was not rapidly hydrolysed as the rest. Further research involving ileostomy patients demonstrated that a portion of the starch ingested from cereals,

bananas and potatoes was passing through the small intestine, to be fermented in the colon.[135, 136] Thus, the physiological definition of resistant starch (RS) was defined as the "sum of starch and starch degradation products not absorbed in the small intestine" of healthy individuals. Analytically, measurement of foods glycaemic properties is based on the progressive enzymatic removal of starch; several methods proposed over the years were based on measurement of dietary fibre, total carbohydrates and RS.

However, measuring the metabolic quality of carbohydrates such as starch presents challenges as the ideal *in vitro* procedure would simulate *in vivo* conditions, where several forms of inaccessible starch can be present during digestion *in vivo*.

The source of starch resistance to digestion has also been object of research. Initially, Englyst and Cummings (1987)[135] identified three main forms of RS: (1) RS1 is starch that is physically inaccessible to digestive enzymes (trapped in food matrix such as intact cells or partly milled or whole grains or seeds); (2) RS2 is native (uncooked) starch in the form of granules that are resistant to digestion because of their physical and chemical structure at the molecular and granular levels (e.g, granule surface area and structure, the amount of crystalline material); and (3) RS3 is retrograded starch occurring in processed foods. A number of other resistant forms of starch have also been identified such as chemically modified starches (RS4) and resistant forms linked to the presence of other dietary components such as proteins, lipids, phenolic compounds and water-soluble NSP forming complexes hindering starch glucan chains from the action of amylolytic enzymes. The formation of amylose-lipid complex leads to increased amylolytic resistance by restricting the granule swelling during cooking; this is identified as RS5.

From a food technology point of view, measurement of RS can be challenging because the RS content of foods can vary greatly depending on the choice of raw material and processing conditions. For instance, measurement of RS in potatoes and potato products showed that in raw potato resistance to digestion was due to the structure of the starch granules whereas in potato cooked in low moisture (e.g., biscuits), starch resistance to digestion was mainly attributable to the retrogradation of starch. Furthermore, a common analytical method for the measurement of RS *in vitro*, proposed by Berry in 1986[137], is based on the definition of RS as "starch that survives exhaustive digestion with amylolytic enzymes". The method was shown to accurately estimate the amount of starch likely to

escape digestion and absorption in the small intestine. However, the 'exhaustive' digestion does not replicate physiological conditions, it does not distinguish between undigested starch residues and native granules, which biochemically are different, or consider that the starch resistance can be due to loss of enzymatic activity because of an extended incubation period. Measurement of RS alone can provide a rough indication of the amount of starch unavailable to digestion however, it does not provide sufficient information on the potential glycaemic effect of starchy foods. Research on high-amylose rice and pea seeds varieties has shown that measuring amylose content alone does not provide sufficient information regarding the rate of starch digestion or glycaemic response.[138, 139] Varieties with similar amylose contents can differ in physico-chemical properties such as gelatinisation behaviour because of the fine structure of starch, influencing starch digestibility and blood glucose response.

Thus, measurement of the starch converted into absorbable products provides a better understanding of the effect of resistant forms of starch on digestibility, complementing the RS and amylose measurements. This can be achieved using an enzyme-kinetic approach to study the mechanistic digestibility of starch-rich food. An advantage of this method is that it does not require sample homogenisation prior to the analysis which disrupts the food structure, a major factor influencing the postprandial response. Simple biochemical models typically involve substrate incubation (e.g., isolated starch, flour or homogenised starch-rich food) with one or more enzymes (primarily amylase but often also amyloglucosidase at physiologically relevant concentrations). The inclusion of both α-amylase and amyloglucosidase in the initial incubation step may be more relevant for uncooked starches that maintain their granular form than for cooked ones. Furthermore, the amyloglucosidase available for experimental purposes is usually of fungal origin (*Aspergillus niger*), which may not be physiologically relevant. The digesta is sampled at various points throughout the incubation to monitor the formation of digestion products. The digestibility measurements obtained can be fitted to a first-order reaction typical of enzyme kinetic to explain the mechanisms of digestion of complex food systems.

1.2.12 A novel wheat for lower glycaemic responses

Consumption of resistant forms of starch, such as high amylose starches, has been reported to reduce glucose and insulin responses.[140] There is accumulating evidence that high amylose foods could contribute to reducing risk factors for chronic diseases, including type 2

diabetes and cardiovascular diseases.[141] Increasing amylose content of foods can be achieved during food production by combining flour with amylose-rich starch from various botanical sources (e.g. maize)[50] or chemically modified starches or by increasing starch resistance to digestion in the food matrix by modulating the food processing. At the crop level, starch characteristics can be altered by both transgenic and non-transgenic approaches to modulate starch structure, by targeting genes encoding enzymes involved in starch synthesis in the grains. In wheat, plants with resistant forms of starch were obtained by downregulation of *starch branching enzyme II* (SBEII) genes that led to wheat grains and flour with higher amylose content than conventional wheat.[11, 12, 39, 40] Starch properties can be modified post-extraction by chemical or physical treatments, however techniques such as TILLING (section 1.2.4) allowed the generation of starches with a wide range of properties during grain development, through changes in genes encoding enzymes of starch synthesis.

The *sbeII* white flour used in this study is characterised by a higher amylose and RS content than WT flour and can be used to produce a range of food products. The use of wheat with altered starch characteristics obtained by TILLING has potential to produce wheat-based foods with low starch susceptibility to amylolysis suitable for human consumption, since the approach does not make use of transgenic technology. However, more evidence is needed in regard to the changes in starch structure leading to altered starch physico-chemical properties and how these can be exploited by food scientists to develop new foods eliciting lower glycaemic responses.

1.2.13 The nutritional relevance of *sbeII* wheat mutants and relevance of the project

The molecular composition, granular structure, and functional behaviour (e.g. thermal and pasting) of starch in *sbeII* mutant wheat have been previous investigated.[48, 142] The *sbeII* mutant starch is usually associated with high amylose or high RS, typically measured on flour but very little is known of *sbeII* starch susceptibility to hydrolysis when processed into different foods and the consequent effect on glycaemic response.

The type of RS and the food structure that delivers it, have a great impact on the extent of the response.[143, 144] So far, only three studies have reported on the structural characteristics of *sbeII* wheat starch in foods (pasta and bread) and their potential glycaemic response. Hazard *et al.* (2015)[145] reported good end-use quality of *sbeIIa/b* durum wheat producing pasta of acceptable quality with improved firmness after cooking. Furthermore, Hazard and

colleagues in the same publication reported the results of an animal study showing a positive fermentation response in mice fed with *sbeII* mutant wheat flour in the form of a pellet. They showed an increase in the production of SCFA and lower colonic pH after 4 weeks of intake, compared to pellet made with WT control flour. While a higher amylose and RS content was reported in raw *sbeII* flour, hydrothermally processing flour into pellets likely modified starch characteristics which were not further investigated by Hazard and colleagues, leaving a gap in the understanding of processed *sbeII* starch susceptibility to hydrolysis. Considering the known altered properties of *sbeII* mutant starch, it seems likely that depending on the type of processing, *sbeII* starch susceptibility to hydrolysis will be deeply affected.

Recently, two studies have investigated glycaemic response to pasta and bread made from *sbeII* mutant wheat. Sissons *et al.* (2020)[146] reported that pasta made from *sbeIIa* durum wheat was of acceptable quality and showed that *sbeIIa* spaghetti containing ~7% RS and ~58% of amylose per serving led to a lower starch susceptibility to hydrolysis *in vitro* and lower GI measured *in vivo*, compared to the wild-type control. However, it is not clear if the RS was measured on dry or cooked pasta: during cooking, starch can gelatinise altering dramatically the content of RS. Nonetheless, the authors reported that the starch susceptibility to hydrolysis measured on cooked pasta was lower in *sbeIIa* pasta compared to the WT control. Here, starch susceptibility to hydrolysis was measured using a two-enzyme system, α-amylase and amyloglucosidase that, as previously discussed, may not be as relevant from a physiological point of view. Pasta is an interesting food product with a compact microstructure and a complex enzyme kinetic of digestion where starch is embedded in a gluten matrix.

In a study on the effect of breads made from *sbeII* mutant wheat flour on postprandial glycaemia, Belobrajdic *et al.* (2019)[147] showed that substitution of conventional wheat flour low in amylose content with a high-amylose wheat flour lowered the postprandial glycaemic response of bread by 39%. However, the low and high amylose breads used by the authors of the study were characterised by a variable starch content, which could have biased the results of their study: thus, a direct comparison between the glycaemic responses of the two bread types is not possible.

This demonstrates the need for an accurate characterisation of the starch material when raw and when hydrothermally processed, to allow for comparisons of different foods across studies. Robust studies should also ensure that food products are nutritionally matched, particularly when designing the portion size and the carbohydrate load between treatments and controls.

Furthermore, other characteristics of the starch fine structure could play a role in starch susceptibility to hydrolysis. Factors such as the starch granule size distribution and the physico-chemical properties of the different populations of granules have not been extensively investigated in *sbeII* mutants.

From this literature review it is evident that further studies are needed to better understand the many factors that influence starch digestibility. So far, progress has been made in understanding some of the structural characteristics and physico-chemical properties of *sbeII* mutant wheat, which showed potential to produce food with lower glycaemic response. There is still a lot of uncertainty about the mechanism regulating starch digestion when found within a food structure, which is of great relevance to improve understanding of sugars uptake from food. Although research has focused on developing high amylose crops, particularly wheat varieties, there is no clear evidence of the physiological effect of starchy foods made from *sbeII* wheat on blood glucose response. In this project, efforts were made to overcome the limitations of previous studies, by using a combination of *in vitro* and *in vivo* methods to characterise starch susceptibility to digestion after different hydrothermal processes. The *sbeII* wheat processing performance was evaluated to identify an *sbeII* wheat product able to elicit lower glycaemic response than conventional wheat-based foods.

1.3 Aims and objectives

The overall aim of this PhD project was to study the effect of *sbeII* mutant wheat-based foods on glycaemic response of healthy individuals. To achieve this aim, the project was broken down into the following key objectives:

1.3.1 Chapter 3: to determine the starch and non-starch polysaccharide characteristics of *sbeII* mutant wheat that may play a role in starch digestibility and glycaemic response. Hypothesis: It is hypothesised that the increase in amylose proportion in *sbeII* starch is the main driver of reduced susceptibility to digestion by amylolytic enzymes.

1.3.2 Chapter 4: to determine the consequential effects of physical and hydrothermal processing on starch susceptibility to hydrolysis of *sbeII* mutant wheat.
Hypothesis: It is hypothesised that the processing conditions greatly affect *sbeII* starch susceptibility to amylolysis.

1.3.3 Chapter 5: to measure starch susceptibility to hydrolysis *in vitro* in *sbeII* semolina pudding and explore its potential *in vivo* GI.
Hypothesis: It is hypothesised that semolina pudding made from retrograded *sbeII* starch has a lower GI compared to the WT control semolina pudding.

1.3.4 Chapter 6: to evaluate the effect of *sbeII* bread with low starch susceptibility to amylolysis on the post-prandial glycaemic response of healthy individuals.
Hypothesis: It is hypothesised that bread made from retrograded *sbeII* starch elicits a lower glycaemic response compared to a WT control bread.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Food materials

Tetraploid durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.) and hexaploid bread wheat (*Triticum aestivum*) carrying combined mutations in paralogous genes encoding *Starch Branching Enzyme IIa* and *Starch Branching Enzyme* II*b* (*SBEIIa* and *SBEIIb)* used in this thesis were developed by Hazard *et al*. in 2012[11] and Schönhofen *et al.* in 2016[12]. A brief description of the development of these *sbeII* mutant lines is reported in this chapter as background on the materials used. For this project, I used durum wheat that is characterised by two genome copies (A and B) and a bread wheat, characterised by three genome copies (A, B and D). Genes encoding *SBEII* are present in each genome, this is a characteristic of wheat. For this project, I used two wild-type (WT) wheat controls; one WT durum wheat for the experiments involving *sbeII* durum wheat and one WT bread wheat, for the experiments involving *sbeII* bread wheat. The WT control wheats corresponded to the same variety of the durum and bread wheat carrying mutations in *sbeII* genes and were grown in the same environmental conditions.

All food materials were prepared in a food grade area and have documentation evidencing microbiological safety and nutrient composition. Foods for consumption were prepared by a member of the research team with formal Level 2 training in food safety, in compliance with Environmental Health Guidelines.

Food ingredients were purchased from food manufacturing companies based in the UK (Sainsbury's Ltd, Waitrose & Partners and Tesco).

2.1.1.1 Tetraploid durum wheat

The mutant *sbeII* durum wheat used in this project was supplied by Dr Brittany Hazard. Mutant wheat lines carrying mutations in *sbeII a* and *b* genes were selected from a TILLING population of the Desert durum® variety 'Kronos' (Arizona Grains Inc., C Grande, Arizona, USA). Using Sanger-sequencing, lines carrying mutations in the *SBEIIa* and *SBEIIb* genes in the homoeologous A and B genomes were identified, backcrossed to Kronos to reduce the number of background mutations and crossed to each other to combine the different mutations according to the crossing scheme in Figure 2.1.[11]

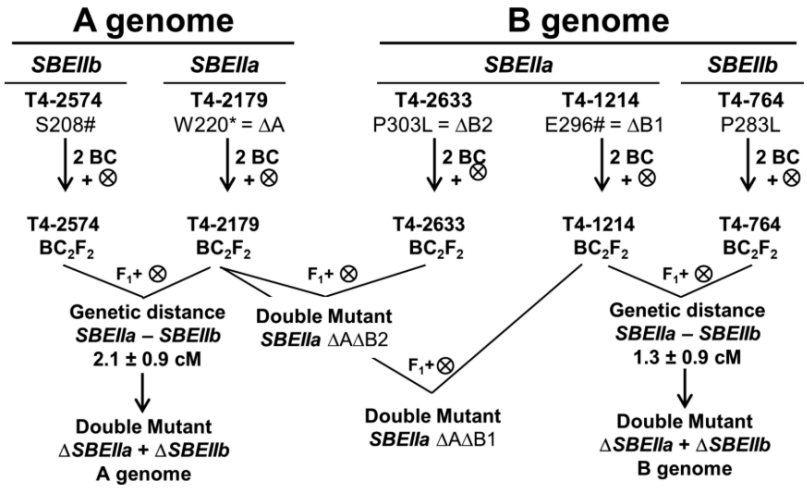


Figure 2.1 Crossing scheme from Hazard et al. 2012 used to obtain sbeIIa/b-AB mutant wheat. *2 BC indicates two backcross generations to the wild type Kronos line and ⊗ indicates self-pollination. Mutation effects are described using a number for the position of the amino acid change, a letter on the left describing the original amino acid, and a letter on the right indicating the new amino acid (* indicates premature stop codon and # a deleted splicing site). Mutations were followed at each stage by sequencing the segregating lines. Figure reproduced here with permission of the authors.*

To bulk seeds, *sbeIIa/b*-AB durum wheat and a WT durum wheat control (Kronos) were grown at the University of California, Davis field station and harvested in Summer 2015. Grains were harvested, shipped to the United Kingdom and stored in food safe area until milling.

Milling of durum wheat grains was carried out by ATC Milling (Berkshire, UK) in August 2016. Grains were conditioned using a two-stage water addition. The samples were de-branned using a Satake laboratory de-branner prior to milling into semolina using a laboratory Bühler mill (method adapted for durum wheat milling). Milled product characteristics were analysed using in-house methods at ATC Milling and a milling report was returned with the material milled (Table 2.1). The extraction rate is the percentage weight (yield) of flour obtained from the grain milled. A 100% extraction rate is expected from wholemeal flour where the entire grain is milled to flour. Refined, white flours have usually a lower extraction rate (~70%) as the bran and germ are discarded before or after milling.[148]

Table 2.1 Milling characteristics of sbeIIa/b-AB wheat grains and WT control wheat grains provided by ATC Milling *(source data not available)*

Parameter	**WT**	***sbeIIa/b*-AB**
Grain moisture (%)	10.2	10.0
Bran dust from de-branning (%)	7.9	10.0
Semolina extraction rate (%)	70.5	62.5
Bran (%)	16.3	20.6
Ash (%) (2h, at 900^{o}C)	0.66	0.67
Bran (specks per cm^{2})	175	247

2.1.1.2 Hexaploid bread wheat

To generate bread wheat *sbeII* mutant lines, durum wheat *sbeIIa/b*-AB mutants were backcrossed twice to Lassik (plant variety protection No. 200800176), a hard-red spring cultivar developed by the University of California, Davis. A mutation in the D-genome copy of *SBEIIa* (*sbeIIa*-D) was selected from a TILLING population of the hard-red spring bread wheat breeding line UC1041+*Gpc-B1/Yr36*, developed by the University of California, Davis[149] and backcrossed to Lassik for five generations. The backcrossed *sbeIIa-D* mutant was then crossed with the backcrossed *sbeIIa/b*-AB hexaploid Lassik line to develop the hexaploid Lassik line with five *SBEII* mutations (*SBEIIa/b*-AB, *SBEIIa*-D), Figure 2.2[12]. This work was carried out by Dr Andre Schönhofen who kindly supplied the mutant grains for this study.

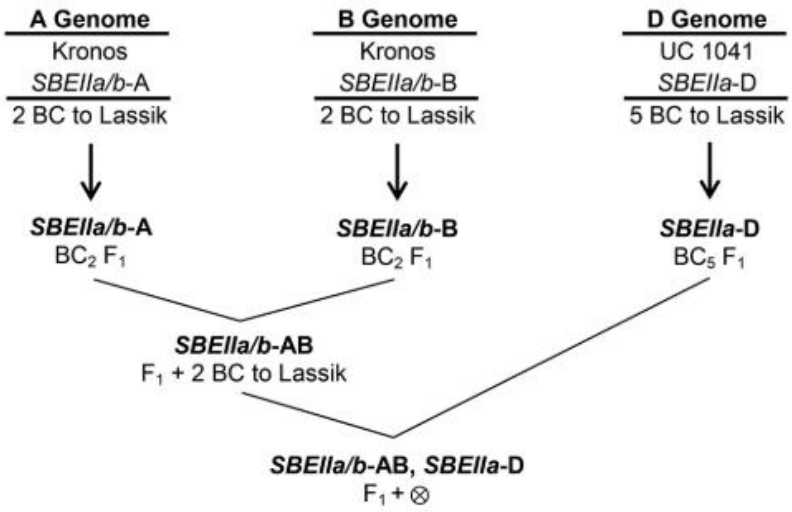


Figure 2.2 Crossing scheme from Schönhofen et al. 2016 used to obtain sbeIIa/b-AB, sbeIIa-D mutant wheat. *BC indicates backcross generations to Lassik; ⊗ indicates self-pollination. Mutations were selected in each cycle by sequencing the segregating lines. Figure reproduced here with permission of the authors.*

sbeIIa/b-AB*, sbeIIa*-D wheat and WT control wheat grains were grown for multiplication at the John Innes Centre Church Farm field station (Bawburgh, Norwich, UK) in 2016 in 6 m^2 plots, coordinated by Dr Oscar Gonzalez. Grains were harvested, bulked according to genotype, then milled by Campden BRI using a Bühler mill after conditioning the grains to 16.4% moisture.

Flour quality analysis carried out by Campden BRI, results were returned in form of a report as presented in Table 2.2. Flour end-use quality is within range reported for other wheat flours analysed using Farinograph method.[150] Based on these results, the food products described in chapter 4 were developed. Flour colour was measured using the CIELAB scale where L* indicates 'brightness/darkness', a* indicates redness/greenness and b* indicates yellowness/darkness. The Falling number[a] measured was higher than what previously reported by Schönhofen *et al.* (2017)[151] for 'Lassik' *sbeIIa/b*-AB, *sbeIIa*-D mutant wheat and WT control however, the Falling number difference between *sbeII* and WT control is consistent to what previously reported. From the Brabender Farinograph analysis, water absorption can be estimated as reported by Sarker *et al.* (2008).[152] Stability and development time are correlated with flour strength and mixing tolerance of the

[a] Falling number is a measure of α-activity/sprout damage in the grain; a low falling number indicates good protein for baking

flour. Development time is similar to what previously reported for 'Lassik' *sbeIIa/b*-AB, *sbeIIa*-D mutant wheat and WT control by Schönhofen *et al*. (2017)[151] however, the water absorption and dough stability where lower in this batch of grains, for both *sbeIIa/b*-AB, *sbeIIa*-D and WT control. The BranScan gives an indication of the amount of bran and aleurone left behind after milling. The bran % appeared to be higher in *sbeII* flour compared to the WT control.

Table 2.2 End use quality of bread flour obtained from sbeIIa/b-AB , *sbeIIa-D and WT control grain; quality analysis was carried out by Campden BRI (source data not available)*

Parameter	**Method**[b]	**WT**	***sbeIIa/b*-AB, *sbeIIa*-D**
Extraction rate (%)	Accredited method by Laboratory Bühler Milling TES-CM-01 (UKAS)	81.2	74.5
CIELAB tristimulus colour	L*	89.42	87.81
	a*	0.63	0.82
	b*	9.93	11.93
Hagberg Falling Number (secs)	TES-CM-106 based on CCAT 06 derived from ICC Standard Method 107/1	440	502
Water absorption (%)	TES-CM-104 based on CCAT 04	54.6	73.5
Dough development time (min)	based on ICC Standard Method 115/1 using a Farinograph	4.5	5.0
Dough stability (min)		5.5	5.5
Ash content (%)	TES-AC-086 by incineration	0.58	0.70
Amount of bran (%)	BranScan TES-CM-064 (non UKAS)	0.50	0.79
Speck count (specks per cm^2)	BranScan TES-CM-064 (non UKAS)	4.6	7.3

A field trial of the *sbeIIa/b*-AB*, sbeIIa*-D mutant and WT control wheat (Lassik) was grown at the John Innes Centre Church Farm field station (Bawburgh, UK) in 2017-2018, coordinated by Dr Oscar Gonzalez. The two genotypes were each sown in eight 6 m^2 plots in a randomised block design in October 2017 ("Autumn" trial) and again in April 2018 ("Spring" trial). Yield and thousand grain weight (TGW) are reported in Table 2.3 as indication of grain performance when grown in the UK. Kernel weight was determined using a Marvin Seed Analyser (Marvitech GmbH) with a sample of approximately 300 grains per plot. The harvested grains were stored in the field trial station until October 2018, when both grain batches (Spring and Autumn) were milled into refined flour by Campden BRI using a Bühler mill.

[b] Techniques used/ Equipment/ Standard specifications (TES)

Table 2.3 Average kernel weight and yield of sbeIIa/b-AB, sbeIIa-D and WT control wheat. *Milling extraction rate is reported here. Values are reported as Means ± SEMs, n=8.*

Genotype	Sowing season	TGW (g)	Yield (tons/ha)	Extraction rate (%)
sbeIIa/b-AB, *sbeIIa*-D	Spring	32.2 ± 0.54 [a]	3.5 ± 0.117 [b]	73.15 ± 0.30 [a]
WT control	Spring	33.8 ± 0.71 [a]	3.9 ± 0.16 [a]	77.97 ± 0.22 [a]
sbeIIa/b-AB, *sbeIIa*-D	Autumn	40.3 ± 0.24 [b]	5.6 ± 0.129 [d]	66.47 ± 1.09 [b]
WT control	Autumn	42.8 ± 0.36 [b]	6.8 ± 0.155 [c]	76.33 ± 0.67 [a]
Source of variation				
Genotype		0.0003	<0.0001	<0.0001
Season		<0.0001	<0.0001	<0.0001
Genotype x Season		0.4	0.0059	0.0008

Different lowercase letters (a, b, c) are significantly different from each other ($p < 0.001$, Two-way ANOVA with Tukey's post-hoc comparison)

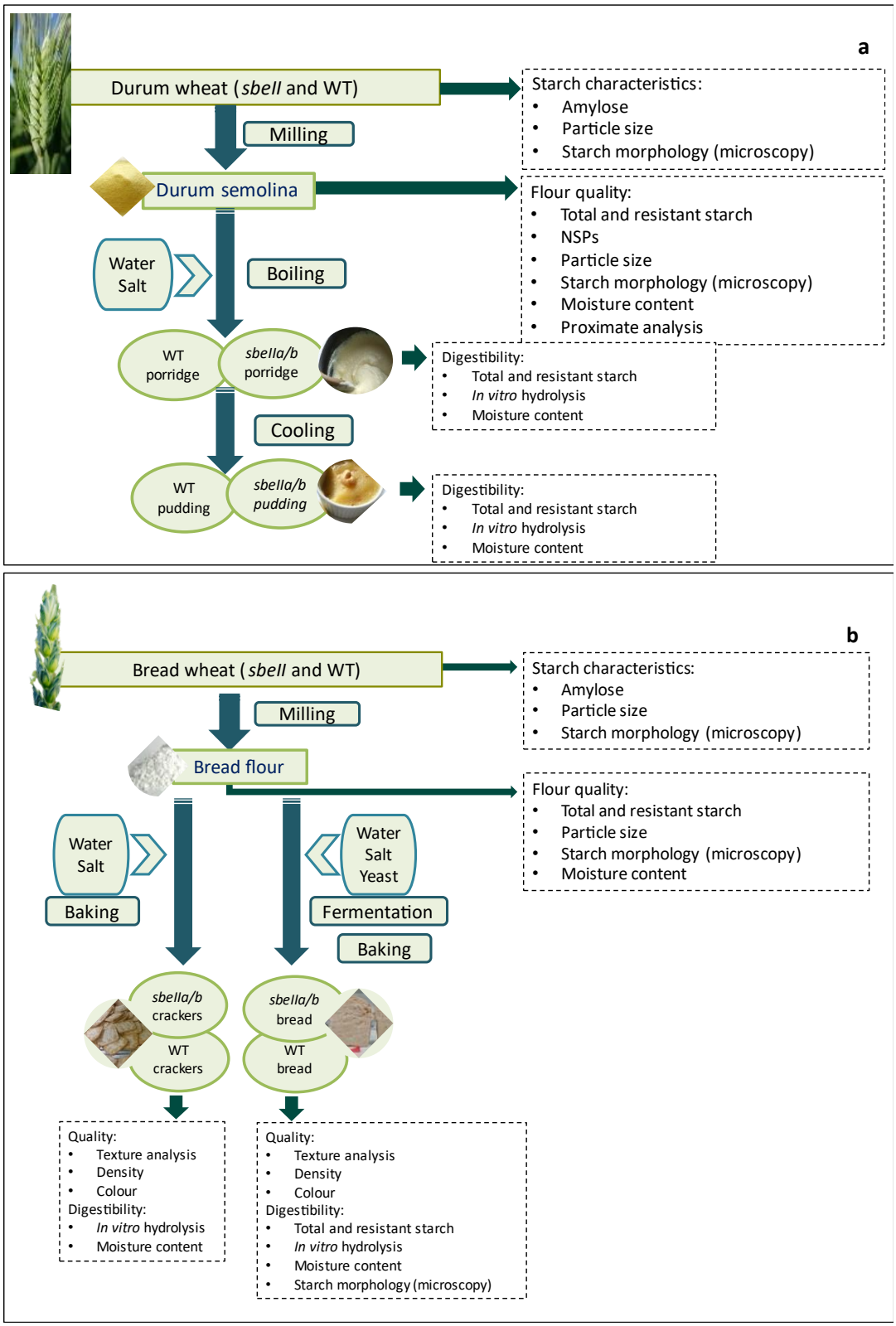
In the following chapters, *sbeIIa/b*-AB will be referred to as '*sbeII* durum wheat' while *sbeIIa/b*-AB, *sbeIIa*-D will be referred to as '*sbeII* bread wheat', to improve clarity.

2.1.2 Chemical reagents

This study used deionised water (Milli-Q water, Millipore, Bedford, MA, USA) throughout the experiments. All the chemicals used were purchased from Thermo Fisher Scientific (Waltham, US-MA) or Sigma Chemical Co. (St Louis, MO, USA), further details are reported for each experiment in the following chapters.

2.2 Methods

Figure 2.3. Methods diagram used at different stages of processing a. durum wheat (sbeII and WT control) and b. bread wheat (sbeII and WT control)



2.2.1 Size distribution analyses

2.2.1.1 Particle size distribution analysis by laser diffraction

Particle size distributions of milled wheat (semolina, flour and starch) were determined using laser diffraction analysis with the Beckman Coulter LS 13 320 Particle Size Analyzer (Beckman Coulter Inc. Brea, CA, US).[153]

Laser diffraction is based on the principle of light scattering: the electrons bound in a material absorb light and re-emit it in a different direction with different intensity. When there is no loss of energy between light absorption and emission, elastic scattering occurs and the intensity of the scattered light can be measured as a function of the particle dimensions and optical properties.[154] Scattered light is used to measure particle dimensions at a standard light wavelength and sample concentration to obtain a clear signal while the refractive index of the media and density of particles are assumed to be uniform throughout the particulate system. Therefore, the scattered intensity is assumed to be a function of the scattering angle, particle shape and size, for particle sizes in the range of microns to millimetres. The LS 13 320 uses the diffraction pattern of a laser beam passed through an object to measure the object's geometrical dimensions. Static light scattering techniques such as laser diffraction are used to measure the volume weighted distribution where the contribution of each particle in the distribution relates to the volume of that particle. The *volume moment mean diameter* (*De Brouckere mean diameter*) is reported in this study to reflect the size of the particles that constitute the bulk of the sample volume, assuming that the particles as spherical.[155]

The instrument set up included a standard cycle of auto-rinse and de-bubble. Offsets were measured (the electrical noise in the detector channel) and the detectors aligned. Then, the background was measured to be below 1.5×10^5. Once the instrument set up was completed, sample suspensions were prepared using deionised water to a concentration of 10 mg/mL. Samples were loaded into the instrument cell with the pump running at 34%, the optical parameters chosen were a particle (starch) and dispersant (water) refractive index of 1.456 and 1.330, respectively.

Size channels in the LS 13 320 are spaced logarithmically and are therefore progressively wider in span toward larger sizes. Statistical calculations were made based on the logarithmic centre of each channel.

2.2.1.2 Size distribution analysis of starch granules by electrical sensing zone

Starch granule size distributions of *sbeII* and WT starch from bread wheat were obtained using the Multisizer 4e Particle Size Analyzer (Beckman Coulter Life Science).

The method is based on the analysis of voltage pulses generated by suspended particles passing through a microscopic opening (aperture), under an electrical current. The amplitude of this pulse is proportional to the volume of the particle. Particles suspended in a weak electrolyte pass through the aperture set between two electrodes with an electric current. When a particle passes through the aperture, it displaces a certain amount of electrolyte that causes a jump in impedance (Figure 2.4). This results in a small change of voltage in the amplifier, which converts variance in current into a pulse. The height of the pulse is proportional to the particle size that is then converted into volumetric units to generate the particle size distribution.

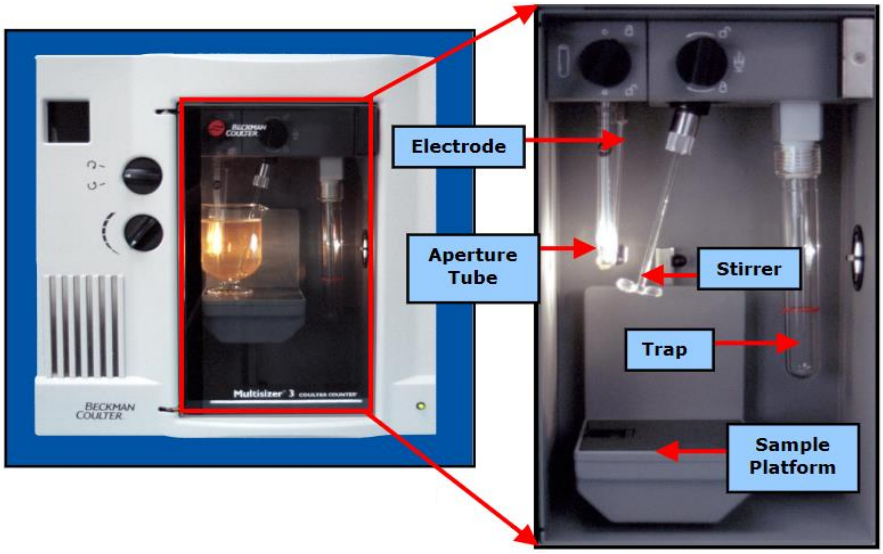


Figure 2.4 Sample compartment of the Multisizer 4e Particle Size Analyzer *highlighting the different instruments components, image from the Beckman counter Multisizer 3 operation manual*

2.2.1.3 Size separation of starch granules by Percoll centrifugation

Size separation of A-type and B-type wheat starch granules is of interest to quantify the percentage of A and B granules and further study their individual physico-chemical characteristics. The separation can be achieved by centrifugation through Percoll. Centrifuging through two Percoll solutions (70 and 90%, v/v) produced purified populations of both A- and B-starch granules. Percoll consists of colloidal silica particles of 15-30 nm diameter (23% w/w in water) coated with polyvinylpyrrolidone (PVP) and it is typically used to isolate to isolate cells, organelles and viruses by density separation.[156] It is also used to

establish non-toxic low viscosity, low osmolarity density gradients to separate cells and subcellular particles.[2] A method was adapted from Peng *et al.* 1999[3] to separate A- and B-granules based on their particle size. The published method required optimisation as the authors did not specify which buffer was used to dilute Percoll or the starch recovery rate after separation. The method also required large volumes of Percoll for granule separation, so a smaller scale method was devised to reduce the cost of the experiment.

2.2.2 Starch composition analyses

2.2.2.1 Total starch

The total starch (TS) is measured as total glucose released by enzymatic digestion from a starch substrate. This method involves the solubilisation and subsequent hydrolysis of starch in the presence of a thermostable α-amylase to produce a range of soluble linear and branched maltodextrins. Maltodextrins are then quantitatively hydrolysed with amyloglucosidase (AMG) to D-glucose, which is measured colorimetrically with a glucose oxidase/peroxidase (GOPOD) reagent.[157] Figure 2.5 shows the enzyme cascade reaction.

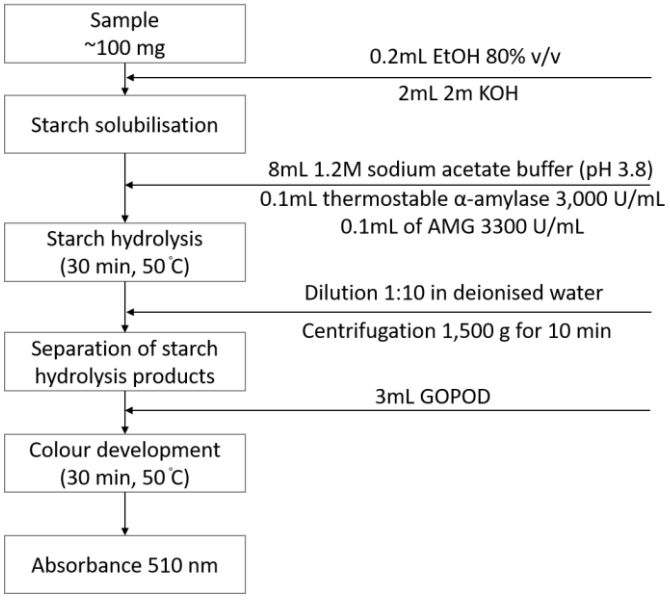


Figure 2.5 Total Starch (TS) method flow diagram

The total starch content of semolina and semolina food products discussed in chapters 3, 4 and 5 was determined using Megazyme's Total Starch Assay Kit. TS of milled materials was measured using the suggested procedure for the determination of starch in samples that do not contain D-glucose and/or maltodextrins (KOH format, AOAC 996.11 and DMSO format - AOAC Official Method 996.11). The potassium hydroxide (KOH) format was used for the

analysis of durum wheat semolina and the durum wheat food products as in most cases a treatment with 2 M KOH is sufficient to release any non-hydrolysed starch. Exception to this are samples containing strong cellular structures or hard particles such as whole wheat grains or legumes. TS content of semolina was later measured using dimethyl sulfoxide (DMSO)[158] (data shown in Appendix A, Figure A2 1). The use of DMSO instead of KOH allowed to measure the finer differences in TS content. Durum wheat is milled to a coarser particle size (semolina) than bread wheat because of the hardness of the grain (as discussed in chapter 3). The use of a stronger solvent like DMSO, may result in a more accurate measurement of the TS content of semolina.

To start the assay, approximately 100 mg sample were weighed into 12 mL glass tubes and wet with 0.2 mL of aqueous ethanol (80 % v/v) to aid dispersion by mixing on vortex mixer (Vortex-Genie® 2, Cole-Palmer). A magnetic stirrer was added to each sample tube and placed in an ice water bath over a magnetic stirrer. Once stirring vigorously, 2 mL of 2 M KOH were added and left to solubilise for at least 20 minutes. After this incubation time, 8 mL of 1.2 M sodium acetate buffer (pH 3.8) were added to each tube while stirring on the magnetic stirrer followed by 0.1 mL of thermostable α-amylase (3,000 U/mL on Ceralpha reagent at pH 6.5 and 40°C or 1600 U/mL on Ceralpha reagent at pH 5.0 and 40°C) and 0.1 mL of amyloglucosidase (AMG, 3300 U/mL on soluble starch at pH 4.5 and 40°C). Tubes were then placed in a water bath at 50°C for 30 minutes and mixed on a vortex mixer every 10 minutes. At the end of the incubation, samples that were expected to have more than 10% total starch were diluted 1:10 using a volumetric flask, before centrifuging them at 1,500 g for 10 min. For the colorimetric determination, 0.1 mL of the diluted solution (supernatant) was incubated with 3 mL of GOPOD reagent at 50°C for 20 minutes. GOPOD reagent was prepared by dissolving the GOPOD Reagent Enzymes (glucose oxidase plus peroxidase and 4-aminoantipyrine, supplied as freeze-dried powder) in the GOPOD Reagent Buffer (pH 7.4, p-hydroxybenzoic acid and sodium azide 0.4 % w/v). In the colorimetric step, D-Glucose is oxidised to D-gluconate with the release of equimolar amounts of hydrogen peroxide (H_2O_2) that was quantitatively measured in a colorimetric reaction employing peroxidase and the production of a quinoneimine dye[159, 160]. Replicates of 0.1 mL of D-glucose standard and blank solution (deionised water) were also included in the colorimetric step. Absorbance of samples and standards were read at 510 nm against the reagent blank using a Biochrom™ WPA Biowave II UV-Vis Spectrophotometer (Biochrom Ltd. Cambridge,

UK). Starch content on a dry weight basis was calculated by converting the absorbance of samples to the amount (µg) of glucose released during digestion, taking into account the incubation volume and any dilution steps, the amount of sample weighed and the moisture content of the sample when weighed.

2.2.2.2 Resistant starch

Resistant Starch (RS) is defined as the portion of the starch that resists hydrolysis by amylolytic enzymes[161]. Numerous methods have been developed for the *in vitro* measurement of RS. The Megazyme assay kit (AOAC 2002.02) is a straightforward method widely used across disciplines to measure starch resistance to digestion. Because it is widely used, it allows for comparison of RS data generated by different research groups, representing a standardised method for carbohydrate characterisation in cereals and cereal-based products. The method involves the incubation of samples with pancreatic α-amylase and AMG for 16 hours at 37°C, during which time non-resistant starch is hydrolysed to D-glucose by the combined action of the two enzymes. The reaction is stopped by the addition of an equal volume of ethanol and the RS is recovered as a pellet on centrifugation. The RS pellet is then solubilised using KOH and the pellet is hydrolysed to D-glucose using AMG. D-Glucose is measured with glucose oxidase/peroxidase reagent (GOPOD) as described in the TS method. Figure 2.6 shows the enzyme cascade reaction. This method was used to quantify RS in semolina and semolina food products discussed in chapters 3, 4 and 5.

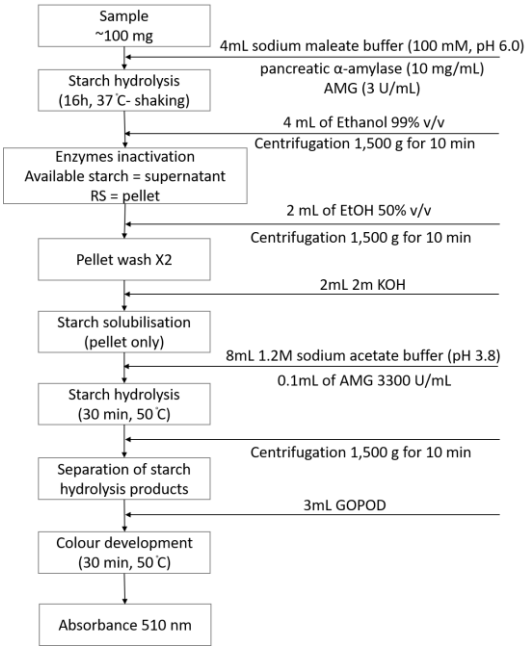


Figure 2.6 Resistant Starch (RS) method flow diagram

To start the assay, approximately 100mg sample were weighed into 12 mL glass tubes and 4 mL of pancreatic α-amylase (10 mg/mL) containing AMG (3 U/mL) in sodium maleate buffer (100 mM, pH 6.0) was added to each tube to hydrolyse available starch. The tubes were mixed on vortex mixer (Vortex-Genie® 2, Cole-Parmer Instrument Co. Ltd, UK) and incubated for exactly 16 h at 37℃ with continuous linear motion mixing (200 strokes/min). At the end of the incubation period, hydrolysis was stopped by adding 4 mL of ethanol (99% v/v) and stirring vigorously on a vortex mixer. Tubes were then centrifuged at 1,500 g for 10 min to separate the pellet (RS) from the products of digestion in the supernatant (available starch). The pellet was re-suspended in 2 mL of 50% ethanol and centrifuged (Eppendorf™ 5810R Centrifuge) at 1,500 g for 10 min and the supernatant was decanted. This step was repeated a second time, after which, the pellet was left to dry by inverting the tubes on paper to drain the excess of liquid. A magnetic stirrer was added to each sample tube and the tubes were placed in an ice water bath over a magnetic stirrer. Once stirring vigorously, 2 mL of 2M potassium hydroxide (KOH) was added and left to solubilise the RS for at least 20 minutes. After this incubation time, 8 mL of 1.2 M sodium acetate buffer (pH 3.8) was added to each tube while stirring on the magnetic stirrer followed by 0.1 mL of amyloglucosidase (AMG, 3,300 U/mL). Tubes were then placed in a water bath at 50°C for 30 minutes and mixed on a vortex mixer every 10 minutes. At the end of the incubation, samples were centrifuged at 1,500 g for 10 min. For the colorimetric determination, the same GOPOD method described previously for TS determination was used. RS content on a dry weight basis was calculated by converting the absorbance of samples to the amount (µg) of glucose released during digestion, taking into account the incubation volume and any dilution steps, the amount of sample weighed and the moisture content of the sample when weighed.

2.2.2.3 Small scale method for TS and RS measurements

TS and RS content of the fractionated starch granules described in chapter 3 and bread rolls described in chapter 6 were measured using a modified version of the Megazyme starch quantification method as described by Edwards *et al.* 2014.[162] This small-scale high-throughput method required less sample and reagents than the Megazyme assay. As the Megazyme assay, this method is also based on the Englyst assay[161] requiring α-amylase and AMG incubation for 16 hours. After 16 hours of incubation, the soluble fraction (the supernatant) containing the product of digestion categorised as 'available starch' was

separated from the pellet, the 'resistant starch'. The pellet was solubilised in DMSO (99%, v/v) and then hydrolysed by α-amylase. Both pellet and supernatant were then incubated with AMG and the product of digestion were quantified using colorimetric determination (full description below). By hydrolysing the pellet with α-amylase before AMG, the RS concentration detected in the WT control and *sbeII* samples was higher compared to the RS measured with the Megazyme assay. Without the α-amylase hydrolysis step, the Megazyme assay may have resulted in underestimated RS content of wheat materials.

To start the analysis, each sample was suspended in 1 mL of pancreatic α-amylase and amyloglucosidase (AMG), mixed on vortex mixer (Vortex-Genie® 2, Cole-Palmer) to ensure suspension and incubated at 37°C in an incubator (New Brunswick™ Excella® E24/E24R Shaker) for 16h with end over end mixing using a blood rotator (Grant rotator, settings: 40 rpm orbital, 02; 35-degree reciprocal, off; 5-degree vibration, off).
The enzyme solution was prepared by adding 250 µL of AMG (Megazyme K-TSTA-100A, 300 U/mL on soluble starch at pH 4.5 and 40°C) to a 10mg/mL pancreatic α-amylase solution (Pancreatin, 10 g, 3 Ceralpha Units/mg) in sodium maleate buffer (100 mM, pH 6.0 plus 5 mM calcium chloride).
At the end of the incubation time, samples were centrifuged for five minutes at 17,000 x g to separate the supernatant containing mostly the products of digestion, from the pellet, containing the starch that resisted digestion. After centrifugation, the supernatant was decanted carefully to avoid pellet contamination and it was kept for quantification of "available starch" concentration. The pellet was re-suspended in 100 µL of ethanol (50% v/v deionised water) and centrifuged (Heraeus™ Fresco™ 17 Microcentrifuge) for five minutes at 17,000 x g to precipitate the undigested fraction. After centrifugation, the ethanol fraction containing only the shorter chains or 'sugar' was discarded. The washing step was repeated using 300 µL of ethanol (100% v/v). After discarding the supernatant, the pellet was left to dry for 30 minutes to allow the ethanol to evaporate. The pellet and supernatant previously obtained were used to quantify the resistant and available starch present in the sample according to a total starch method adapted from Megazyme. The pellet was solubilised by adding 200 µL of DMSO and incubated in boiling water bath (Grant Instruments™ SUB Aqua Pro Water Bath) for 16 minutes with intermitting mixing on a vortex mixer (Vortex-Genie® 2, Cole-Palmer). At the end of the incubation, 300 µL of thermostable α-amylase (Megazyme cat. no. E-BSTAA, 100 U/mL on Ceralpha reagent at pH

6.5 and 40°C) was added to the pellet samples and incubated for 6 minutes at approximately 85°C using a water bath. At the end of the incubation time, pellet samples were transferred to a 50°C water bath to equilibrate. Meanwhile, 100 µL of the supernatant samples obtained after the 16 hours incubation with AMG and α-amylase were diluted with 400 µL of sodium acetate buffer (100 mM, pH 5.0) plus calcium chloride (5 mM) and transferred to a 50°C water bath to equilibrate. To complete the digestion process, 5 µL of undiluted AMG (3,300 U/mL on soluble starch at pH 4.5 and 40°C) was added to both sets of tubes (pellet and supernatant) and incubated at 50°C in a water bath for 30 minutes.
The glucose produced from starch digestion in the available fraction (supernatant) and resistant fraction (pellet) were quantified using a colorimetric glucose oxidase assay.
For the colorimetric determination, 1 mL of GOPOD reagent, prepared according to the Megazyme assay procedure (described above), was added to 33 µL of sample and incubated at 50°C in a water bath for 30 minutes. Prior to incubation, samples were diluted depending on the expected starch content. The available starch fractions (supernatants) were diluted 1:4 with sodium acetate buffer (100 mM, pH 5.0 plus calcium chloride 5 mM) as a lower concentration of reducing sugars was expected to be released from this fraction compared to the resistant fractions (pellet) that were diluted to 1:15 with sodium acetate buffer.

2.2.2.4 Starch isolation

Starch can be isolated from intact grains or from milled grains (flour). Isolated starch was used for amylose determination, microscopy, granules separation by size and granule size analysis later described.

To isolate starch from wheat grains, approximately 50 wheat grains were left in a petri dish on moistened filter paper overnight at 4°C: this allowed the grains to absorb water, facilitating the removal of the husk and the germ. They were then ground using a pestle and a mortar with approximately 5 mL of deionised water, after removing the husk and embryo manually. The extract was then filtered through a layer of Miracloth (475855, Calbiochem®) by washing with 30 mL of deionised water.

When using flour, approximately 10 g of flour was washed through a layer of Miracloth with 30 mL of deionised water, to separate the starch form the larger particles such as aleurone or cells debris.

The suspension was centrifuged, at 2000 g for five minutes and the pellet recovered was washed with 30 mL of 2% (w/v) aq. sodium dodecyl sulfate (SDS). SDS is used to remove the non-starch components such as proteins and lipids. The pellet was washed and centrifuged twice in 5 mL of 2% SDS. The resulting starch pellet was washed a further three times with 5 mL of acetone and left to dry overnight in the fume hood to ensure complete evaporation of the solvent.[163]

2.2.2.5 Amylose determination

Determination of the amylose content of starch can be achieved by measuring the blue amylose-iodine complex colorimetrically. The formation of this complex requires triiodide (I_3^-) ions to initiate the reaction. DMSO solubilises starches and iodine to form triiodide ions.[164, 165] After an overnight incubation of starch in a solution of DMSO and iodine, an aliquot of the solution is diluted in deionised water to allow the amylose-iodine complex to form. From the absorbance reading, the apparent amylose content is calculated using a standard curve obtained from pure amylose. The amylose detected is then adjusted for amylopectin binding iodine as explained by Knutson *et al*.,[164, 166] according to Equation 1

$$\%\ Amylose = \frac{\%\ apparent\ amylose - 6.2}{93.8}$$

Equation 1: Calculation of amylose % adjusted for amylopectin binding iodine

To measure amylose content of starches, a 0.05% (w/v) suspension of starch (isolation as described above) in 6mM iodine solution (0.229 g of re-sublimed iodine in 270 ml of DMSO and 30 mL of deionised water) was left to dissolve on a vertical multifunction rotator (PTR 35, Grant BIO) providing end over end mixing overnight (Figure 2.7). Samples were then diluted 1:10 with deionised water and left for 30 min to allow the iodine-amylose complex to form a stable colour prior to reading the absorbance at 600 nm (Benchmark™ Plus Microplate Reader, Bio-Rad) against the reagent blank. Apparent amylose content was calculated using a standard curve of the amylose standards (1-5 mg potato amylose type III, Sigma-Aldrich)[167, 168].

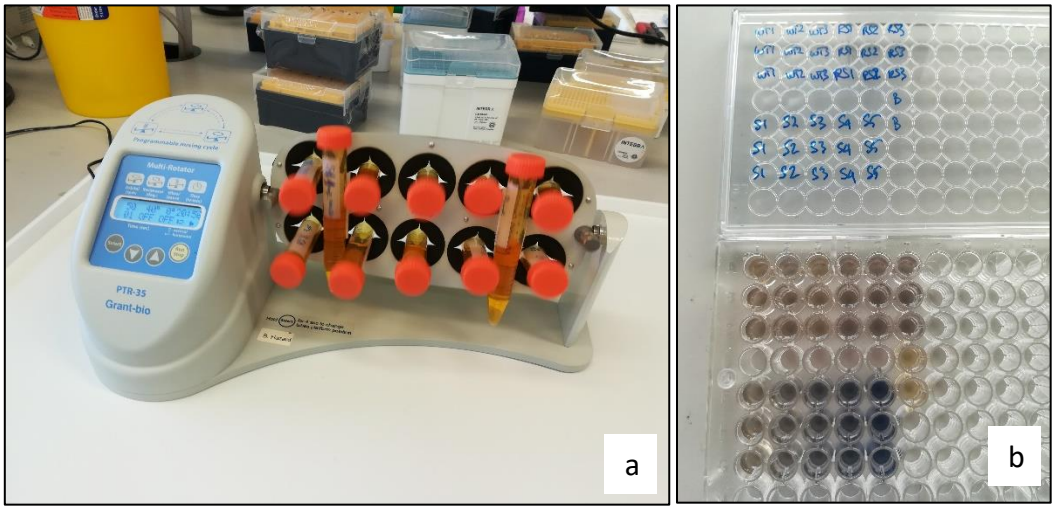


Figure 2.7 Amylose determination assay set up*: a. overnight incubation with end over end mixing and b. colour development in a 96 well-plate. The bottom 3 lanes are the amylose standards (1-5 mg potato amylose type III).*

2.2.2.6 Moisture

Moisture content of the raw and hydrothermally processed semolina and flour was determined using the AACC (44-15A) air oven method, one stage procedure, involving the measurement of sample weight before and after drying to determine moisture content of the sample.[169] A modified version of the same method was implemented for small amounts of flour/semolina material. Disposable aluminium tins were washed, dried and stored in a heated cabinet until needed. The tins were placed in the oven at 130℃ for one hour and placed in a desiccator for hour to cool down. Each tin was weighed, and the weight was recorded. About 100 mg of sample was placed in each tin, each sample was analysed in triplicate. The tins containing the samples were kept in a desiccator while measuring the samples to prevent moisture absorbance from the environment. The samples were placed in an air-oven at ~140℃, a thermometer was placed inside the oven to monitor internal temperature. Once the oven reached 140℃, the timer was set for 16 hours. After 16 hours, the tins were carefully removed from the oven and placed immediately in the desiccator to cool for at least one hour; the weight was recorded. Variation between replicates was <0.2%.

2.2.2.7 Starch morphology

2.2.2.7.1 Widefield light microscopy

Widefield light microscopy was used to visualise starch granule shape and size.

In the Olympus BX60 upright microscope, the whole specimen is exposed to light and the image is captured by a digital camera.

Isolated starch and wheat semolina or flour were used as is (dry) as well as in a suspension with deionised water. Starch and grain sections (obtained using a microtome) were stained with Lugol solution (aqueous iodine solution, 4% v/v) to visualise the increase in amylose content of starch granules from *sbeII* wheat. Iodine is used to stain high-amylose starches because of the prevalence of 1,4-linkages in amylose chains resulting in a right-handed helix. Because of its structure, amylose forms strong complexes with iodine producing a very intense blue colour visible at λmax = 620 nm or using a light microscope.[170]

2.2.2.7.2 Scanning Electron Microscopy

Scanning Electron Microscopy was used to visualise starch granules within the grain as well as isolated starch.

The Zeiss Supra55 VP FEG Scanning Electron Microscope (SEM) uses a focused beam of electrons to generate detectable signals of a solid specimen. When the electrons generated from the "gun" reach the sample targeted, they interact with it generating a series of signals: secondary electrons originated from the atoms of the sample (Figure 2.8a). They have lower energy that the electrons emitted as a result of inelastic interactions between the electron beam and the sample and they are used to produce SEM images capturing morphology and topography of samples' surfaces.[171]

Biological samples are often non-conductive and therefore require a coating of a conductive material to improve the quality of the image. A layer of gold coating is added by sputtering using a high-resolution sputter-coater (Agar Scientific Ltd) to remove the "charging" effect that creates artefacts when imaging (Figure 2.8b). Samples were imaged on the same day of coating to avoid coating degradation. To image the samples, stubs with samples were mounted on the stage inside the microscope chamber. To open the chamber door, N2 gas was introduced in the vacuum chamber to bring it to atmospheric pressure. One all samples were mounted; the chamber was closed and the pump was activated to re-create the

vacuum. Using the internal camera, the stage was placed closer to the lens. The electron high tension (EHT) was turned on with low accelerating voltages, and the secondary detectors were selected to image the specimen.

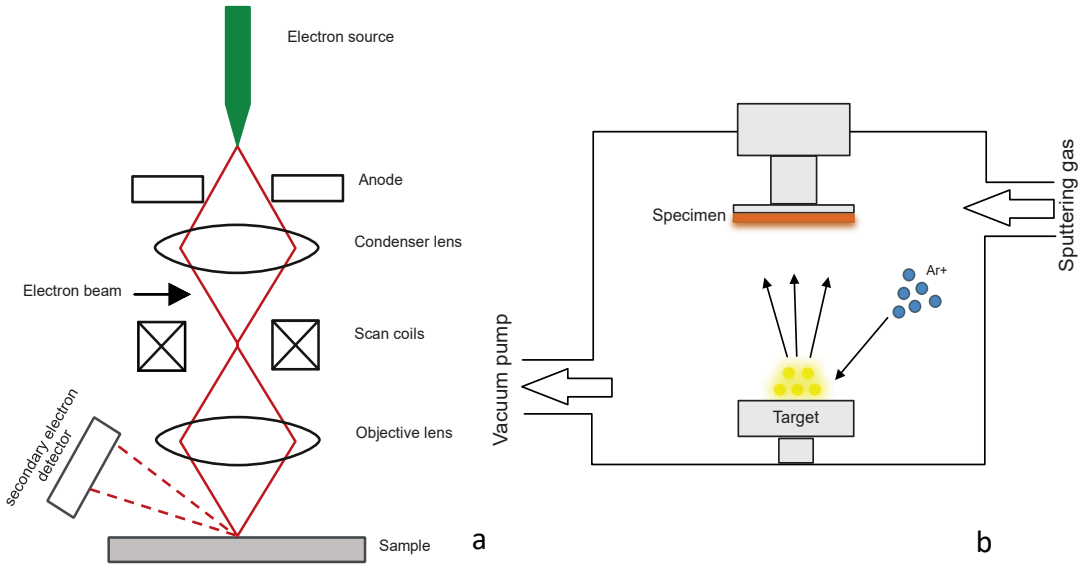


Figure 2.8 SEM diagram. *a. Electron flow from the source ("gun") to the sample. Secondary electrons are detected to create the SEM image. b. Sputter coating process: gold target is bombarded with heavy gas atoms (Ar^+) and metal atoms are ejected from the target by the ionized gas, they cross the plasma to deposit onto any surface within the coating unit including the specimen.*

2.2.3 *In vitro* starch amylolysis

Analysis of starch amylolysis is of great interest to determine starch susceptibility to digestion and to predict glycaemic index (GI) in a range of carbohydrate foods. An enzyme-based method can be a powerful tool to screen different foods for their potential GI, before investing in clinical studies. The rate of starch amylolysis measured *in vitro* was shown to correlate with the increase in venous glucose concentration observed *in vivo.*[85, 172]

Starch amylolysis follows a pseudo first-order reaction, in which the rate of hydrolysis slows down over time because of the increased concentration the products of starch digestion and substrate exhaustion.[173] In a single-enzyme system, the rate of the reaction is dependent on the enzyme-substrate ratio. To measure differences in starch susceptibility to amylolysis, the enzyme-substrate ratio is standardised, based on the catalytic ability of the enzyme. A unit of amylase activity is defined as the amount of α-amylase that catalyses the conversion of one micromole of substrate per minute under the specified conditions of the assay method, as explained below.

A Michaelis-Mentel model can be used to calculate the rate of the reaction by fitting a first order equation to the experimental data (Equation 2), where the end point of the reaction is expressed as C_t representing the percentage of starch digested.[174] However, as the rate of the reaction decreases over time, plots of the hydrolysis product formed (or starch digested) against time are logarithmic. A Logarithm of Slope (LOS) can be used to determine starch amylolysis of the first order equation (Equation 2) in logarithmic form (Equation 3), which gives a linear plot describing the relationship between LOS digestibility constants, *k* and C_∞, and time of amylolysis, *t*.[162]

$$Ct = C_\infty (1 - e^{-kt}))$$

Equation 2: First order equation where Ct is the concentration of product at a given time (t), C_∞ is the product concentration at the end of the reaction, and k is the amylolysis rate constant

$$\ln\left(\frac{dC}{dt}\right) = - kt + \ln(C_\infty k),$$

Equation 3: Logarithmic form of the first order equation (eqn 2)

Here, *Ct* represents the concentration of the digestion product at time (*t*), C_∞ is the product concentration at the end of the reaction time and k is the digestibility rate constant. C_∞ can also be expressed as the percentage of starch digested over the total starch content of the sample. When endogenous sugar due to processing is present at the start of the reaction, the baseline sugar value (Y0) is summed to C_∞ to estimate the percentage of starch digested in time (*t*).[173]

The use of a Logarithm of Slope analysis has the advantage that rate and extent of hydrolysis can be determined reflecting the proportion of starch digested within a certain amount of time.[34] These parameters have been shown to correlate well with glycaemic index of starch-based foods as measured *in vivo.*[174] Another parameter that was shown to correlate well with GI measured *in vivo* is the percentage of starch digested after 90 minutes (C_{90}) that can be used for direct comparison of starch susceptibility to hydrolysis *in vitro*.[174]

To measure starch amylolysis, samples were suspended in 5 mL of PBS (Phosphate Buffer Saline tablets pH 7.4 at 25C, Sigma-Aldrich P4417) and allowed to equilibrate at 37 °C for 10 min with continuous end over end mixing using a blood rotator (Grant rotator, settings: 30 rpm orbital, 02; 35-degree reciprocal, off; 5-degree vibration, off). A 'blank' aliquot (100 µL) of each sample was taken into 1.5 mL microfuge tubes containing 100 µL of 0.3 M Na_2CO_3

(pH 9). The details of the sample preparation can be found in the following chapters' method sections.

To start the assay, porcine pancreatic α-amylase (EC 3.2.1.1, supplied in a DFP-treated suspension of 2.9 M NaCl containing 2 mM $CaCl_2$, A6255, Sigma-Aldrich Co. Ltd, Poole, UK) was added to achieve an activity of 2 U/mL in the digestion mixture (i.e., containing ~5 mg/mL starch). The tubes were promptly returned to the rotary mixer and incubated at 37 °C for the duration of the digestion. After 3, 6, 9, 12, 15, 30, 45, 60, 75, 90 min, aliquots (100 µL) of the digestion mixture were sampled and diluted 1:2 in 0.3 M Na_2CO_3 to stop the reaction. Aliquots were centrifuged at 12,500 g for 5 min to separate any starch remnants from the supernatant used for analysis of the starch amylolysis products. The aliquots collected from the digestion mixture were analysed for total reducing sugars (i.e. starch digestion products) using the PAHBAH assay that is sensitive to maltose and maltotriose products of amylolysis. The supernatants were diluted 1:10 in deionised water, and 100 µL of the diluted sample was added to 1 mL of freshly prepared PAHBAH working reagent (250 mg p-hydroxybenzoic acid hydrazide dissolved in 4.75 mL of 0.5 M HCl and made up to 50 mL with 0.5 M NaOH). Standards containing known concentrations of maltose (0 - 1000 µM) were included. Samples and standards were incubated at ~99 °C for 5 min and subsequently equilibrated for 10 min at room temperature before transferring them to a clear plastic flat bottom 96-well plate to measure absorbance (λ = 405 nm) in a microplate reader (Bio-Rad Benchmark Plus, Waukegan, Illinois, USA). Reactions of reducing sugars with hydrazides of benzoic acid derivatives, in strong alkali, gives a yellow colour, linked to the formation of anionic forms of carbohydrate hydrazones. Reducing sugars were expressed as maltose equivalents by reference to a standard curve. Blank values taken for each assay prior to enzyme addition (Y0) represent the baseline sugar value and were used to account for any endogenous sugar.

2.2.3.1 Amylase activity

Amylase activity was determined by assaying hydrolysis of potato starch using the starch amylolysis method previously described. One unit of activity liberates 1 mg maltose from starch in three minutes at pH 6.9, 20 °C that corresponds to 1 IU/mg protein at 20 °C.[49] Since the amylolysis assay is carried out at 37 °C, the activity of the enzyme was also tested at 37 °C as described below.

A 5 mg/mL suspension of soluble potato starch was heated with continuous stirring in a boiling water bath (~ 90 °C) for 20 minutes to ensure complete gelatinisation of starch (Figure 2.9).

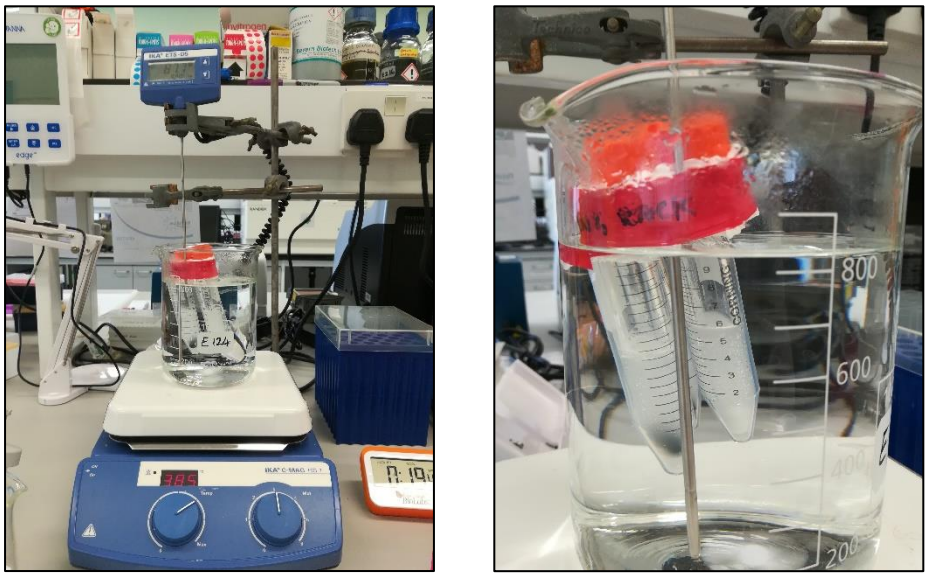


Figure 2.9 Starch gelatinisation set up. *To reach complete and uniform starch gelatinisation, triplicates of a starch in water suspension were placed in a beaker with boiling water. Water was heated using a hot plate stirrer (IKA® C-MAG HS hotplate stirrers), continuous stirring ensured a homogeneous water temperature. Each tube contained a magnetic stirrer (flea), water temperature was controlled*

For the activity determination, 5 mL of the gelatinised starch suspension was transferred in triplicates to 15 mL Falcon tubes and left to equilibrate at 37 °C for 10 minutes with continuous end over end mixing using a blood rotator (Grant rotator, settings: 30 rpm orbital, 02; 35-degree reciprocal, off; 5-degree vibration, off). A 'blank' aliquot (100 µL) of each sample was taken into 1.5 mL microfuge tubes containing 100 µL of 0.3 M Na_2CO_3 (pH 9). To start the assay, 100 µL of porcine pancreatic α-amylase (EC 3.2.1.1, supplied in a DFP-treated suspension of 2.9 M NaCl containing 2 mM $CaCl_2$, A6255, Sigma-Aldrich Co. Ltd, Poole, UK) working solution (prepared as 5uL of supplied stock into 5mL PBS) was added to the digestion substrate. The tubes were promptly returned to the rotary mixer and

incubated at 37 °C for the duration of the digestion. After 3, 6, 9 and 12 minutes, aliquots (100 µL) of the digestion mixture were sampled and diluted 1:2 in 0.3 M Na_2CO_3 to stop the reaction. Aliquots were centrifuged at 12,500 g for 5 min to exclude any starch remnants and prepared for subsequent analysis of starch amylolysis products. The aliquots collected from the digestion mixture were analysed for total reducing sugars (i.e., starch digestion products) using the PAHBAH assay as previously described.

Reducing sugars were expressed as maltose equivalents by reference to a standard curve and the linear rate of maltose released every 3 minutes (mg/mL) was calculated.

2.2.4 End-use quality

End-use quality of wheat-based foods products is determined by the flour composition (protein content, starch properties, etc.) as well as the processing used to produce a certain food. Texture Profile Analysis (TPA) of baked foods, such as bread or crackers, is a useful test to determine foods textural properties.[175] A texture analysis test requires compressing the test food using on a Texture Analyser (TA-XT2, Stable Micro Systems, Godalming, UK) to mimic the food behaviour when chewed. Briefly, a specific probe is fitted to the TA-XT2 and a section of food is mounted on the platform below the probe, Figure 2.10 (a,b). Distance between probe and sample, speed for compression and withdrawal are set based on the type of sample analysed: more details of the settings can be found in chapter 4 and chapter 6 together with a description of the samples used.

From the TPA trace, several parameters can be calculated depending on the type of food (solid or semi-solid) and the texture feature of interest.[176] In baked foods, the hardness, springiness, cohesiveness chewiness and resilience are the most common (Figure 2.10c). For baked goods with very low moisture content, fracturability is also a commonly estimated parameter. The *hardness* value is the peak force that occurs during the first compression. The *springiness* indicates how well a product physically springs back after it has been deformed; it is measured as the distance (as time difference or actual distance) of the detected height during the second compression divided by the original compression distance (Distance 2 / Distance 1 or Time 2/Time 1). The *cohesiveness* is a measure of product structural integrity and it is calculated as the area of work during the second compression divided by the area of work during the first compression and indicates how well a product withstands a second deformation (Area 2/Area 1). The *chewiness* is

calculated as *gumminess* (Hardness * Cohesiveness) * *springiness*. Since most baked foods in this study were solid, the gumminess was not reported as it applies only to semi-solids. *Resilience* indicates how well a product regains its original height after compression and it is calculated as the upstroke energy of the first compression divided by the downstroke energy of the first compression (Area 4/Area 3).

TPA does not only provides insight in the textural properties of a food but may also provide objective measures of food sensory characteristic, in an objective manner. Some of the TPA parameters described above are known to relate with *in vivo* sensory attributes.[177] However, the interpretation of such relationship should be treated with caution. For example, while hardness and springiness are linearly correlated *in vitro* and *in vivo*, the relationship between sensory and instrumental data certain parameters, such as cohesiveness and chewiness, is non-linear and requires data transformations or non-parametric statistical tests. Furthermore, the accuracy of the sensory data also relies on the accuracy of the panellists' perception of texture, often not trained to recognise specific attributes.[178]

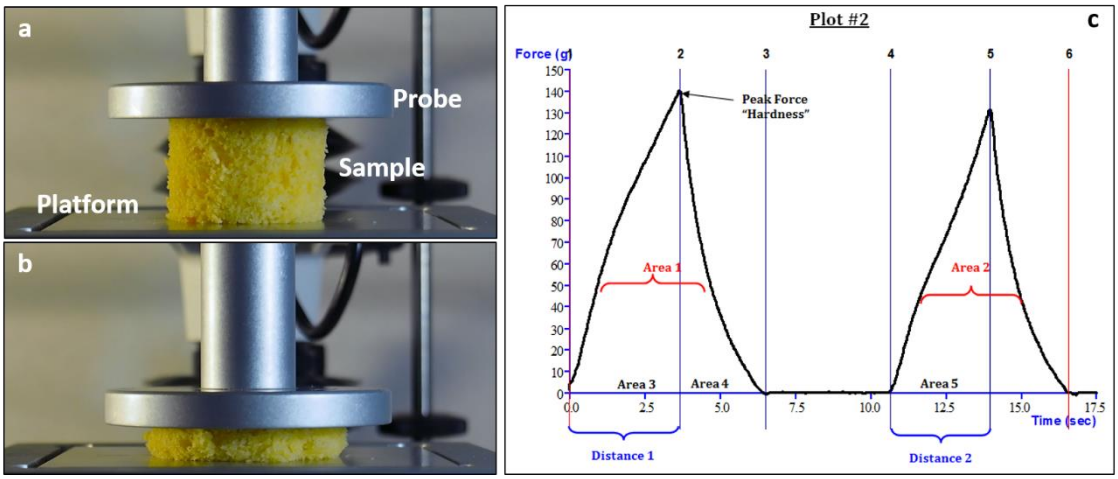


Figure 2.10 Texture Profile Analysis *compression test (a. before compression, b. after compression). c. Texture profile trace used to calculate the parameters described above. Images from Stable Micro Systems, https://texturetechnologies.com/.*

2.2.5 Statistical analysis

Statistical analyses, graphs and incremental areas under the curve (iAUC) were obtained with R.[179] Datasets were curated using the *reshape2*[180] package and plots were obtained using the *ggplot2* and *ggpmisc* package.[180-182] Further details of data analysis and statistical tests are described for each experiment in the following chapters. Data are presented as means ± SEMs unless stated otherwise.

Chapter 3 Determinants of *sbeII* wheat physico-chemical properties: a focus on starch and non-starch polysaccharides

Abstract

In wheat, starch branching enzymes (SBEs) play a major role in defining the starch molecular structure. Mutations in *sbeII* genes are known to cause alterations in starch structure; visual assessment of starch morphology and measurements of amylose and RS content of wheat can be used to detect such changes (Figure 3.1). In this chapter, these parameters were investigated in *sbeII* durum and bread wheat, together with another starch characteristic: the granule size. Besides starch, NSP contribute to the carbohydrate content of milled wheat, originating from the starchy endosperm or bran left behind after milling. These are a minor component in refined flour or semolina, yet they can influence the viscosity of carbohydrate-based foods and therefore their physiological impact on digestion. The NSP molecular structure of milled *sbeII* wheat was determined in this study.

Gaining an understanding of the chemical and physical properties of carbohydrates in novel wheats like *sbeII* wheat may allow better understanding of their importance in human physiology and health.[183]

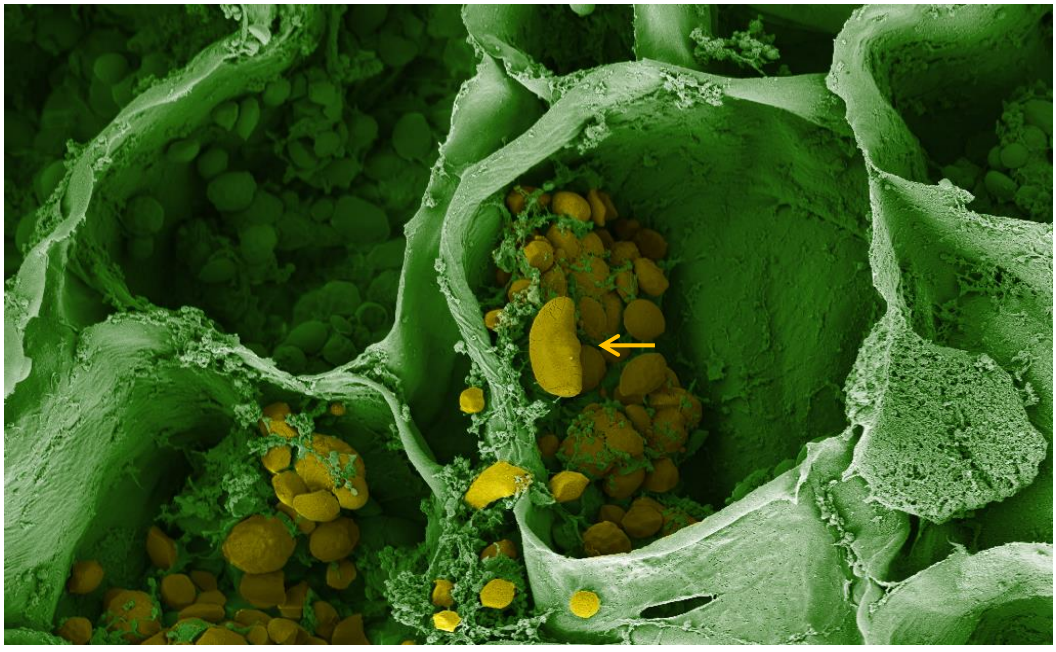


***Figure 3.1 Chapter 3 visual abstract**: sbeII wheat grain section during development (9 days post anthesis). One starch granule already shows the characteristic change in morphology from flat disk to crescent shape (yellow). The SEM image was obtained from a grain section (EHT = 3.00 kV, Mag = 6.00 KX). Grains were fixed by critical point drying before slicing in half and mounting on a SEM stub for sputter coating. The image was coloured using photoshop to highlight the cell wall in green and the starch granules in yellow.*

3.1 Introduction

This chapter presents some of the intrinsic characteristics of *sbeII* wheat that may play a role in designing novel wheat foods with lower starch susceptibility to hydrolysis aimed to elicit lower glycaemic responses. Intrinsic properties refer to the structural characteristics of starch and non-starch polysaccharides in the grain while extrinsic properties refer to effects of processing on starch properties,[184] discussed in chapter 4.

3.1.1 Intrinsic starch composition: structure and morphology

Starch is a complex carbohydrate made of amylose and amylopectin packed into granules of different sizes. Starch granules have a complex internal structure as shown in Figure 3.2; they are thought to have an amorphous core of mostly amylose, surrounded of alternating semi-crystalline and amorphous regions (100–500 nm), organised radially into growth rings. The semi-crystalline growth rings are made up of blocklets (25–100 nm), which consist of amylopectin and amylose arranged into alternated crystalline and amorphous lamellae with a periodicity of 9 nm on average.[28] The crystalline lamellae are formed by double helices of adjacent amylopectin chains; amylopectin branch points at the base of the cluster, amylopectin chains that are not organized into helical configurations, and long linear amylopectin chains that interconnect the clusters form the amorphous lamellae. Amylopectin is the main polymer in starch, it makes up approximately 75% of the granule. It is a large, branched molecule consisting of clusters of α-1,4-linked glucose chains (~12–20 units) emerging from regions rich in branch points (α-1,6 linkages). Amylose is the minor component of starch consisting of long α-1,4-linked glucose chains with few branch points.[18] It is thought to occur mostly as single unbranched helices in the amorphous regions of the matrix[185] and to not be involved in the organization of the granule matrix.[28] Their chemical and molecular characteristics are described in detail in Chapter 1.

The structural and chemical properties of these two polymers and their type of crystalline packing within the granule confer specific physico-chemical characteristics to the starch.[186] During starch synthesis in the grain, the various starch branching enzymes (SBE) isoforms in plants greatly impact structural and functional properties of starches. The activity of SBE determines branching pattern in amylopectin and the polymodal distribution of chain lengths that defines the cluster structure.[29] In wheat, mutant plants lacking SBE activity are characterised by starch with altered amylose to amylopectin ratio, due to changes in the polymeric chain length and molecular weight.[29] These changes can affect starch granule

morphology and functionality in foods because of altered gelatinisation and viscosity,[187, 188] with impact on the starch susceptibility to hydrolysis and consequently, starch-based foods digestibility.[52]

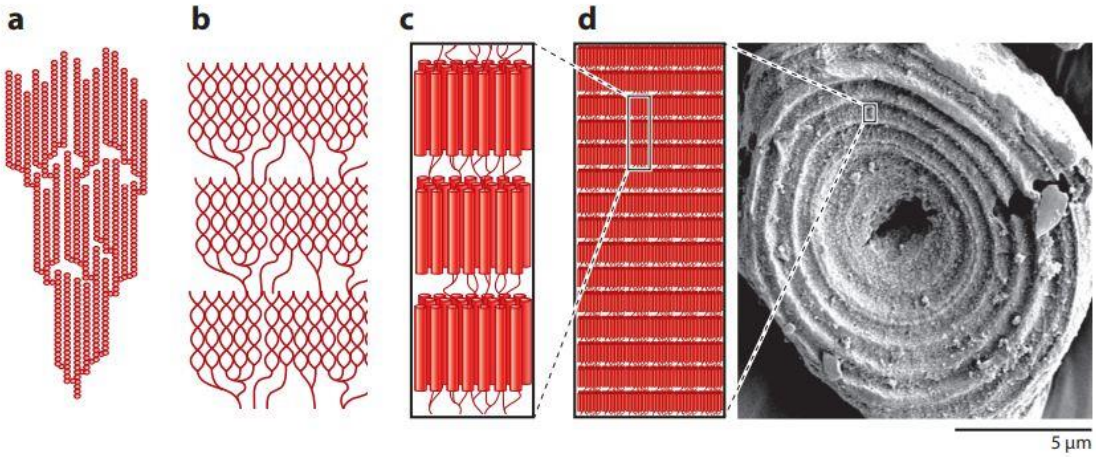


Figure 3.2 The structure of a starch granule. *a. Part of an amylopectin molecule: clusters of linear chains of α-1,4-linked glucose residues connected by α-1,6 linkages. b. Double-helix formation between adjacent linear chains within clusters. c. Representation of repeated crystalline arrays formed by the packing of double helices, interspersed with amorphous lamellae where the α-1,6 linkages are located, with periodicity of 9–10 nm. d. An inner face of a maize endosperm starch granule etched to show the growth rings. Each ring spans tens of the semicrystalline repeats shown in panel c. Figure obtained from Smith and Zeeman (2020),[28] with Authors permission.*

3.1.2 Properties of A and B starch granules

In wheat, starch is made of granules of different sizes and shapes. The most common are A-granules characterised by a 'lenticular' shape and spherical B-granules.[19] During endosperm development, production of starch granules results in a bimodal size distribution: large, lenticular A-granules (10-35 µm in diameter at maturity in wheat) that initiate early during grain development, and smaller, spherical B-granules (2–7 µm in diameter) that initiate later.[189] A-granules account for more than 70% of the starch by weight in wheat endosperm, but for less than 10% of the granules by number, while the B-type account for over 90% of the granules by number, but less than 30% of the total starch by weight.[190]

The biosynthesis and composition of B-granules is also thought to differ from that of A-granules, mainly with respect to the amylose content. Amylose and amylopectin components of starch both increase as the kernel matures.[191] The amylose content of wheat starch, expressed as a percentage of the total starch, increases with granule size and with granule maturity thus, it is generally accepted that A-granules have a larger proportion of amylose than smaller B-granules.[192, 193] Studies reported approximately a 10% difference in amylose content

between A and B granules in starch from wheat, with A granules having higher amylose content compared to B granules of the same wheat genotype.[194-196]

B-granules are also thought to differ from A-granules in their physiochemical properties that can determine the rate and extent of starch susceptibility to hydrolysis. Previous research has shown that the relatively larger surface area of small granules (B-type) correlated with a greater initial enzymatic hydrolysis compared to the larger granules.[197, 198] Salman *et al.*[199] measured the digestibility of A and B granules by incubating starch granules with porcine pancreatic α-amylase and amyloglucosidase to quantify the amount of starch digested within 24 hours. They reported that B-granules were digested at a faster rate initially, compared to A granules, possibly because of the larger surface area of small granules.[199] After 24 hours, the A-granule fraction was digested to a greater extent however, these results should be interpreted with care as the statistical analysis of differences in starch hydrolysis was not clearly reported. Several studies have shown that A and B starch granules isolated from wheat had different pasting properties, with B granules exhibiting relatively lower pasting temperature and viscosity compared to A granules and a lower retrogradation rate after storage. [195, 196, 200] Smaller B granules are associated with greater water absorption and swelling compared to A-granules, possibly because of the lower crystallinity of the starch polymers.[200] A greater specific surface-area may also contribute to the higher water absorption of B-granules as well as higher amylolysis rates compared to larger A-granules.[201]

3.1.3 Non-starch polysaccharides

Non-starch polysaccharides (NSP) derived from the grain cell walls completely resist enzymatic digestion in the small intestine.[202] During milling, most of these components that are categorised as dietary fibre are removed however, a small percentage of NSP can be found in refined flour and semolina and can have implication when developing wheat foods for improved glycaemic control. NSP are complex carbohydrates made of hexose sugars (glucose, galactose, mannose), deoxy-hexoses (rhamnose, fucose, glucuronic and galacturonic acid) and pentose sugars (arabinose and xylose);[203] they make up 2 - 3% of the dry weight in refined wheat flour[204] and 1.4 - 1.8% of the dry weight in durum wheat semolina.[205] In wheat, arabinoxylans (AX) constitute the main source of dietary fibre as they account ~70% of the cell wall polysaccharides. The remaining NSPs are (1→3,1→4)-β-D-glucan (mixed-linkage β-glucan or MLG) (~20%), cellulose (2%) and glucomannan (7%). AX have a main backbone of β-D-xylopyranosyl residues linked by (1→4) glycosidic linkages and some residues are substituted

with α-L-arabinofuranosyl residues at position 2 and/or 3.[4, 204] The soluble fraction of dietary fibre (comprised of AX and β-glucans) is considered to have health benefits,[206, 207] Figure 3.3.

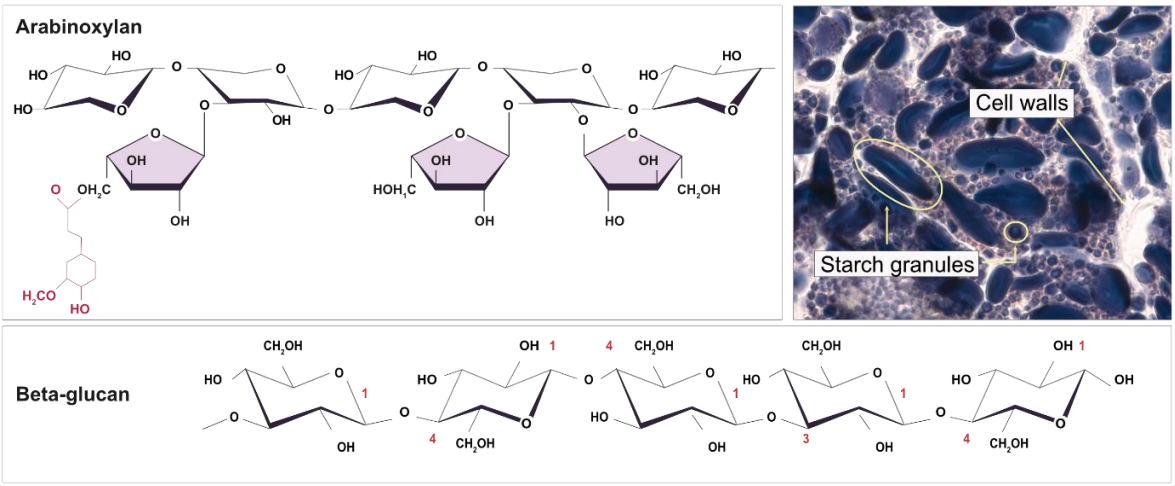


***Figure 3.3 Chemical structure of the main NSPs in wheat**, AX and B-glucans. Light micrograph shows a cell from the starchy endosperm of a wheat grain, imaged from a grain section stained with Lugol's solution. Starch granules are surrounded by cell wall. Figure adapted from Shewry et al. (2020) [2] with Authors permission.*

At high concentrations, AXs, particularly water-soluble AXs, can contribute to an increased meal viscosity, lower starch gelatinisation and decreased starch amylolysis by reducing the diffusion rate of enzymes, having implications for postprandial glycaemia.[208, 209] Wheat has a relatively low content of fructans, another NSP component. However, because of the high intake of wheat-based products, it is the main source of fructans in UK diets.[210] Fructans are oligo- or polysaccharides with a complex, branched structure; they have a backbone of β (2→6)-linked fructosyl monomers with β(2→1)-linked fructose branches and a terminal glucose molecule. They are usually classified according to their degree of polymerisation (DP): polymers of 2–9 units are referred to as oligofructose and those with >10 units as inulins.[211] Nutritionally, fructans are of interest because of their prebiotic effect. Dietary supplementation studies have shown that fructans increase gastrointestinal bifidobacteria and reduce appetite.[212, 213] On the other hand, a high intake of fructans (e.g. 20 g/day) can cause symptoms of bloating and flatulence that are mild in healthy subjects but severe in those with irritable bowel syndrome.[214, 215]

3.2 AIM: Determine the starch and non-starch characteristics of *sbeII* wheat that may play a role in starch digestibility and glycaemic response

This chapter aims to investigate the intrinsic properties starch and non-starch polysaccharides of *sbeII* wheat that may influence digestibility of starchy foods.

The morphology, amylose and RS content of starch from s*beII* wheat were previously investigated[11, 39, 151] however, this is the first time that *sbeII* grown in the UK has been characterised and its starch characteristics are reported in this chapter.

Another aim of this chapter was to explore further other intrinsic *sbeII* wheat properties, such as the starch granules size and NSP composition. These *sbeII* wheat characteristics had not been investigated previously but may play a role in determining starch susceptibility to hydrolysis.

3.3 Materials

In this chapter, starch and non-starch characteristics of *sbeII* durum wheat semolina and bread wheat flour are reported.

Semolina samples were obtained from bulked durum wheat grains (2015) and flour samples were obtained from bulked bread wheat grains (2016), as explained in chapter 2.

Particle size analysis of milled fractions showed interesting differences between *sbeII* wheats (durum and bread wheat) and WT controls. Further analysis of starch granule size distribution was carried out on isolated starch from the *sbeII* and WT bread wheats grown in 2017-2018 (described in chapter 2).

Samples from each plot were analysed for TS and RS content. Based on the RS content, three plots per genotype were selected for further starch characterisation. The plots selected showed the maximum, median and minimum content of RS and were thought to be representative of the overall RS content per genotype, per growing season. The study design is represented in Figure 3.4 and Figure 3.5.

***Figure 3.4 Bulking layout and sampling of durum wheat and bread wheat**. Durum wheat 'Kronos' variety 2014-2015; WT control wheat and sbeII wheat; bread wheat 'Lassik' variety 2015-2016 WT control wheat and sbeII wheat.*

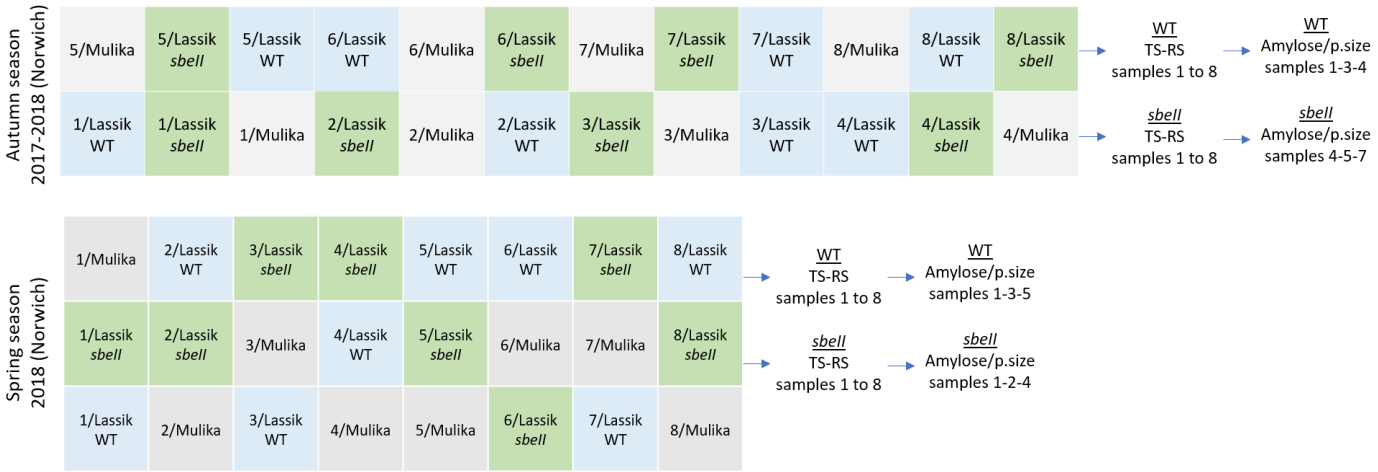


***Figure 3.5 Field trials layout and sampling of bread wheat** 'Lassik' variety; WT control wheat and sbeII wheat during two growing seasons. Plot size = 6 m², 'Mulika' is a standard wheat variety used as internal control*

3.4 Methods

3.4.1 Starch composition

3.4.1.1 Starch granule morphology

Starch granules were visualised using widefield light microscopy and Scanning Electron Microscopy (SEM), as described in chapter 2.

SEM images of isolated starch were obtained from durum wheat grains and bread wheat grains by Mr Brendan Fahy (Research Assistant in the Hazard Group). Starch as a powder was distributed on a microscope stub using a spatula and sputter coated with ~15 nm gold particles as described in chapter 2. Samples were imaged at 3 kV on the same day of preparation to prevent coating degradation.

Light microscopy images were obtained from grain sections, isolated starch and flour.

Grain sections of durum wheat were obtained by slicing mature grains on a microtome set on 2 µm for thickness. The micrographs were captured by Mr Brendan Fahy (Research Assistant in the Hazard Group). Sections were stored in a petri dish and mounted on a microscope slide by applying a drop of deionised water to the slide and laying one grain section on it. A cover was applied over the grain section and the excess water was removed with paper tissue.

Bread wheat flour and isolated starch from bread wheat flour were laid on light microscope slides using a spatula. Starch samples were stained with Lugol's solution (5%) while bread wheat flour was imaged in deionised water. A cover was applied over the samples and the excess water/staining solution was removed with paper tissue. Bread wheat flour samples in water were imaged under polarizing light to observe the birefringence patterns.

3.4.1.2 Total and resistant starch of semolina and flour

TS and RS content of durum wheat semolina and bread wheat flour were quantified using Megazyme assay kits, KOH format, described in chapter 2 (2.2.2). Amylose content was measured on isolated starch from semolina and flour as described in chapter 2 (2.2.2.5).

3.4.2 Non-starch polysaccharides composition in semolina and flour

NSP analyses were carried out at Rothamsted Research by Dr Alison Lovegrove and her group as a collaboration within the Designing Future Wheat Institute Strategic Programme, funded by BBSRC UKRI.

3.4.2.1 Fructans

Samples were prepared according to Verspreet *et al*.[216], where 10 mg of sample (flour or semolina) was suspended in 1mL of deionised water and heated at 80°C for 60 minutes on a heat block (Eppendorf ThermoMixer) at 300 rpm. Samples were left to cool down and then centrifuged at 9000 g for 10 minutes to collect the supernatant. For each sample, 50 uL of supernatant was diluted with 950 µL of deionised water. Another 50 µL of supernatant from each sample was used for acid digestion with 3.19 µL of 1 M HCl, followed by a heating step at 70°C for 90 minutes. The digestion was stopped by adding 2 µL of 1 M Na_2CO_3 and the samples were diluted to 1 mL with deionised water. Both digested and non-digested samples were then filtered and injected onto Dionex 3000 chromatography system using a Fructan PA100 column, together with arabinose, galactose, glucose, fructose, sucrose, melibiose and raffinose standards (1-30 µM).

3.4.2.2 Enzymatic fingerprinting

Enzymatic fingerprinting was used to measure arabinoxylan oligosaccharides (AXOS) and mixed-linked β-glucan oligosaccharides (GOS) released following enzymatic digestion of semolina and flour samples from WT control and *sbeII* durum and bread wheat. The method used was as described by Ordaz-Ortiz *et al.* (2004)[217]; except that recombinant enzymes were used for digestions. The method used for identification of oligosaccharides was described in Ordaz-Ortiz and Saulnier (2005).[218] Semolina from WT control and *sbeII* wheat was digested with endo 1,4 β-xylanase (E.C.3.2.1.8), a xylanase of the GH11 group from *Neocallimastix patriciarum* (PRO-E0062, Promix Limited, UK,) and endo 1,3(4) glucanase ('lichenase') (E.C.3.2.1.73) from *Clostridium thermocellum* NCIB 10682 (PRO-E0017, Promix Limited, UK) to digest AX and MLG, respectively. Following digestion, samples were filtered, diluted in 10 µM melibiose (internal standard), and analysed using a CarboPac PA1 analytical column (guard column: 2 x 50 mm x analytical column: 2 x 250 mm) on a Dionex ICS-3000 (Thermo Scientific). The peak areas of the oligosaccharides released by enzyme digestion with endo 1,4 β-xylanase were expressed as percentages of the total peak areas of all AXOS. Two major GOS were released by enzymatic digestion with endo 1,3(4) glucanase: G3 and G4. An estimate of 'total' MLG was therefore calculated as the sum of G3 + G4 peak areas, and G3 to G4 ratio was calculated.

3.4.2.3 Total (TOT) and water extractable (WE) monosaccharides

Briefly, for 'Total', 5 mg of semolina and flour samples from WT control and *sbeII* durum and bread wheat were suspended in 10 mL of deionised water (0.5 mg/mL) and homogenised in a

glass/Teflon homogeniser. Triplicate aliquots of 200 µL (equivalent to 100 µg of original sample) were taken and freeze-dried overnight. For WE- monosaccharide analysis 5 mg of semolina sample from WT and *sbeII* wheat was resuspended in 1 mL of deionised water and incubated on a Denley spiromix for 30 minutes at 25℃. The suspension was centrifuged at 2,500 g for 10 minutes and the supernatant collected, aliquoted and freeze-dried overnight. Both TOT and WE samples were hydrolysed in 2 M trifluoroacetic acid (TFA) as described by Bromley *et al*.[219]. Hydrolysates were diluted to 0.25 µg/µL in deionised water. If samples were not analysed immediately, they were stored at -20℃. Three technical replicate samples were aliquoted and further diluted in 10 µM 2-deoxy-galactose solution (internal standard) to a content of 0.05 µg/µL.

Neutral monosaccharides were separated by HPAEC-PAD (using a Dionex ICS-5000+ equipped with eluent generator with (EGC 500 KOH cartridge); Thermo Scientific, US) in a 23.5 min run on a CarboPac PA-20 column (guard column: 3x30 mm, analytical column: 3x150 mm; Thermo Scientific, US) at 30°C. Twenty-five µL of diluted sample was injected onto the column, using the following conditions: Isocratic 4 mM KOH, 14.5 min; 100 mM KOH, 15-18 minutes; 4 mM KOH, 18.5-23.5 minutes; the flow rate was 0.5 ml/min. Authentic monosaccharide standards were used for peak identification; they were subjected to the same acid hydrolysis as the experimental samples to allow for any destruction of sugars during hydrolysis prior to the generation of calibration curves. All experimental samples were randomised prior to HPAEC analysis. Calibration standards were run at the beginning, middle and end of each analytical run cycle. All data were processed with the Chromeleon analytical software (version 7.2SR4; Thermo Scientific, US) to calculate the amount of analyte in an unknown sample using external standards/calibration curves. Total and WE AX were calculated as the sum of xylose and arabinose minus an adjustment for arabinogalactan peptide [220, 221] and multiplied by 0.89 to convert monosaccharide values, for arabinose and xylose, to the polysaccharide, arabinoxylan.

3.4.3 Particle size distribution analyses

3.4.3.1 Particle size distribution analysis by laser diffraction

3.4.3.1.1 Durum wheat semolina isolated starch

Isolated starch from durum wheat semolina was analysed immediately following the addition of deionised water to samples. Following the set up previously described (chapter 2, 2.2.1), a 40% Polarization Intensity Differential Scattering (PIDS), indicating the sample concentration, was

used for this analysis. A 40% PIDS is normally used for materials of coarse particle size such as semolina of flour to improve sampling. The analysis was set to return the particle size distribution as the 'Volume moment mean diameter' (D [4, 3]).

3.4.3.1.2 Bread wheat flour isolated starch

Isolated starch from bread wheat flour was suspended in deionised water and sonicated for 15 minutes. This analysis was performed using an obscuration limit (sample concentration) of 10%. The obscuration limit was used instead of the PIDS because the small particle size of starch was expected to be below 50 µm. A larger obscuration limit could have negatively affected samples smaller than 10 µm by creating multiple scattering. The analysis was set to return the particle size distribution as the 'Volume moment mean diameter' (D [4, 3]).

3.4.3.2 Size distribution analysis of starch granules by electrical sensing zone

Extracted starch was suspended in Isoton II Diluent (supplied by Beckman Coulter Life Science) to 10 mg/mL solution. Samples were sonicated in a water bath for 15 minutes; then 250 µl of suspension was filtered through a 70 µm nylon cell strainer into Multisizer cup and further diluted by adding 100 ml of Isoton II. The analysis was standardised by the total count of 10000 particles and set to return the particle size distribution as 'Volume percent' to highlight any size differences between samples. Volume percent is calculated as follows: the number of particles counted per particle diameter class analysed (µm) represents the area of a "bin" of the distribution (as the bins in a barchart). The Multisizer 4e Particle Size Analyzer software then averages the number of particles counted to the total bin volume and returns the Volume Percent for a specific particle size. The advantage of this method for particle size analysis is that, while the laser diffraction method is prone to overestimate non-spherical objects[222] such as A-granules in the *sbeII* starch, the electrical sensing zone method is not affected by either granule shape or density as the instrument relies on an electrical resistance method to discriminate among particles by how they affect the electrical resistance of an electrically conductive liquid where the particles are suspended.[223]

3.4.4 Size separation of starch granules by Percoll centrifugation

Undiluted Percoll (Pharmacia Biotech, Quebec) osmolality was adjusted to make isotonic Percoll (90%) by adding 9 parts (v/v) of Percoll to 1 part (v/v) of 1.5 M NaCl. This solution was further diluted using 1.5 M NaCl to prepare 70% (v/v) Percoll.

A 2.5 mL starch suspension in water (0.1 g/L) was sonicated for 15 minutes. The sonicated starch suspension was gently laid on top of 5 mL Percoll 70% without mixing and centrifuged for 10 minutes at 10 g with minimum acceleration/break, at 15℃. Following centrifugation, the supernatant containing mostly B-granules was separated and the pellet containing mostly A-granules was resuspended in 2.5 mL of deionised water.

This step was repeated twice as described above. Each time, pellet and supernatant were collected and kept separate for analysis.

The pellet obtained was treated as described above using an isotonic Percoll (90% v/v).

The pellet and supernatants were left at room temperature overnight to allow all starch particles to deposit at the bottom of the tube. Samples were then centrifuged at 450 x g for 5 minutes to decant and discard the supernatant (mostly Percoll solution).

The samples were then placed in an evaporator (GenevacTM EZ-2 Elite evaporator) on an 'aqueous' setting at 30℃ until completely dry (approximately 2.30 hours). The dry pellets were then re-suspended in water for particle size analysis. Widefield microscopy was used to visualise the two fractions at the end of the process. A variable degree of purity and of starch content was measured across the different supernatants collected, especially when washing with 90% Percoll. Impure and low-recovery samples were excluded from the analysis.

3.4.5 Statistical analyses

TS, RS, amylose and NSP contents of *sbeII* wheat were compared to the WT controls using independent two sample t-tests.

Particle size distributions of milled durum and bread wheat (semolina and flour) were analysed quantitatively using independent two sample t-tests to identify mean differences between the *sbeII* wheat materials and the WT wheat controls.

The particle size of isolated starch showed a mixture distribution (two distinct peaks overlapping) of A and B-type granules for both *sbeII* and WT control starch when measured using the electrical sensing zone method. The individual distributions forming the mixture are known as mixture components and can be identified by fitting a mixture model. This analysis was carried out by Dr George Savva (QIB statistician). Mixture models are a type of clustering analysis returning the probability distribution of observations in the overall population. Finite mixture distributions model grouped data by the method of maximum likelihood using a combination of a Newton-type algorithm and the Expectation–Maximization (EM) algorithm. The EM algorithm is an iterative method to find maximum likelihood estimates of parameters in statistical models,

where the model depends on latent variables. The mixture components distributions were estimated using constrained values of granule size for each group of granules (A and B-type) across genotypes. Granules with size of <10.0 µm were classified as B-type granules whereas those between 10.1–35.0 µm in diameter were categorised as A-type starch granules. Granules with diameters >35.0 µm were considered to be impurities or compound granules and excluded from the analysis[224].

Once identified, the two mixture compenents were fitted to a nested linear mixed model to determine differences in the distribution of A and B-granules of *sbeII* starch compared to the WT control. The model accounted for the random effect of the sample replicates (plots) including a interaction term of genotype (*sbeII* or WT control) by field trial (Autumn or Spring), and p-values were obtained by Satterthwaite approximation.

R packages *plyr*[225], *mclust*[226] were used for data curation; *mixdist*[227] was used for mixture analysis; *lme4*[228] and *emmeans*[229] were used to model and estimate means of mixture components; *ggplot2*[181, 230] was used for data visualisation.

Data are presented as means ± SEMs unless stated otherwise.

3.5 Results

3.5.1 Starch composition

3.5.1.1 Starch granule morphology

Starch from *sbeII* and WT durum wheat and bread wheat were visualised using widefield light microscopy and SEM as described in chapter 2. SEM images of starch isolated from durum wheat grains showed the two main granule size populations typical of wheat starch in *sbeII* and WT starch: the larger A-type granules and the smaller B-type granules (Figure 3.6, a and b). A fraction of *sbeII* starch granules showed a characteristic crescent shape as compared to the regular lenticular shape of WT starch granules (Figure 3.6 c and d), in both durum and bread wheat (only starch images from durum wheat are shown as the granules appeared very similar). Starch from *sbeII* wheat presented a marked longitudinal grove and surface erosion or pores extending towards the centre of the granule (Figure 3.6 e and f).

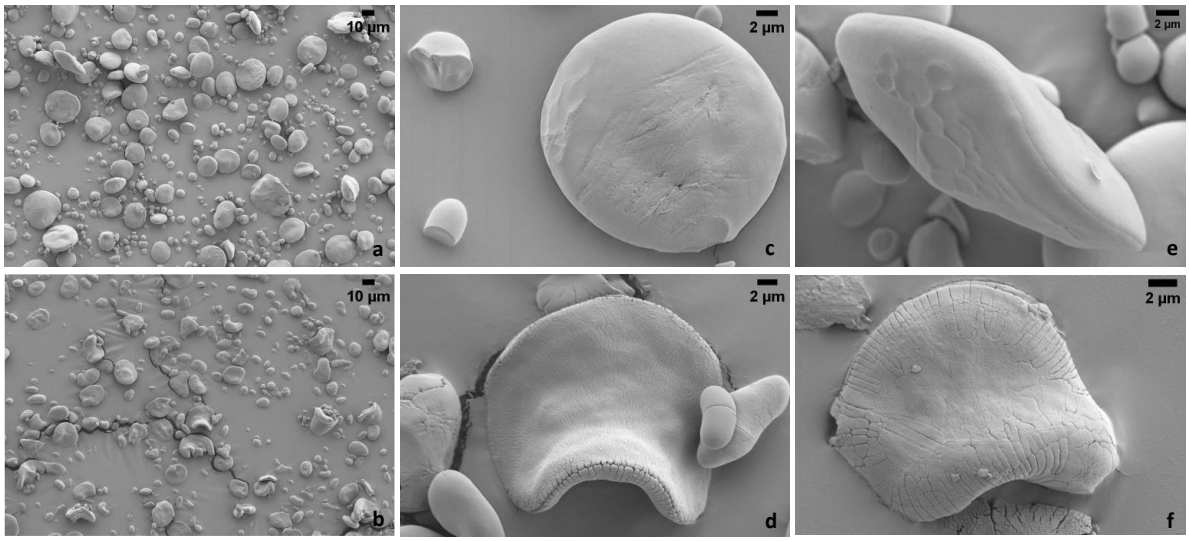


Figure 3.6 SEM images of starch *isolated from WT control (a, c, e) and sbeII (b, d, f) durum wheat grains. Images by Brendan Fahy.*

Changes in granule morphology were also visible in widefield light microscopy images. Using sections of durum wheat grains in deionised water, it was possible to observe the growth rings on the granules' edge in WT durum wheat starch, as the polymers are organised in a radial fashion within the granule, and a darker region at the hilum, the centre of the granule, when granules were stained with iodine (Figure 3.7 a). The polymer chains of *sbeII* durum wheat starch may be organised differently from the WT starch, as perpendicular lines are visible instead of the typical radial growth rings (Figure 3.7 b).

The change in morphology and the superficial irregularities were visible in both starch granules within the grain sections (durum wheat, Figure 3.7 a and b) and isolated starch from bread wheat (Figure 3.7 c and d).

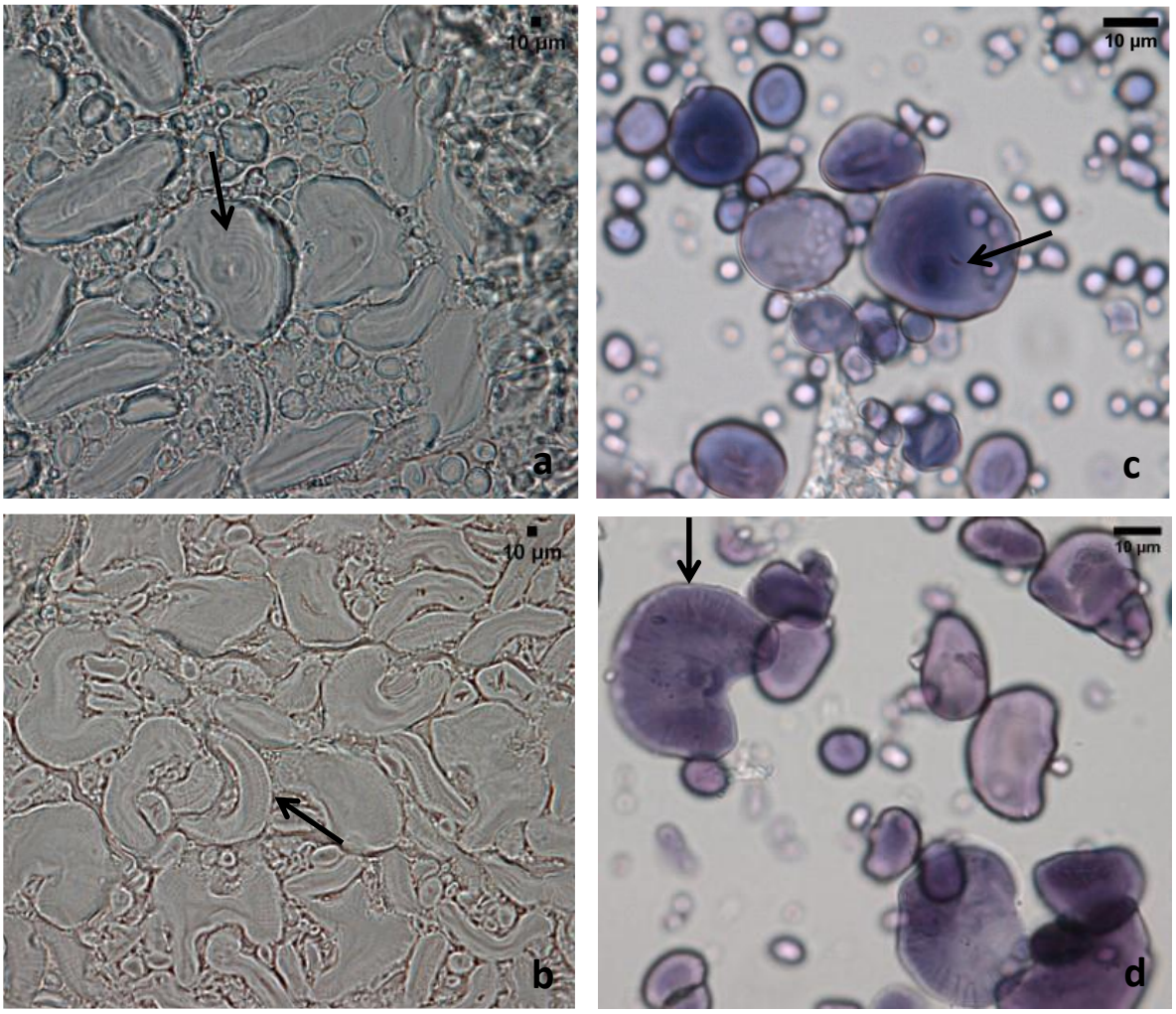


Figure 3.7. Light micrographs of durum wheat grain sections *in water by Brendan Fahy, a. WT control starch and b. sbeII starch. Light micrographs of isolated starch from bread wheat flour stained with 5% Lugol's solution c. WT control starch and d. sbeII starch. Arrows indicate the hilum (a and c) and the altered morphology and perpendicular lines (b and d).*

Due to the semi-crystalline nature of amylopectin molecules in the granule, WT starch granules showed a birefringence pattern known as 'Maltese cross' when viewed with polarized light. Figure 3.8 shows WT bread wheat flour (a and c) and *sbeII* bread wheat flour (b and d) in deionised water under with and without a polarizing filter. The reduced birefringence pattern visible in *sbeII* starch in response to polarized light may reflect the disrupted starch radial ordering, possibly because of the increased ratio of amylose to amylopectin.

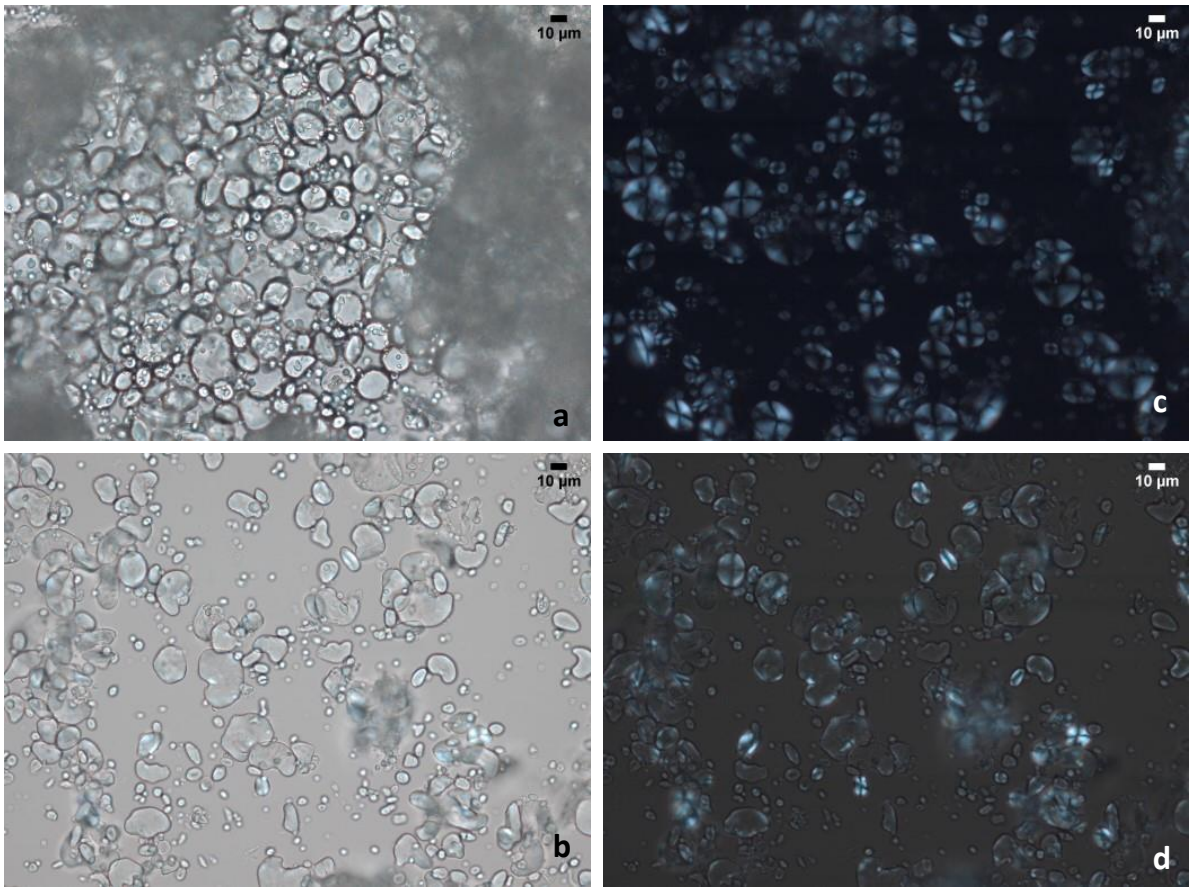


Figure 3.8 Starch in bread wheat flour WT control (a and c) and sbeII (b and d) light micrographs. *Images c and d show the birefringence pattern when the polarised filter is used.*

3.5.1.2 Starch composition of durum wheat semolina and bread wheat flour

TS content of bulked *sbeII* and WT control durum wheat semolina did not significantly differ, they were 60.65 ± 3.84 g/100 g on a dry weight basis (dwb) and 67.90 ± 1.44 g/100 g dwb, respectively (unpaired two-sample t-test, p = 0.11).

Resistant starch content of raw *sbeII* semolina (4.87 ± 0.6 g/100 g starch dwb) was higher than the WT control (0.77 ± 0.34 g/100 g starch dwb, two sample t-test, p = 0.004). The apparent amylose content of starch isolated from *sbeII* semolina was also higher than the WT control (32.98% ± 0.69% w/w, vs. 23.05% ± 1.5% w/w), (unpaired two-sample t-test, p = 0.004).

Preliminary analysis of bread wheat flour showed a similar trend in RS content between *sbeII* and WT wheat harvested in 2016 as shown in Figure 3.9. Apparent amylose content of starch

isolated from *sbeII* and WT control bread wheat grains was 26.02% ± 3.17% and 18.72% ± 1.617% (w/w), respectively.

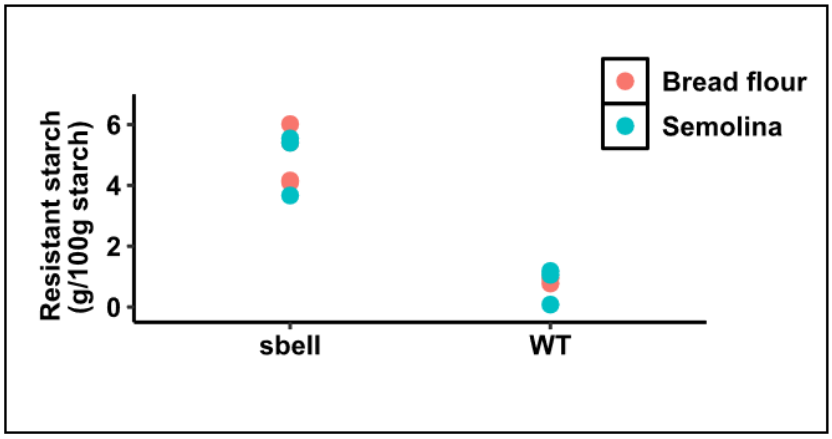


***Figure 3.9 Resistant starch (RS) content of milled fractions**: RS is expressed as a percentage of starch sbeII and WT control wheat, on dwb. The sbeII and WT control semolina was obtained from durum wheat grains harvested and milled in 2015; the sbeII and WT control bread flour was obtained from bread wheat grains harvested and milled in 2016.*

Further analysis of flour from bread wheat grown in 2017-2018 showed greater RS content than the WT control, as previously seen in the bread flour obtained from grains of the 2016 season, shown in Figure 3.9. The RS content of *sbeII* bread wheat was higher in the 'Spring' trial compared to the 'Autumn' trial.

The TS and RS content of flour from each season are reported in Table 3.1. There were no significant differences in TS between genotypes or seasons, while the RS proportion of starch was singificantly higher in *sbeII* flour compared to the WT control, particularly in the flour otbained in the Spring field trial. Apparent amylose content is also reported here as the mean ± SEM of three selected plots. The plots selected showed the maximum, the minimum and the median content of RS and are thought to be representative of the eight-plot field trial. Amylose percentage was singificnatly higher in *sbeII* starch isolated from wheat grown in different season and no effect of season was indetified here.

Table 3.1 Total Starch (TS) content on dwb, Resistant Starch (RS) and amylose proportion of starch in sbeII and WT control bread wheat flour sown in Autumn 2017 and Spring 2018.

Sample	Season	Genotype	TS (g/100g flour)	RS (% of TS)	Amylose (%)
Bread wheat	Spring	*sbeII*	73.9 ± 1.2 [a]	6.4 ± 0.5 [c]	39.06± 1.1 [b]
		WT	72.1 ± 2.9 [a]	0.6 ± 0.1 [a]	25.73± 0.7 [a]
	Autumn	*sbeII*	72.3 ± 1.6 [a]	3.8 ± 0.1 [b]	38.32 ± 0.5 [b]
		WT	77.1 ± 1.8 [a]	0.4 ± 0.0 [a]	25.43 ± 0.2 [a]
Source of variation					
Genotype			0.4	< 0.001	< 0.001
Season			0.3	< 0.001	0.4
Genotype x Season			0.1	< 0.001	0.7

Means ± SEMs, n = 8. Apparent amylose (%), Means ± SEMs, n = 3. Different lowercase letters (a, b, c) are significantly different from each other ($p < 0.001$, Two-way ANOVA with Tukey's post-hoc comparison).

3.5.2 Particle size distribution

3.5.2.1 Size distribution of isolated starch measured by laser diffraction

The granule size distributions of isolated starch from durum and bread wheat grains were also characterised using the laser diffraction method on the LS 13 320 particle sizer by Beckman Coulter previously described. Starch granule size distribution are shown in Figure 3.10. Starch from both *sbeII* and WT wheats had two main granule size populations characteristic of wheat starch including the larger A-type granules between 10 - 30 µM in diameter and the smaller B-type granules of diameter between 2-7 µM. The starch from *sbeII* durum wheat followed a bimodal size distribution, like the WT control (Figure 3.10a). However, the shape of the distribution of granules sizes from *sbeII* bread wheat distribution was different from the WT control, suggesting a difference in the proportion of A and B starch granules from *sbeII* wheat, compared to the WT control (Figure 3.10, b and c). This difference in B-granule peak was not apparent in starch isolated from durum wheat grains, possibly due to a larger variation between sample replicates.

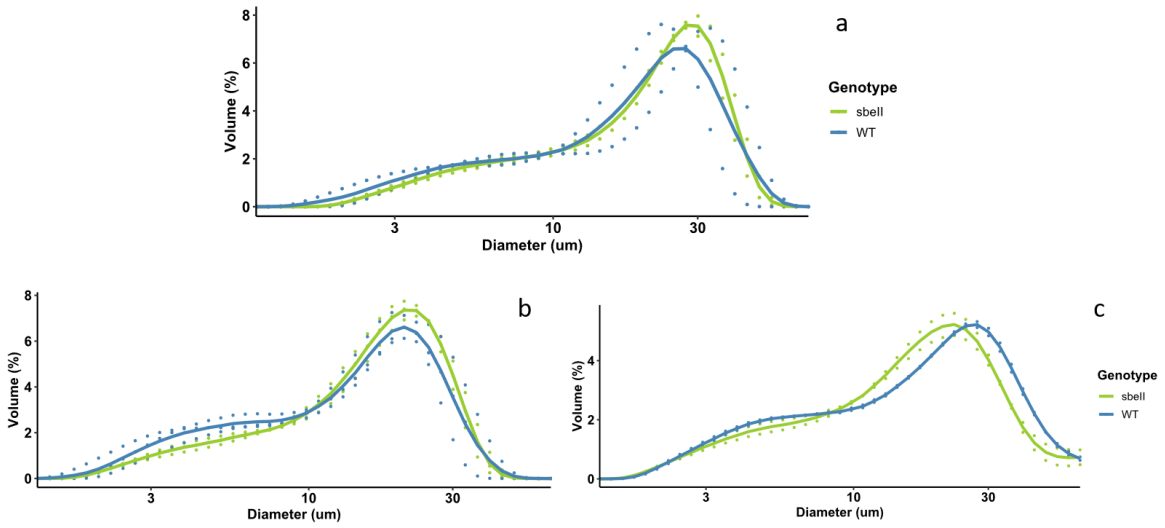


Figure 3.10 Granule size distribution of starch from sbeII and WT control wheat obtained by laser diffraction analysis. *a. Starch isolated from durum wheat grains (sbeII and WT control), data points represent n = 2 replicates of bulked semolina and curves represent the mean distribution on a log diameter scale. b/c. Starch isolated from sbeII and WT control bread wheat sown in Spring 2018 (b) and Autumn 2017 (c). Data points represents the independent replicates of wheat grains grown in a randomised block design: n = 3 in plot b and n = 2 in plot c. Curves represent the mean distribution on a log diameter scale.*

3.5.2.2 Size distribution of starch granules measured by electrical sensing zone

The granule size distribution of starch isolated from *sbeII* and WT control bread wheat from two frield trials was further characterised using the electrical sensing zone method on the Multisizer4e by Beckman Coulter (Figure 3.11). The distribution data were fitted to a finite mixture distribution model. The granule size frequency was modelled as diameter of each particle weighted by its volume. The model parameters were set to the expected mean granule size based on values reported in literature[39] (B-granules = 5 µm and A-granules = 18 µm) but with no constraints on mixture proportions. This allowed for the two overlapping peaks of A- and B-type granules to be estimated as reported in Table 3.2.

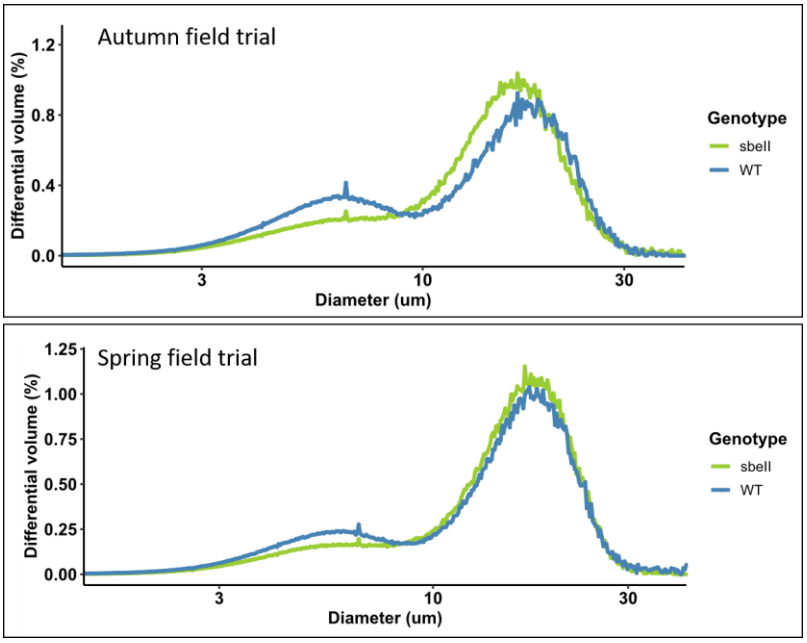


Figure 3.11 Size distribution of starch from sbeII and WT control bread wheat obtained by electrical sensing zone analysis *from Autumn and Spring trial, n = 3 independent replicates, per genotype, per field trial, data expressed on a log-diameter scale.*

The components of the mixture identified using the mixture model were compared across genotypes (*sbeII* against WT control) using linear mixed model described in the Methods section of this chapter (3.4.5). The proportion of B-granules in *sbeII* starch was found to be 10% smaller than in the WT control (p-value <0.0001). The model showed that proportion of B-granules was affected by the genotype and by the sowing season (p-value = 0.0145). In the Autumn trial, *sbeII* starch had 12.8% ± 0.014% less B-granules compared to the WT control (p-value <0.0001) while in the Spring trial, *sbeII* starch had 7.8% ± 0.014% less B-granules compared to the WT control (p-value <0.0001, mean and SEMs of 3 independent replicates), (Figure 3.12).

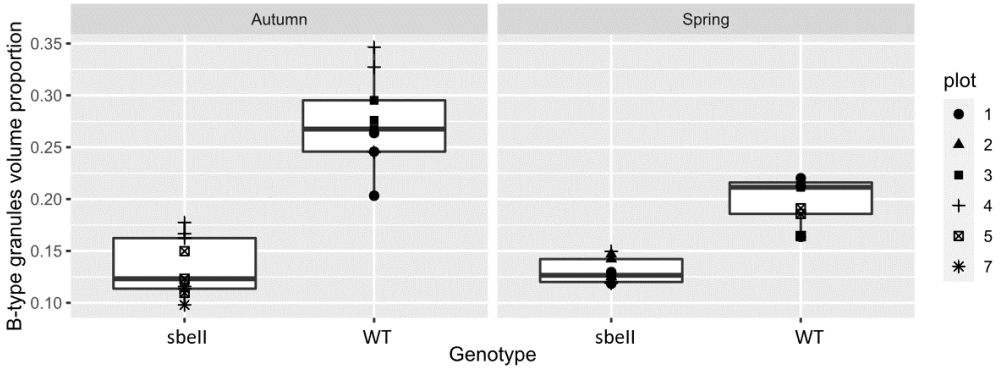


Figure 3.12 Volume proportion of B granules in sbeII and WT control starch*, n = 3 independent replicates per trial (plots).*

Table 3.2 Volume percent of A and B starch granules per cropping season.

Season	Genotype	Starch granule type	Mean (%)	CI (%)
Autumn	*sbeII*	A	86.1	(83.4, 89.9)
		B	13.9	(11.1, 16.6)
	WT	A	73.3	(70.6, 76.0)
		B	26.7	(24.0, 29.4)
Spring	*sbeII*	A	87.6	(84.9, 90.4)
		B	12.4	(9.6, 15.1)
	WT	A	79.8.6	(77.1, 82.5)
		B	20.2	(17.4, 22.9)

Means and confidence intervals (CI), n= 3.

3.5.2.3 Size separation of starch granules

Isolated starch from bread wheat grains was fractionated using Percoll centrifugation to separate A-granules from B-granules. Figure 3.13 shows the particle size distribution obtained by laser diffraction analysis of the two fractions obtained after Percoll centrifugation of WT starch, the pellet and the first supernatant decanted (70% Percoll). Laser diffraction analysis was used to evaluate the efficacy of the separation: the pellet fraction, with mean particle diameter of 17.47 µm ± 1.16 µm, contained mostly A-type granules, and the supernatant, containing mostly B-type granules, with a mean diameter of particles 4.58 µm ± 1.26 µm. Some Percoll could also have been detected by laser diffraction as it consisted of silica particle of 15–30 nm diameter. Figure 3.14 shows the unfractionated starch in water containing both A and B size granules (a,d), the pellet fraction in water containing particles of a broad range of diameter, mostly A-granules (b,e), and the supernatant fraction in water containing smaller monodispersed B-granules (c,f).

Laser diffraction analysis was possible only for the WT starch; due to COVID-19 lockdown and technical issues with the instrument it was not possible to complete the analysis on the *sbeII* starch.

Starch recovery after fractionation was analysed using the small-scale TS method described in chapter 2. After Percoll centrifugation, approximately 61.7 g ± 16.4 g /100 g of sample used was starch recovered from the WT pellet and 39.3 g ± 2.19 g /100 g of sample used was starch recovered from the supernatant (expressed as the mean and SEM of n = 2 independent samples). For the *sbeII* starch recovery was similar for the pellet fraction (60.2 g ± 1.9 g /100g

sample) however, a much lower recovery rate was found for the supernatant fraction (29.7 g ± 1.15 g/100 g sample).

Apparent amylose content was measured on both fractions to identify differences in the starch molecular composition of A and B-granules. The amylose content of the *sbeII* starch pellet (A granules) was higher than the WT control starch pellet (35.44% ± 1.6% and 22.38% ± 0.7%, respectively, expressed as the mean and SEM of n = 3 independent samples), consistent with previous measurements in unfractionated *sbeII* and WT starches (section 3.5.1.2). Determination of amylose content for the supernatant fraction did not lead to conclusive results, possibly because of the low and variable starch recovery after Percoll centrifugation.

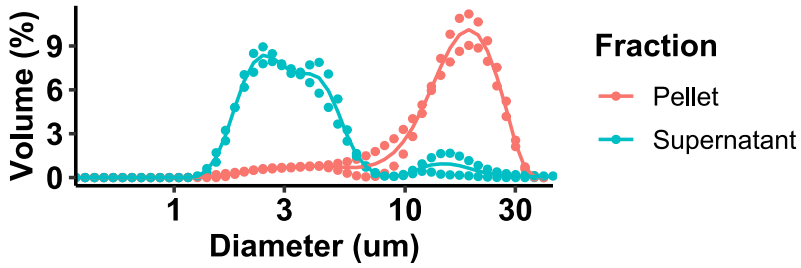


Figure 3.13 Particle size distribution of WT control fractionated starch after Percoll centrifugation obtained with laser diffraction technique *(LS 13320 as described in chapter 2). The 'pellet' fraction contained mostly A granules; the 'supernatant' fraction contained mostly B granules, n= 2 independent replicates.*

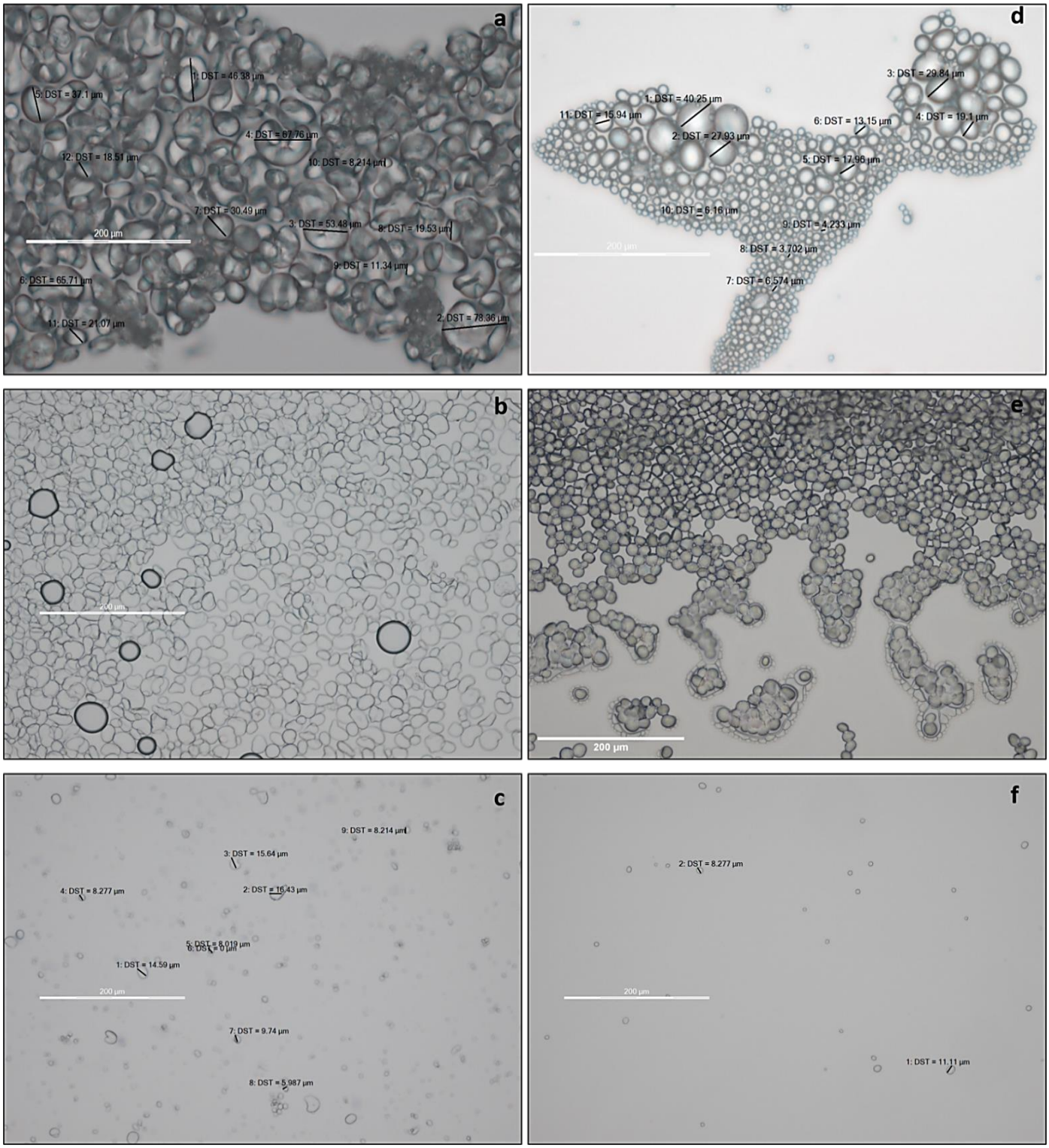


***Figure 3.14 Widefield light microscopy images of the 'pellet' and 'supernatant' fractions after Percoll centrifugations**, white scale bar = 200 µm. a. sbeII starch; b. sbeII pellet fraction containing mostly A granules. c. sbeII supernatant fraction containing mostly B granules. d. WT control starch. e. WT pellet fraction containing mostly A granules. f. WT supernatant fraction containing mostly B granules*

3.5.3 Non-starch polysaccharides composition of semolina and flour

NSP content and composition were measured in *sbeII* durum wheat semolina as well as in *sbeII* bread wheat flour with respective WT controls to explore the contribution of other nutritional factors to digestibility and glycaemic response.

Fructans (w/w) and fructans degree of polymerisation (DP) were greater in flour from *sbeII* durum and bread wheats compared to the WT controls but these were still within the range reported for refined wheat flour by the HEALTHGRAIN study (0.6% - 1.2%)[4] (Table 3.3). Fructans in durum wheat are not widely characterised; however, fructans content of whole-wheat grain has been reported to vary from 0.84% to 1.85%.[231] The *sbeII* semolina fructans content was within this range.

***Table 3.3 Fructans percentage and degree of polymerisation** (DP) in durum wheat semolina and bread wheat flour.*

Sample type	Genotype	Fructans (%)	SE	DP
Semolina	*sbeII*	1.028	0.034	3.22
	WT	0.659	0.026	2.89
Flour	*sbeII*	1.098	-	4.81
	WT	0.796	-	3.95

Means ± SEMs are reported for semolina samples from bulked durum wheat grains (n = 3) and for bread wheat flour (n = 2) from bulked bread wheat grains. For bread wheat flour samples, the average of two technical replicates is reported without SEMs.

Total AX content was found to be significantly higher in the *sbeII* semolina compared to the WT control (Two sample t-test, p-value = 0.0069), but this difference was not as marked when comparing total AX of *sbeII* and WT control bread wheat flour (Two sample t-test, p-value = 0.33). Water-extractable AX (WE-AX) content was greater in *sbeII* bread wheat flour compared to the WT control (Two sample t-test, p-value= 0.05), but not for the durum wheat semolina samples (Two sample t-test, p-value= 0.32), (Table 3.4).

Table 3.4 Total (TOT) and Water Extractable (WE) Arabinoxylans (AX) *of sbeII durum wheat semolina and bread flour compared to the WT controls.*

Parameter (mg/g sample dwb)	Semolina WT control	Semolina *sbeII*	Flour WT control	Flour *sbeII*
WE-AX	1.17 ± 0.06	1.51 ± 0.29	3.59 ± 0.17	4.48 ± 0.17*
TOT-AX	13.27 ± 0.44	20.72 ± 1.01***	10.71 ± 1.27	12.07 ± 0.27

Means ± SEMs of independently analysed samples from bulked durum wheat semolina (n = 3) and samples from bulked bread wheat flour (n = 3). Arabinoxylans were calculated as the sum of arabinose and xylose corrected for arabinogalactan (AGP) content and multiplied by 0.89 to convert monosaccharide values, for arabinose and xylose, to the polysaccharide, arabinoxylan. [47, 232, 233] Complete monosaccharide profiles are reported in

Table ***A3 1*** in appendix A. Parameter values that are significantly different between the two semolina types or flour types (*sbeII* compared to the WT control) are indicated with an asterisk: *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired Students t-test).

Enzymatic fingerprinting experiments showed no significant difference in total AXOS (sum of peak areas of all known AXOS peaks) in *sbeII* and WT semolina (Two sample t-test, p = 0.07). However, there were differences in AXOS substitution pattern between *sbeII* and WT control semolina (Table 3.5). Substituted AXOS were higher in *sbeII* semolina compared to the WT control (Two sample t-test, p = 0.0016), but no significant differences in unsubstituted AXOS were detected (Two sample t-test, p = 0.26). To describe the structure of MLG and the distribution of β1-3 and β1-4 bonds, an estimate of total MLG was obtained by summing G3 and G4 GOS peak areas and the ratio of G3 to G4 GOS determined as the ratio of G3 to G4 GOS peak area[205]. The mean of G3+G4 of *sbeII* semolina was greater than that of the WT control (Two sample t-test, p = 0.028, Table 3.5) and the ratio of G3 to G4 was 2.4 for *sbeII* semolina vs. 2.2 for the WT control.

Table 3.5. AXOS calculated as 'Sum of peaks relative to the internal standard' of sbeII durum wheat semolina compared to the WT control.

Parameter	Semolina WT control	Semolina *sbeII*
TOT-AXOS	10.02 ± 0.74	12.64 ± 0.97
AXOS Unsubstituted	5.39 ± 0.6	6.61 ± 0.79
AXOS Substituted	4.63 ± 0.15	6.03 ± 0.2**
MLG (G3+G4)	2.29 ± 0.12	2.78 ± 0.11*

Means ± SEMs of independently analysed semolina samples (n=4) from bulked semolina. Parameter values of *sbeII* that are significantly different from the WT control are indicated with an asterisk; *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired Students t-test).

Semolina was later used to develop a food model (pudding) as described in chapter 4 for a glycaemic index (GI) pilot study (chapter 5). The NSP content and composition were measured on freeze-dried pudding samples prepared alongside pudding portions served in the GI pilot study; these results can be found in the Table A3 2, Table A3 3(appendix A). AXOS composition of bread wheat flour samples is reported in Table A3 4(appendix A) as the average of two replicates for *sbeII* and WT control flour.

3.6 Discussion

This chapter presents some novel characteristics of carbohydrates in *sbeII* wheat that may pay a role in starch digestibility. The intrinsic properties described in this chapter may influence the processing, the end-use quality of wheat-based food products, their starch susceptibility to hydrolysis and ultimately, their effect on glycaemic response (discussed in chapter 4, 5 and 6 of this thesis).

3.6.1 Amylose proportion and starch resistance to digestion

Starch in *sbeII* durum and bread wheats used in this study were characterised by altered starch properties compared to the WT control, consistent with the *sbeII* characteristics reported previously by other authors. The RS content of *sbeII* and WT control wheats (durum and bread wheat) used in this study were similar to those reported by Hazard *et al*. in 2015 and by Schönhofen *et al.* in 2017.[145, 151]

The amylose and resistant starch contents of *sbeII* durum and bread wheat were greater than that of the WT controls, even though some variation was observed between growing seasons. The *sbeII* bread wheat grown in Norwich in Spring 2016 showed a lower RS content than the same bread wheat genotype grown in Norwich in Spring 2018. This is probably due to environmental factors during wheat growth that other authors have shown to greatly affect the starch characteristics of wheat.[57] This was also evident from the yield measured in the two consequent field trials in 2017 and 2018, reported in chapter 2 (Table 2.3), where there was an effect of season of growth and of the genotype on the yield obtained, as well as a genotype by season interaction. The use of wheat cultivars not adapted to the UK environment may also have affected the levels of RS and amylose measured in this study. The amylose content of *sbeII* and WT control durum wheat used in this study were lower than those measured using the

same cultivars by Hazard *et al*. (2015),[145] reporting 44.8% ± 2.03% amylose content in *sbeII* (mean and SEM of wheat grown in three different locations) and 27.9% ± 0.92 in the WT control, and by Schönhofen *et al.* (2017),[151] who reported 45.16% ± 0.49% amylose in *sbeII* wheat across three growing locations and 30.7% ± 0.28% amylose in the WT controls (mean ± SEMs, n = 3), using the same bread wheat cultivar used in this study. It is possible that by using UK adapted wheat varieties carrying mutations in *sbeII* genes, higher amylose wheat lines could be obtained, even though this is a relatively lengthy process as several crosses are required to generate *sbeIIa* and *b* wheat lines.

The different amylose concentrations detected could also be due to differences in the analytical methods. The values reported by Hazard *et al*.[145] and Schönhofen *et al.*[151] were obtained using the Amylose/Amylopectin assay kit by Megazyme on wheat semolina and flour rather than on isolated starch; the use of flour instead of purified starch can lead to increased variability of the results.[234] The Megazyme kit is based on amylopectin precipitation by Concanavalin A (Con A), which leaves the amylose in the supernatant. After separation the amylose is enzymatically hydrolysed to D-glucose that is then quantified colorimetrically. Both methods (amylose-iodine and Con A) are subject to uncertainties. It has been suggested that the use of the Megazyme kit allows for high-throughput analysis of a variety of samples (flour and starches) however, the amylose-iodine method requires less steps and less reagents to quantify amylose once starch has been isolated. Furthermore, the amylose-iodine method can also be designed to be high-throughput as the only limitation is the number of tubes to be incubated per assay run (so the number of slots on the rotator or the number of rotators). It has been debated whether the need to apply an empirical correction for amylopectin–iodine complex formation (described in chapter 2), when measuring amylose-iodine binding, would lower the reliability of the method; however, comparable results in measured amylose content were reported by Batey and Curtin (1996)[235] and by Gérard *et al.* (2001)[236] when SEC, iodine-binding, DSC and lectin-binding methods (including Con A) were compared. Therefore, the use of an iodine-binding method to determine amylose content of starch was deemed appropriate in this study.

It should be noted that the starch structure of *sbeII* is radically altered not only in the amylose fraction but also, in the amylopectin fraction. A study by Tuncel *et al.* (2019)[237] showed that starch from potato carrying mutations in SBE genes was characterised by altered chain length distribution of amylopectin. They identified an increased proportion of long, unbranched

amylopectin chains that are likely to be measured as "amylose" in most assays, including iodine and lectin-binding methods,[28] resulting in tubers or cereals with increased amylose content compared to the WT control.

The changes in *sbeII* starch morphology observed in this study were also comparable to the starch morphology of *sbeII* starch described by Slade *et al.* in 2012.[39] Changes in the starch structure of *sbeII* wheat had likely altered the starch crystallinity, affecting the radial organisation within granules; this could have resulted in crescent-shaped starch granules with reduced birefringence pattern, particularly visible in A granules, as observed by microscopy. Altering the amylose to amylopectin ratio can introduce defects into the crystalline lamellae, affecting the stability of the crystals in the wheat starch granules and their altered morphology.[19, 238]

3.6.2 Non-starch polysaccharides composition

Content of NSPs in wheat is generally of interest as this dietary fibre component of flour and semolina can exert health benefits via mechanisms linked to gut fermentation (prebiotic activity).[239] Wheat semolina or refined flour with increased NSP is of interest to develop high-fibre wheat-based food products. However, the content AX and B-glucans content of white flour is usually very low (2%-3% of dry weight) and normally it is reported together with other dietary fibre components, including RS.[2] Thus, to understand the effect of food products with greater level of amylose and RS, it is important to characterise well the starting material used so that any physiological effect observed after consumption of a novel food products (like *sbeII* wheat-based foods) can be attributed to the right source.[240] The NSP, particularly soluble AXs and fructans, are known to act as prebiotics and provide physiological benefits linked to gastric emptying and satiety, hormonal responses and glucose homeostasis and gut fermentation.[241]

The *sbeII* durum and bread wheat were characterised by an increased content of AX and altered MLG composition, derived from cell wall components. The soluble fraction of AX was significantly higher in *sbeII* bread wheat flour compared to the WT control and the proportion of substituted AXOS was greater in *sbeII* than the WT control semolina. It has been suggested that under certain conditions (such as high molecular weight and high content) some NSP components, especially the soluble fraction, may influence the viscosity of a food matrix and therefore alter the interaction between digestive enzymes and substrates.[208, 209] The higher ratios of G3 and G4 residues in *sbeII* refined flour compared to the WT control and the greater proportion of substituted AXOS suggest a change in AXOS and MLG structures in *sbeII* wheat. However, the

overall total and WE-AX contents measured in *sbeII* wheat were still within the ranges reported by Ward and colleagues in 2008[242] and by Andersson *et al.* in 2013[231] for bread wheat. The increase in the MLG content (G3+G4) and change in structure (G3:G4) measured in raw *sbeII* semolina are still within the range indicated by De Santis *et al*. (2018) for other durum wheat cultivars.[243] As AX solubility depends largely on molecular weight and substitution degree,[244] a change in AX composition could correspond to a change in viscosity properties.

Wheat semolina and refined flour usually have very low contents of NSP as the majority of AXs and MLG are in the bran that is removed during milling. Milling extraction rate indicates how much of the kernel is recovered in flour or semolina. A lower extraction rate could indicate that less of the bran fraction was combined with the finer milled fraction to give flour or semolina therefore, a lower content of NSPs. [148, 245] While a lower extraction rate was observed for *sbeII* wheat flour and semolina compared to the WT controls (Materials, chapter 2), the milled *sbeII* materials had marginally higher bran content (Materials, chapter 2, 2.1.1.1 and 2.1.1.2), which could explain the increase in NSP content. In this case, the lower extraction rate could be due to a less effective milling process by rollers, possibly due to other factors such as increased grain hardness, as reported by Schönhofen *et al.* (2017).[151] It remains unclear whether the elevated levels of NSP are a direct pleiotropic effect of *sbeII* genes or an effect of other external factors such as the environment or the milling process.

Overall, the difference in NSP content in *sbeII* wheat is an interesting observation and future studies should take into account their potential role in gut fermentation.

3.6.3 Size distribution of starch granules measured by laser diffraction

Starch from *sbeII* cereals is known to be characterised by a relatively higher resistance to amylolytic enzymes (RS). However, no study to date has reported on the *sbeII* starch granules size distribution and how altered proportion of A vs B granules may affects starch physico-chemical properties.

In this study, particle size analysis by laser diffraction showed a mixed distribution of *sbeII* granules, typical of wheat starches.[39] However, the shape of the distribution of *sbeII* starch appeared different from that of the WT control, in bread wheat, with differently shaped distributions corresponding of A granules (20-30 µm) and B granules (< 10 µm). This suggested possible differences in the proportion of A and B-granules in *sbeII* wheat that was not previously reported. However, the starch distribution of samples analysed by laser diffraction analysis were characterised by a large variation in particle size, particularly for starch isolated

from durum wheat semolina. The analysis of starch from durum wheat was carried out using 40% PIDS, which is usually used for materials of larger particle size (such as semolina); the sample concentration used may have been too high to detect smaller particles (10 µm and smaller) because of multiple scattering. The analysis should have been repeated using a lower obscuration as the one used for starch isolated from bread wheat.

Some of the variability observed in the analysis could also be the result of impurities left behind during the starch isolation process or other analytical factors linked to the LS 13 320 instrument. Starch from the Autumn trial presented a right tail of few but larger particles that contributed to broadening the distribution; this probably explains the lower volume (%) detected compared to the starch from the Spring trial (Figure 3.10c). The tail represented particles of up to 170 µm, that were trimmed to improve data visualisation. These are unlikely to be large starch granules but probably clusters of granules that did not separate completely during sonication.

Further experiments could help clarify whether experimental factors may have confounded the results. Repeating the starch isolation could provide insight on the present of other particles interfering it the analysis. Other isolation methods could also be considered to ensure that the protein component is removed completely so that starch granules are not aggregated into clusters of different size.

3.6.4 Size distribution of starch granules measured by electrical sensing zone

In order to explore further the possible differences in the proportion of A and B granules observed in *sbeII* and WT control bread wheat starch, granule size distribution was analysed using electrical sensing zone analysis on a Multisizer4e Coulter Counter. The advantage of this technique, compared to laser diffraction, is that particles is determined by impedance, improving measurements precision for non-spherical particles.

This analysis showed that the proportion of B granules in *sbeII* starch was lower than that of the WT control starch. Overall, the proportions of A and B-granules reported here are still within the ranges reported by Zhang *et al.* in 2016 in Chinese wheat cultivars.[224]

The altered proportion of A and B granules observed in *sbeII* starch could be an indirect effect of *sbeII* mutations however, as with other starch characteristics, it cannot be excluded that environmental factors played a role in determining the ratio of A and B granules.[246] The percentage of B-granules varied depending on the season of growth as shown by the particle size distribution analyses by laser diffraction and electrical sensing zone analyses of starch from

bread wheat. Starch isolation, sample preparation and instrument performance are common sources of confounding effects; these were controlled by using a good number of independent replicates that were analysed within 24 hours of sample preparation. Starch was isolated from 3 different plots per genotype, per field trial, and each sample replicate was analysed in triplicates. Thus, it is unlikely that the differences observed were caused by experimental factors. The use of *sbeII* and WT control starch from other field trials could provide more information regarding the difference in A vs B-type granules observed in this study, this confirm whether a true difference in the proportion of B granules exists between *sbeII* starch and the WT control.

3.6.5 Physico-chemical properties of A and B starch granules

Another aim of this study was to determine the composition of A and B starch granules from *sbeII* wheat, to explore the specific contribution of A- and B-type granules to the overall starch composition and behaviour of *sbeII* wheat. To achieve this objective, it was deemed necessary to separate the A-granule fraction from the B-granule one.

Separation of A and B granules can be challenging and often leads to only partly pure fractions. Salman *et al.*(2009)[199] attempted the separation, reporting that their B-granule fraction contained approximately 18% A granules at the end of the process.

Several separation methods can be found in the literature, including sedimentation, micro-sieving, filtration and Percoll separation, as described by Peng *et al.*[194] and Park *et al.*[188] Micro-sieving and filtration were not considered suitable for the study presented in this chapter as they can result in an incorrect granule size distribution due to the loss of certain granule fractions, particularly the smaller B-granules.[190] The Percoll separation method by Peng *et al*.[194] required optimisation as the published method did not provide enough detail. It was decided to modify the published method to combine Percoll separation with a sedimentation step to remove most of the Percoll solution, especially from the supernatant phase containing predominantly B-granules. The method was found to be reasonably efficient in separating the two granule fractions, as shown in the results of this chapter, however, a major limitation of the method was the presence of Percoll in the purified fractions, which may interfere with further starch analyses that require binding of enzymes to the substrate or iodine binding for amylose determination. Granule shapes should also be considered; Percoll separation has not been reported on starch granules with altered morphology. It is unclear whether the crescent-

shaped granules of *sbeII* starch may sediment or separate differently from the lenticular or spherical ones.

Due to time restriction and the lockdown during the COVID-19 outbreak, it was not possible to continue this work to obtain conclusive results on the amylose content of B-granules in *sbeII* and WT control starch. The *sbeII* A-granules starch showed amylose levels that are consistent with those measured on unfractionated starch, greater than the WT control starch. To measure amylose content of B-granules, the method required further optimisation to improve starch recovery after Percoll separation. Other experiments that were planned were to measure *in vitro* susceptibility to hydrolysis of the two granule populations.

More research is required to optimise the starch fractionation using Percoll: based on the results presented in this chapter, achieving a higher sample purity during separation and a higher starch recovery rate should be the priorities.

3.7 Conclusions

In order to develop food products with lower susceptibility to amylolysis and potential to elicit a lower glycaemic response *in vivo*, *sbeII* starch characteristics were measured. The *sbeII* materials were characterised by structural changes that can be quantified as increased amylose and RS proportion compared to the WT controls and altered granule morphology. These changes are known to reflect on starch properties (gelatinisation and retrogradation) that could be exploited to develop foods with low susceptibility to amylolysis.

The changes observed in NSP content and composition could contribute to increase the fibre content of *sbeII* foods even though, because of the subtle changes reported in this chapter, the *sbeII* starch properties are expected to act as main driver in the glycaemic response to starchy foods.

For the first time, *sbeII* starch granule distribution was investigated. The differences in the proportion of B granules in *sbeII* starch compared to the WT control could be an indirect effect of the *sbeII* mutations and may contribute to some of the altered starch characteristics measured in *sbeII* wheat. Further research could focus on the optimisation of a separation method to determine amylose content, susceptibility to hydrolysis and other structural characteristics such as the chain length of amylose and amylopectin in A and B granules, of *sbeII* and WT control starches that control starch physico-chemical behaviour. Granule size distribution is a determinant of starch susceptibility to amylolysis, when starch is isolated, as the size and the composition of granules determine the rate and extent of digestion. Smaller granules are digested faster than larger ones; smaller B granules are thought to have generally a lower proportion of amylose conferring to B granules different physicochemical characteristics from A granules. However, starch is consumed after a cooking process therefore the physico-chemical properties of cooked starch play a major role compared to granule size distribution on susceptibility to hydrolysis. This will be explored in the next chapter.

Chapter 4 The effect of processing on *sbeII* wheat end-use quality and starch susceptibility to hydrolysis

Abstract

This chapter presents an analysis of the starch susceptibility to hydrolysis of *sbeII* wheat flour and semolina used in different food-products formulations and processes. Durum and bread wheat *sbeII* and WT control grains were milled to semolina and flour and their particle size analysed by laser diffraction. The milled fractions were used to measure the effect of hydrothermal processing on starch susceptibility to hydrolysis using the *in vitro* amylolysis model previously described. Different hydrothermal processes were considered; semolina was cooked in excess of water to produce a porridge and cooked and cooled to produce a pudding. Bread flour was used to produce bread, a high moisture food cooked in high heat, and crackers, made as a low moisture dough baked briefly at high heat. The processing performance and starch susceptibility to amylolysis of *sbeII* wheat after hydrothermal processing are reported in this chapter (Figure 4.1).

***Figure 4.1 Chapter 4 visual abstract**: crop to food approach to design sbeII wheat-based food products. This chapter presents the starch characteristics of sbeII wheat after processing.*

4.1 Introduction

Wheat grain is a great source of nutrients that are mostly unavailable to digestive enzymes in their raw 'unprocessed' state. To increase nutrients accessibility, wheat grains are crushed during milling and then exposed to heat and water during cooking.[148] To obtain refined flours, the outer layers of the grain are removed exposing the starchy endosperm to the actions of mill stones or rollers. The endosperm cells are broken exposing and releasing starch and other nutrients; the extent of starch availability in milled fractions depends on the milling efficiency. Wheat kernel characteristics and milling efficiency determine the achievable particle size; this is thought to have a direct effect on starch digestibility and glucose response to starch-based foods.[247]

Upon cooking, starch gelatinises (as explained in Chapter 1), becoming more susceptible to the action of digestive enzymes.[186] In wheat grains, the starch molecular structure, as well as other nutrient components, are a key factor in determining digestibility wheat foods. The degree of starch gelatinisation and retrogradation is known to be linked to the nutrient composition of the raw wheat material.[248] The ratio of amylose to amylopectin, starch granules properties and fibre/protein content and type of wheat (bread or durum) determine the properties of the surrounding food matrix and the degree of starch gelatinisation and retrogradation.[201, 249]

4.1.1 The effect of milling

Milling is a process aimed at fracturing the wheat grain endosperm to expose starch granules. Roller milling usually require the grains to be soaked in water for several hours to allow moisture absorption, this process is called tempering. This ensures that the bran can be easily fragmented and separated from the grain while softening the endosperm by weakening the protein-starch bonds.[250] Rollers are selected depending on the grains size, hardness and required end products. After each milling step, the products obtained pass through a series of sieves to obtain coarser semolina or refined flour.[251]

Hard wheats, such as durum and bread wheats, are characterised by a harder kernel compared to soft wheats used for cake or cookie making.[6] Kernel hardness depends on the strength of the protein-starch interactions in the endosperm: when starch and proteins are tightly bound, stronger forces are required to break the bond that can cause starch granules to fracture.[252] The degree of damage depends on kernel hardness (defined as the force required to crack the kernel)[253] and the mechanical process used to crush the grains. Since

starch damage increases with wheat kernel hardness and mechanical work of the rollers, milling hard wheats generally produces more damaged starch than soft wheats, characterised by a softer endosperm that can be reduced into small particles (flour) readily.

The physical damage caused by milling affects starch crystallinity. Damaged starch granules swell quickly in water and have increased water absorption capacity compared to intact granules.[254] Flour water absorption is an important determinant of its end-use functionality, starch rheological properties and viscosity.[255] In a starch-gluten system such as bread, the hydration properties play a major role in the development of the final product and its digestibility.[256]

Wheats with increased kernel hardness (such as durum) require specific milling adjustments to prevent excessive starch damage. This is achieved by milling durum wheat to a coarser particle size compared to bread wheats (>200 µm), i.e., semolina, to reduce the number of fractured surfaces and thereby limit the degree of starch damage.[257] Coarsely milled wheat may contain some intact cells where starch is trapped and inaccessible to digestive enzymes. This is likely to be digested more slowly than a finely milled endosperm lacking intact cells.[258] Milling is therefore an important part of processing and a key factor in determining wheat foods digestibility as changes in particle size can influence the extent of starch digestibility.[34]

4.1.2 The effect of cooking

When starch is suspended in excess of water, the granular crystalline structure can be disrupted by applying heat ('gelatinisation').[52] During hydrothermal processing, water enters the granule and destabilises the hydrogen bonds of the amorphous regions, weakening the crystalline regions nearby. The lack of stability within the granule's regions permits further water entry and granule swelling.[186] As the temperature increases, starch double helices melt causing the granule to become more amorphous.[259] Amorphous granules are more susceptible to digestive enzymes as the loss of crystalline structure exposes more binding sites for amylolytic enzymes.[92] Gelatinised starch cannot reverse to its original structure but, upon cooling, starch chains can re-associate to form new, partially ordered, structures ('retrogradation'). The retrogradation process happens at different rates for the different components of starch, depending on their structure. Short-term storage mainly leads to retrogradation of amylose, while amylopectin recrystallization is a slower process that depends on the ability of the branched chains to re-associate.[257] Amylose retrogradation in

processed foods is thought to contribute to gel strength, dough stickiness, ability to absorb water, and digestibility, whereas amylopectin retrogradation is a more important determinant in bread and cakes staling.[186] The extent of gelatinisation and retrogradation are major determinants of starch susceptibility to enzymatic digestion and foods end-use quality.[257] These greatly depend on the water content and processing temperature of starchy foods.[260, 261] Thus, starch gelatinisation and retrogradation can be manipulated by modifying the amount of water and heat applied during processing as well as the mechanical process applied.[262]

The combination of high moisture levels and high temperatures (e.g. bread making) or high pressure and shearing (as in extrusion cooking) favours starch gelatinisation and, as a consequence, the starch becomes highly susceptible to digestive enzymes.[263] In contrast, in moderate to low moisture foods with shorter cooking times such as biscuits and pasta, a lower degree of gelatinisation or limited starch swelling preserves starch resistance to hydrolysis.[264, 265]

In this chapter, *sbeII* wheat semolina and flour were used to produce different food products, including porridge and pudding from durum wheat semolina, crackers and bread from bread wheat flour.

4.1.2.1 Bread making

Wheat bread is a widely consumed product, characterised by a highly porous structure and gelatinised starch that can be easily broken-down during digestion in the upper GI tract.[266] Breadmaking involves the combination of flour, water and yeast (although not all bread types require it); once flour and water are combined, hydrated flour can be 'kneaded' to strengthen the gluten network and left to ferment. The gas produced during fermentation are trapped by the strong gluten network, which expands during baking giving bread loaves a certain volume. The high temperature of the oven causes starch to gelatinise, increasing bread digestibility.[267]

Bread making is an ancient technique that has evolved over the centuries to produce breads with improved organoleptic characteristics, high volume loafs with a softer and flavourful crumb. As bread became a staple in many UK households, the traditional breadmaking method (or sponge method) requiring several hours of fermentation, was no longer suitable for large scale production. The increased industrialisation of bakeries in the late 1950s focused on the optimisation of a new breadmaking method by the British Baking Industry

Research Association[3] (Chorleywood, Hertfordshire, UK), known as the Chorleywood method in the UK or straight-dough method in the USA. The newly develop method involved a faster process with less manual handling; this allowed bakers to produce soft white wheat loafs, that were cheaper than those obtained with a conventional breadmaking technique, to satisfy the increasing demand for bread. The method relies on increased mixing rate and dough temperature, the addition of extra water to adjust dough consistency and the use of an 'improver' to shorten the fermentation step, usually a combination of oxidising agent (such as ascorbic acid), emulsifiers (to provide dough strength and extend shelf-life) and enzymes (proteases and amylases to boost yeast activity).[268] The increased mechanical manipulation of the dough develops heat and contributes to starch granules swelling, favouring starch gelatinisation, while the short resting period limits flour hydration and gluten development.[269]

4.1.3 Crackers making

Crackers, like biscuits, are characterised by limited starch swelling and gelatinisation. Crackers dough is moderately low in moisture and it is cooked briefly at high temperature.[263] Crackers making requires similar ingredients to bread making: some crackers require a fermentation or resting period, like fermented or soda crackers, or snack crackers. Others are sheeted immediately after mixing.

Soda crackers are characterised by the addition of sodium bicarbonate (1%) which increases the alkalinity of the dough, and 8-10% of shortening. Once the dough is rested, it is sheeted to about 4 mm and then laminated six to eight times. They are typically 4 mm thick and 50 × 50 mm square, flaky but crisp. Typically, soda crackers are cut by making perforation lines and baked as a whole sheet to minimize dough waste.

In the UK, cream crackers are more common than soda crackers. These are characterised by a slightly higher fat content (12–18%) than soda crackers, they are generally hand-cut before baking to a 65 × 75 mm rectangular shape and are slightly thicker than soda crackers (~6.5 mm). Cream crackers have a moisture content of 3–4%, which, along with the increased fat content, make the crackers flaky and relatively soft. [270]

The nature and quantity of each ingredient used, particularly the flour, determines the dough rheological properties and baked characteristics.[271] Cracker production usually requires soft flours (low-protein and fine in particle size) for a tender finished biscuit. Crackers that

[3] Today known as Campden BRI

undergo a fermentation stage often require a strong (high-protein) wheat flour with strong dough properties, such as bread wheat.[272]

4.2 AIM: Determine the consequential effects of physical and hydrothermal processing on starch susceptibility to hydrolysis of *sbeII* wheat

The rate of starch enzymic hydrolysis in durum wheat semolina and bread wheat flour was measured after cooking to evaluate the how the nature of the starch, the changes in temperature and duration of heating, the water availability and the use of mechanical forces affected the starch amylolysis. Semolina was used to produce a porridge and a pudding; these are cooked foods with high moisture content but, while porridge would be consumed immediately after cooking, pudding is left to cool.

Bread wheat flour was used in baking to produce bread that as a dough, it is a relatively high moisture food-system, and crackers, that as a dough, have a lower moisture content than bread dough.

Bread wheat products were developed and produced by Campden BRI. Campden BRI also performed the end-use quality analyses on these products, results were delivered in the form of a report.

Durum wheat products were developed and produced at the Quadram Institute Bioscience.

4.3 Materials and methods

4.3.1 Formulation and processing of products made from *sbeII* durum wheat

4.3.1.1 Durum wheat porridge and pudding

Durum wheat semolina was processed as follows: it was boiled in water (porridge) and the RS and rate and extent of starch hydrolysis were measured. Semolina was boiled in water and left to cool (pudding) and the RS and rate and extent of starch hydrolysis were measured.

The formulation and cooking process were based on recipes of commonly consumed semolina-based foods. Cooked samples were prepared by adding 1mL of water to ~100 mg of semolina, this was boiled for 10 min at 99℃ hand-mixing every 3-4 minutes using a spatula to obtain a “porridge”. Cooled samples were prepared by adding 1mL of water to ~100 mg of semolina and cooking this as described above. After boiling, the cooked semolina was left to cool for 10 minutes at room temperature and then placed on ice for 2 hours to obtain a

"pudding". The same formulation and processing were used for both types of semolina, no extra water was added.

4.3.1.2 Starch composition analyses of porridge and pudding

Semolina porridge and pudding were prepared as described above and total and resistant starch were measured using the Megazyme kit KOH format (described in chapter 2).

4.3.2 Particle size distribution of milled wheats by Laser Diffraction analysis

4.3.2.1 Durum wheat semolina

Semolina particle size was analysed in three different states: raw, hydrated in water and cooked in an excess of water. Raw samples were prepared by adding deionised water to semolina followed by immediate analysis. Hydrated samples were prepared by suspending semolina in room temperature (~21 °C) deionised water for 40 minutes before starting the analysis. Boiled samples were prepared by adding deionised water to the semolina and boiling for 10 min with intermittent mixing by inversion.

Size analysis was carried out using a LS 13 320 described in chapter 2, samples were loaded to reach a 40% PIDS. The analysis was set to return the particle size distribution as 'Volume moment mean' (D [4, 3]) of particle diameter.

4.3.2.2 Bread wheat flour

Bread flour particle size was analysed immediately following the addition of deionised water to the samples. Following the set up previously described (chapter 2), samples were loaded to reach a 40% PIDS. The analysis was set to return the particle size distribution as the 'Volume moment mean' (D [4, 3]) of the particle diameter.

4.3.3 Formulation and processing of products made from *sbeII* bread wheat

4.3.3.1 Campden BRI bread

The bread formulation is reported in Table 4.1 based on a dry weight basis. For bread, water was originally set to a standard 61% however, *sbeII* wheat flour had a higher water absorption (73.5%) compared to the WT control (54.6%) so, it was decided to adapt the recipe based on the flour water absorption for a correct dough development and texture. Failure to adapt the water content would have resulted in increased mixing time and poor dough development, which affect bread volume[273] Bread dough was produced according to the standard Chorleywood Bread Process using a spiral mixer, mixing for 2 minutes at low speed and 8 minutes at medium speed. Each batch was portioned into 24 individual rolls of 250 g to obtain

baked loaves of ~225 g and proofed for 5 minutes before final moulding. Loaves were shaped using a conical moulder and placed in 400 g loaf tins. These were proved in a proofing cabinet at 40˚C (75-80% relative humidity) for 55 minutes. Loaves were baked at 235˚C for 20 minutes and cooled for 3 hours. After cooling, the bread was packed in plastic bags and transported to the Quadram Institute Bioscience in Norwich. Baked loaves had an approximate shelf life of 5 days.

Table 4.1 Chorleywood bread formulation created by Campden BRI*, dry basis percentage corresponds to the baker's percentage where the ingredients are referenced against the amount of flour. The same formulation was used to produce sbeII and WT control breads.*

Ingredient	**% dry basis**
Bread flour	100
Water	N/A[4]
Yeast	2.25
Salt	1.5
Shortening	2
Improver	0.8

4.3.3.2 Campden BRI crackers

Crackers were produced according to a standard recipe used at Campden BRI (Table 4.2). The original recipe included syrup and malt; these were removed from the formulation to obtain a lean recipe without extra sugars as these may have interfered with the starch analyses. The ingredients were combined in a spiral mixer for 2 minutes at low speed. The crumbly dough was kneaded for 10 minutes at medium/high speed. The low hydration of the dough did not allow for a cohesive mass to form, rather a hard and tough dough that could be sheeted using a reversible dough sheeter (LMA floor model). The dough was passed through dough rollers 6 times, each time reducing the rollers gap from 8 to 1 to obtain a dough sheet 0.6-0.7 cm high. Crackers were cut using a cream cracker cutter and baked on wire trays at 250°C for 6-8 minutes. Once baked, the crackers were cooled and packed in vacuum sealed bags for transport to the Quadram Institute Bioscience in Norwich.

[4] Water added was based on water absorption of each flour: 73.5% for *sbeII* flour and 54.6% for WT control flour

***Table 4.2 Cracker's formulation created by Campden BRI**, dry basis percentage corresponds to the baker's percentage where the ingredients are referenced against the amount of flour. The same formulation was used to produce sbeII and WT control breads.*

Ingredient	Mass (g)
Flour	100
Water	40
Shortening	9.5
Salt	1
Sodium Bicarbonate	2

Crackers formulation is reported based on mass basis, the same formulation was used for both flour types, no extra water was added.

4.3.4 Products quality analysis

4.3.4.1 Bread dough quality

Bread dough quality was evaluated visually based on the descriptors in Table 4.3. A 'sticky' dough is not desirable as it can produce a weak side wall and a sticky crumb; this is linked to undesirable browning of the crust and early moulding of baked bread.[267]

Table 4.3 Dough quality scores

Dough score	Dough stickiness
1 Tight	A Sticky
2 V	B Slightly Sticky
3 Optimum	C Not Sticky
4 V	
5 Soft	

4.3.4.2 Bread volume

Bread volume was measured using a VolScan Profiler 600 (Stable Micro Systems, Surrey, UK) using the AACCI Standard Method (reference 10-16.01). The benchtop laser-based scanner measures the volume of bread and bakery products similarly to the seed displacement method (see chapter 6). A whole loaf of bread is mounted on its longest axis inside the scanner chamber by inserting it between the base support and the top arm of the vertical stage. By starting the scan, the loaf rotates to allow for a full 3D image to be acquired. Bread volume was measured twice on two bread loaves for each bread type (*sbeII* and WT control).

4.3.4.3 Bread structure

Bread cell (gas bubbles) structure was analysed using the C-cell Colour imaging system by Calibre Control (Warrington, UK). Cell structure was used to assess the internal structure and external characteristics to ensure consistent quality across bread types. The bread loaf was sliced on the Graef Universal Slicing Machine (WI-BCP-378) to produce 15 mm thick slices. Four slices from the centre of one loaf per genotype were selected for quality analysis. Each slice was inserted in the imaging drawer of the instrument and cell diameters, cell number and wall thickness were determined using the Calibre Control analysis software.

4.3.4.4 Bread texture analysis

Texture Analysis on bread was carried out using a SMS TA-XT2 Texture Analyser (Stable Micro Systems, Surrey, UK). Texture was tested repeatedly during product storage (1, 3, 5, 7, 8 days after baking). For this analysis, a 2 x 10mm section was cut out of each bread slice used in the C-Cell analysis, four slices per loaf were used. The specimen was mounted on the stage and the test was carried out using a P25 cylinder probe (Perspex, 25 mm diameter) with a flat base on 'force in compression' mode (Test speed 5 mm/s, Distance 35% strain, Trigger Auto, 5g, Time 0.1s between the 2 compressions), Figure 4.2a.

The test returned the 1) maximum force on the trace as the predictor of sensory firmness, 2) the cohesiveness from the trace as the predictor of rate of breakdown in the mouth, 3) the springiness from the trace as the predictor of springiness to touch.[274] A detailed description of these parameters and their calculation can be found in chapter 2, section 2.2.4.

4.3.4.5 Cracker texture analysis

Texture Analysis on crackers was carried out using a SMS TA-XT2 Texture Analyser (Stable Micro Systems, Godalming, UK) one day after baking. A 'three-point bend test' was used to determine fracturability and hardness of the crackers: a force was applied to the centre of the sample (as in Figure 4.2b) and the breaking stress was determined. The 'distance to break' gave an indication of the brittleness (fracturability) of the sample, showing how far a sample can be deformed before fracture while the 'maximum force' was used as a measure of hardness.[275]

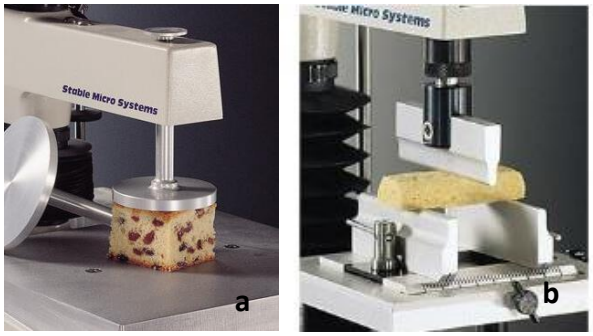


Figure 4.2 a. Compression test for bread or cake. b. Three-point bend test on a biscuit.

Figure modified from www.stablemicrosystems.com

4.3.5 *In vitro* starch amylolysis

In vitro starch susceptibility to hydrolysis was measured using the amylolysis model described in chapter 2 (2.2.3), where samples are incubated with pancreatic α-amylase at 37 °C for up to 120 minutes. The progress of the amylolysis is monitored through reducing sugar analysis (the product of digestion).

Starch amylolysis in **bread** was measured within two days of production because of the very limited shelf-life of the bread at room temperature. Storage at room temperature was preferred as cooler temperature (fridge or freezer) favour starch re-crystallisation.[276]

Bread loaves were sliced to a thickness of ~1.5 cm. Two slices from the centre of the loaf were cut in 4x4.5 cm squares without removing the crust and minced using a food processor (Kenwood CH 180 Mini chopper) for 55 seconds. Three replicates of crumb were taken from each bread, three bread loaves were used for the amylolytic assay.

Crackers packed in vacuum bags were stored for one month at room temperature before measuring starch hydrolysis, as their shelf-life was much longer than bread (up to 1 year). On the day of analysis, three crackers were grinded for 55 seconds in a food mixer (Kenwood CH 180 Mini chopper) to produce small fragments. Three independent samples were used for this analysis.

Porridge was prepared as described in section 4.3.1.1. After cooling at room temperature for 10 minutes, samples were taken using a spatula.

Pudding was prepared as described in section 4.3.1.1. Using a spatula, the core of the pudding was scooped into a garlic press. The pudding was squeezed through the garlic press to produce cylinders of approximately 0.5 cm in length to be used for the amylolysis assay.

4.4 Results

4.4.1 Durum wheat food products

4.4.1.1 Particle size during processing

Durum wheat semolina mean particle size in raw, hydrated and boiled state are reported in Table 4.4. Particle size distribution of raw *sbeII* semolina was larger than the WT control (mean difference = 20.57 µm, [95CI -0.68, 40.46], unpaired t-test, p-value = 0.04). Light micrographs in Figure 4.3 showed an heterogeneous material, with some free starch granules (Figure 4.3 a2, a4) surrounded by proteins (fibrils) as well other granules trapped within residuals of cell wall (Figure 4.3 a1, a2). When hydrated or boiled, there were no differences between *sbeII* and WT control semolina particle size distribution (p-value = 0.07 and 0.06, Table 4.4). A Two-way ANOVA with pairwise comparison showed a significant effect of the processing and the genotype on the semolina particle size distribution but no interaction of genotype by process.

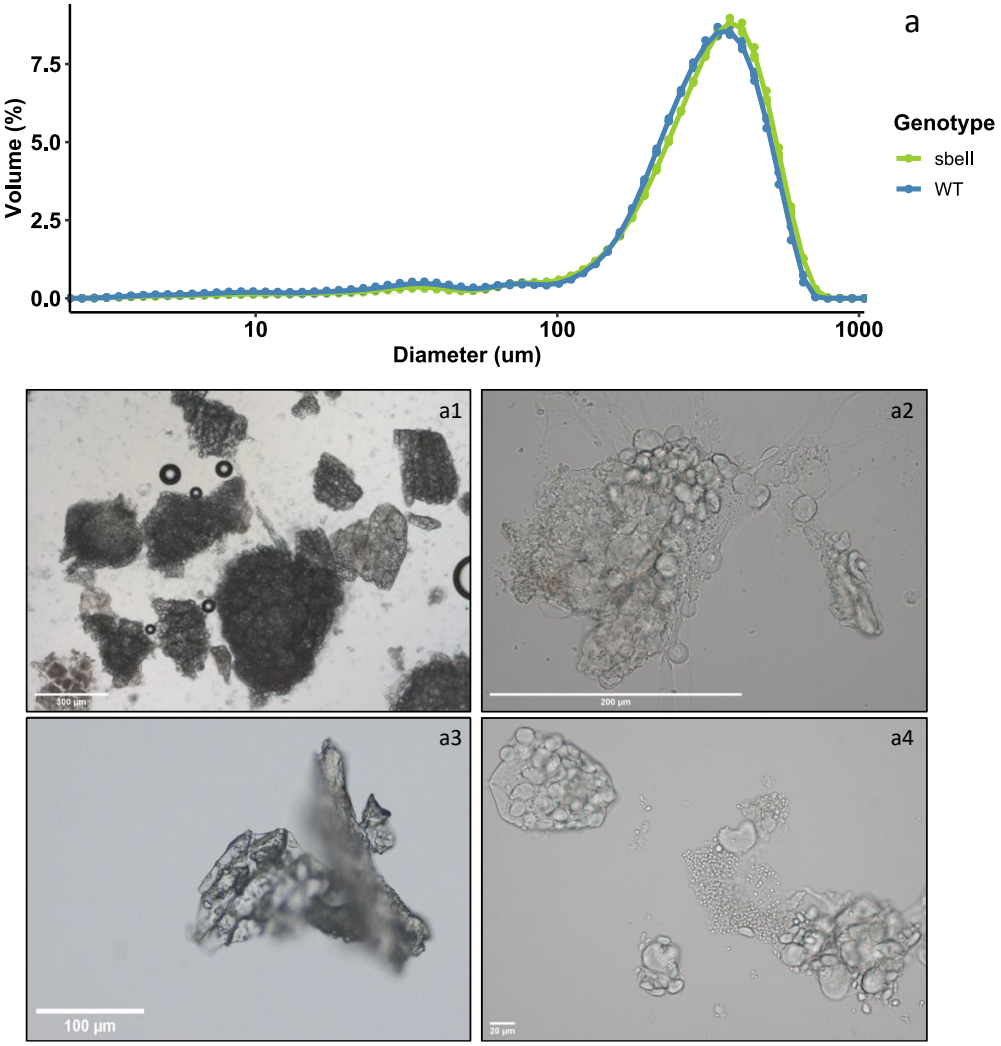


Figure 4.3 Particle size distribution of durum wheat semolina *(a); data points represent n=3 replicates of bulked sbeII and WT control semolina, curves represent the mean diameter on a log diameter scale. a1-a4. Light micrographs of semolina in water showing a range of particles within semolina.*

Table 4.4 Mean particle size distribution of raw, hydrated and boiled semolina (sbeII and WT control, respectively).

Semolina	Process	Mean particle size (µm)
WT control	Raw	258.42 ± 5.53
sbeII		279.00 ± 4.23**
WT control	Hydrated	217.16 ± 3.70
sbeII		228.33 ± 2.04 ns
WT control	Boiled	359.63 ± 8.44
sbeII		386.60 ± 3.89**
Source of variation		
Genotype		0.0004
Process		< 0.001
Genotype x Process		0.3

Mean ± SEMs, n= 3. Two-way ANOVA with genotype pairwise comparisons of estimated marginal means, WT size is used as reference within each group, p-value< '***' 0.001, '**' 0.01, '*' 0.05 '.

4.4.1.2 Starch composition during processing

Total starch content of semolina did not vary with processing and it was used to calculate the proportion of RS over total starch content of processed semolina. Total starch content during processing is reported in Figure A4 1(Appendix A). RS in *sbeII* semolina was found to be different after cooking for 10 minutes, where some 'gelatinisation' occurred, and two hours of cooling, during which time some 'retrogradation' occurred in both *sbeII* and WT semolina, as shown in Figure 4.4. Cooked semolina starch was characterised by a lower RS compared to the cooked and cooled semolina, where some retrogradation occurred. RS was significantly higher in *sbeII* cooled semolina suggesting a higher starch retrogradation in the *sbeII* semolina compared to the WT control. Overall, *sbeII* semolina was characterised by a higher proportion of resistant starch compared to the WT control when cooked and cooled.

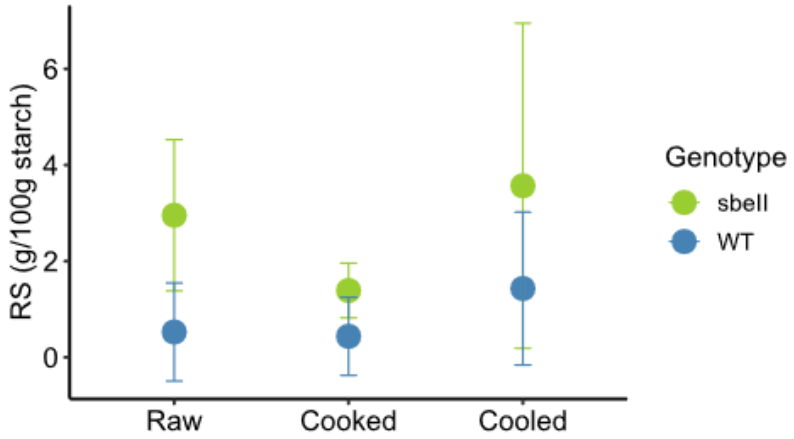


***Figure 4.4. Proportion of resistant starch of raw, cooked and cooled sbeII and WT control semolina**. Means ± SEMs comparing sbeII to WT within each treatment. Mean ± SEMs, n = 3 replicates of bulked semolina that were treated independently.*

4.4.1.3 Starch In vitro amylolysis

There was no difference in starch hydrolysis after 60 minutes of incubation with α-amylase between the *sbeII* semolina porridge (64.4% ± 1.8%) and WT porridge control (68.1% ± 1.9%), (unpaired t-test, p-value = 0.23) nor between the *sbeII* semolina pudding (33.6% ± 8.1%) and WT pudding control (47.7% ± 1.9%), (unpaired t-test, p-value =0.2). The increase in semolina pudding RS was observed after 2h of cooling.

A non-linear regression analysis was used to estimate the *k* and C_∞ parameters. A 2-parameter asymptotic curve (Equation 4) was fit to the experimental data Figure 4.5.

$$(y=a(1-e^{-bx}))$$

Equation 4: Non-linear regression curve

The non-linear model fit was found to be more appropriate than the LOS analysis (not shown) and the calculated *k* and C_∞ are reported in Table 4.5. Starch in *sbeII* pudding was found to be less susceptible to amylolysis based on the parameters *k* and C_∞. The starch hydrolysis of all samples appeared to plateau after the first 15 minutes of incubation. Thus, the parameters calculated using the regression model may not truly represent the rate and extent of digestion. Observing the experimental data points shown in Figure 4.5, processed semolina starch hydrolysis could appear to be a two-phase reaction as the reaction rate slows down after the first 15 minutes of incubation. The LOS analysis did not show a good fit of the model for a biphasic reaction thus, the change in reaction rate is likely due to the ratio of enzyme to substrate used in the experiment.

While no differences were found after 60 minutes of amylolysis, starch in *sbeII* pudding was significantly less susceptible to hydrolysis than the WT control after 15 minutes of amylolysis, before entering the plateau phase (25.6% ± 1.5% and 44.5% ± 1.8% respectively, unpaired t-test, p-value = 0.01). This difference was not observed between *sbeII* porridge and WT control (unpaired t-test, p-value = 0.07).

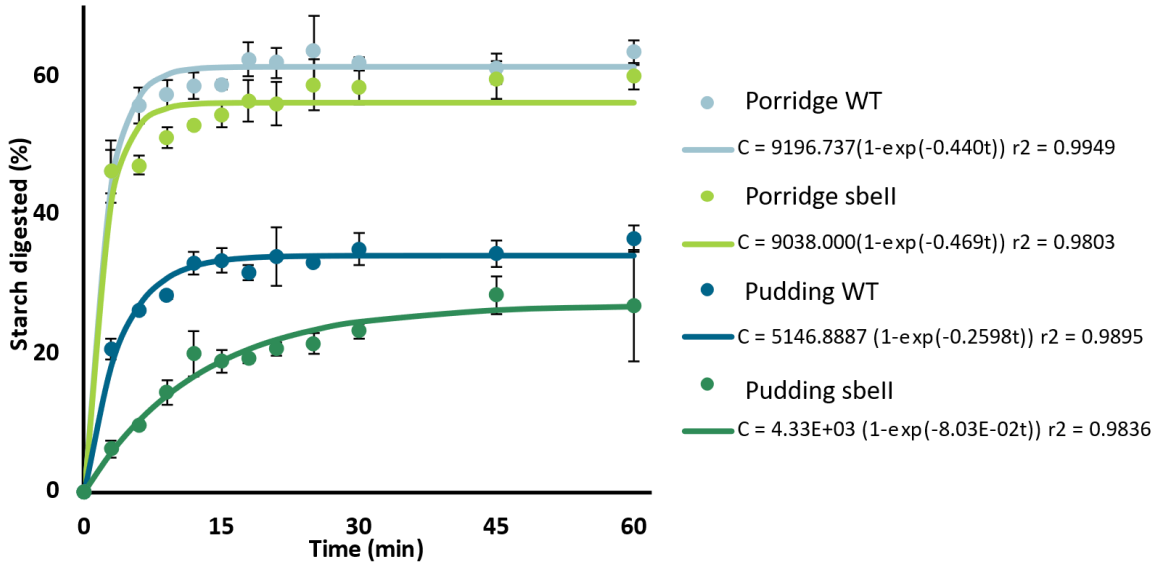


Figure 4.5. Starch amylolysis of sbeII and WT control porridge and sbeII and WT control pudding with fitted non-linear regression curves. *Parameters C_∞ and k were estimated by fitting a first order equation (Equation 4) to the experimental data (data points, Mean ± SEMs, n =3.) Fitted curves are shown by solid line, the equation used to generate them and an estimation of the goodness of fit (r2) are reported in the legend.*

Table 4.5 Variable estimates from the amylolysis curves and LOS analysis of sbeII and WT control porridge and sbeII and WT control pudding.

Semolina	Genotype	Non-linear regression parameter	C_∞ (%)	k (min^{-1})
Porridge	WT control	Estimate	61.4	0.440
		std error	0.6	0.036
	sbeII	Estimate	56.2	0.469
		std error	1.1	0.080
Pudding	WT control	Estimate	33.9	0.260
		std error	0.6	0.025
	sbeII	Estimate	26.8	8.03E-02
		std error	1.2	9.22E-03

Mean ± SEMs, n = 3. C_∞ is the total extent of starch amylolysis accounting for Y_0 (endogenous baseline sugar) and k is the rate constant of the reaction; both parameters were obtained by curve fitting to the starch amylolysis data.

4.4.2 Bread wheat food products

4.4.2.1 Particle size during processing

Volume Moment Mean diameter (D [4, 3]) of *sbeII* bread wheat flour was similar to the WT control flour (means ± SEMs were 149.47 ± 17.53 µm and 123.99 ± 7.94 µm respectively, n = 3 independent replicates of flour, unpaired two sample t-test, p = 0.25). Flour size distribution is shown in Figure 4.6 (b), while Figure 4.6 b1 and b2 show light micrographs of bread flour with clusters of starch granules but no starch trapped within the cell walls.

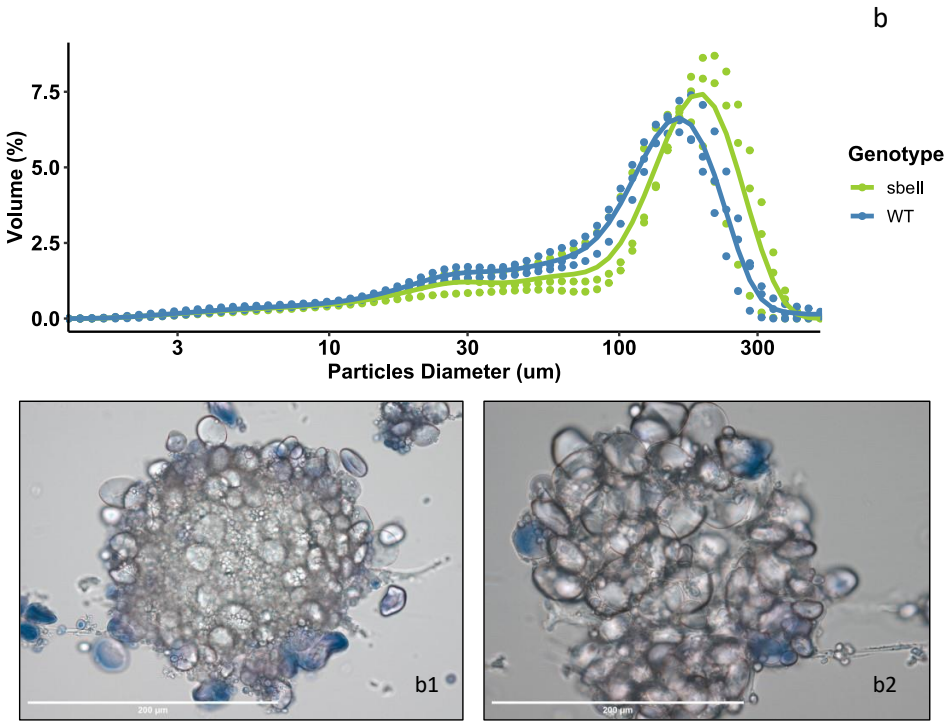


Figure 4.6 Particle size distribution of bread wheat flour, sbeII and WT control *(b); data points represent n= 3 independent replicates of refined flour; curves represent the mean distribution on a log diameter scale. b1, b2. Light micrograph of bread flour in water partially stained with Lugol's solution showing clustered starch granules and protein fibrils (scale = 200 µm).*

4.4.2.2 Bread quality analysis

Dough quality and stickiness were assessed visually before baking. The *sbeII* dough quality was scored a 2, less than optimum but not "tight" compared to the WT control; *sbeII* dough was scored B (slightly sticky) as the WT control.

4.4.2.2.1 Bread structure

Based on the C-cell analysis, similarities between *sbeII* and WT control breads were found for crumb structure but not in wall structure (

Table **4.6**). There were no differences in wall thickness between *sbeII* and WT breads (two-samples t test, p-value = 0.1) however, the crumb of *sbeII* loaves had a marginally lower

number of cells (two-samples t test, p-value = 0.002) and of smaller diameter (two-samples t test, p-value = 0.03), even though this difference does not appear to be important.

Table 4.6 C-cell bread structure measure by Campden BRI

Bread loaf	Cells number	Cell diameter (mm)	Wall thickness (mm)
WT control	4553.2 ± 69.0	2.02 ± 0.08	0.43 ± 0.004
sbeII	4333.7 ± 123.7	1.99 ± 0.04	0.43 ± 0.005

Mean ± SEMs, n = 4.

4.4.2.2.2 Bread volume

Volscan analysis of bread loaves showed differences in volume and density between *sbeII* bread and the WT control bread. Specific volume-mean of 2 *sbeII* bread loaves was found to be lower than the WT control bread loaves (3.6 ± 0.2 ml/g and 5.2 ± 0.13 ml/g, respectively). The *sbeII* bread loaves were found to be denser than the WT control bread loaves (276.16 ± 14.98 kg/m^3 and 193.68 ± 4.86 kg/m^3, respectively).

4.4.2.2.3 Bread texture

Hardness and resilience of loaves were tested at different time points during storage. After one day of storage, there were already clear differences in both hardness and resilience between *sbeII* and WT control loaves. A freshly baked *sbeII* loaf showed greater hardness compared to the WT control. The hardness increased with storage for both bread types while resilience decreased as water evaporated from the loaves however, the decrease in resilience appeared to be more marked in the WT control than the *sbeII* bread (Figure 4.7). Campden BRI was unable to provide the raw data for this analysis so, it was not possible to present the statistical analysis of bread quality changes.

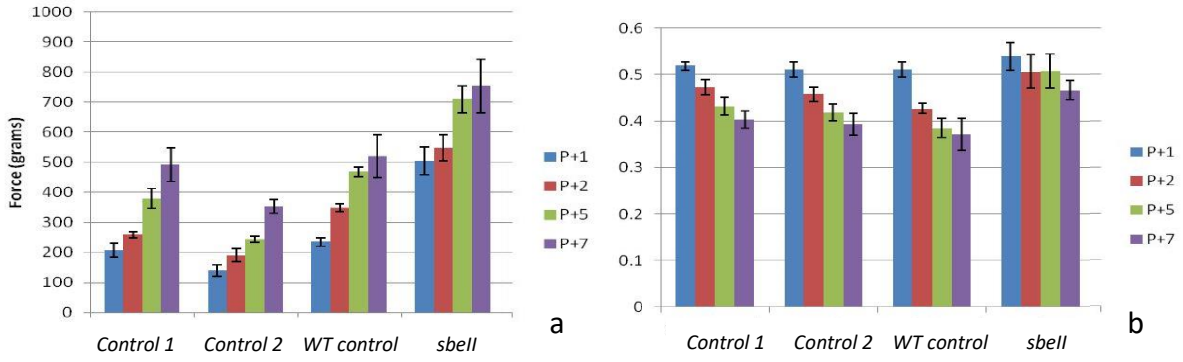


***Figure 4.7. Hardness (a) and resilience (b) of bread during storage**. Figures were provided by Campden BRI (source data not available. 'Control 1' and 'Control 2' are internal controls, "P" corresponds to the baking day, hardness and resilience were measured during storage one, two, five and seven days after baking. Mean values with error bars = SEMs, n = 3.*

4.4.2.2.4 Bread in vitro *amylolysis*

In vitro amylolysis of bread loaves showed a significantly lower starch hydrolysis for *sbeII* bread compared to the WT control (C_{120} mean difference = 16.8 µmol L^{-1} min^{-1}, [95CI 2.67, 31.017], unpaired t-test, p-value = 0.03). LOS plots (Figure 4.8 b, c) showed that a single-phase starch digestion can be described by Equation 3 where the values of k and C_∞ are estimated from the slope and y-intercept of the LOS plot, respectively. The total breakdown of starch represented by C_∞ and the rate constant (k) of starch digestion were determined from the slope and y-intercept of the LOS plot and are reported in Table 4.7. While the hydrolysis was carried out for 120 minutes, C_{90} as well as C_{120} were calculated as previous studies have shown a direct correlation between C_{90} and GI values measured *in vivo.*[174] Both C_∞ and k were lower for *sbeII* bread than the WT control. These kinetic parameters provided a good fit to the experimental data as shown in Figure 4.8. The R^2 values for these fitted models are not optimal ($R^2 < 0.9$), suggesting a low accuracy of the rate of digestion measured by the parameter k.

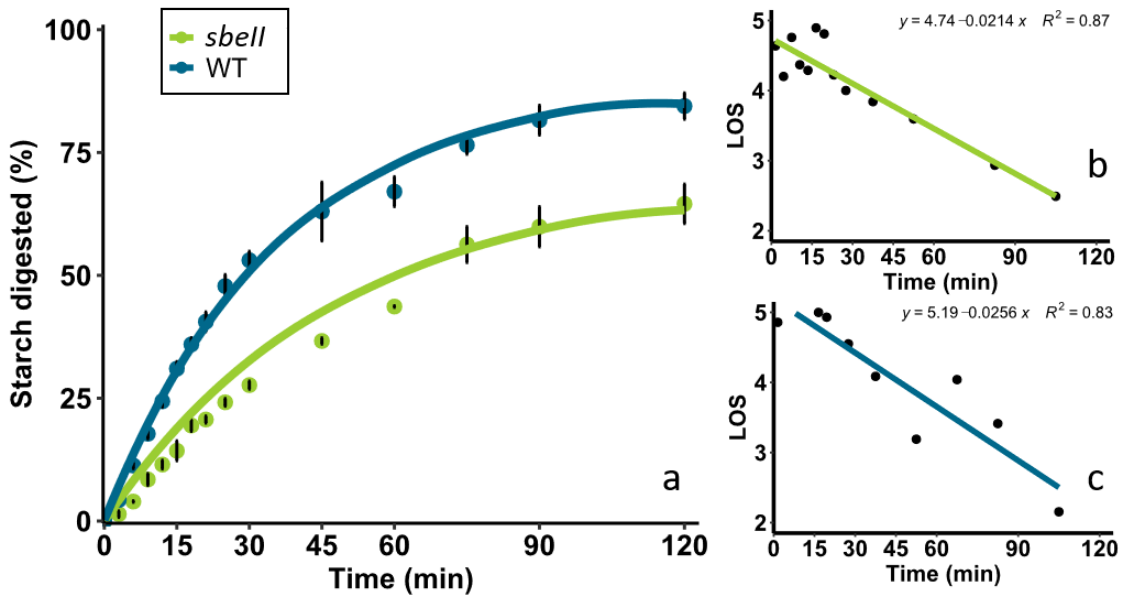


Figure 4.8 a. Starch amylolysis of sbeII bread and WT control. *Starch amylolysis experimental data are shown by fitting a first-order equation (Equation 2) based on the k and C_∞ values obtained from the LOS analysis. Each experimental data point represents the mean value from the analysis of n=3 independent samples with error bars = ± SEMs. b. LOS plot obtained for sbeII bread. c. LOS plot for WT bread. The linear phase is defined using Equation 3 from which k and C_∞ values can be estimated. Legend applies to all panels*

Table 4.7 Variables estimates from the amylolysis curves and LOS analysis of sbeII and WT control breads.

Bread	C_{60} (%)	C_{90} (%)	C_{120} (%)	C_∞ (%)	k (min^{-1})
WT control	75.6 ± 3.0	90.1 ± 3.0	92.9 ± 2.7	88.2	0.028
sbeII	55.3 ± 0.1	67.9 ± 4.1	76.1 ± 4.0	76.1	0.019

C_{60}, C_{90} and C_{120} represent the proportion of starch digested by α-amylase after 60, 90 and 120 min obtained from the starch hydrolysis curves. C_∞ is the total extent of starch amylolysis accounting for Y_0 (endogenous baseline sugar) and k is the rate constant of the reaction; both parameters were obtained applying the LOS analysis to the starch hydrolysis data. Values are reported as means ± SE of n=3 independent replicates.

4.4.2.3 Campden BRI crackers

4.4.2.3.1 Crackers quality analysis

Texture analysis of crackers indicated that crackers made from *sbeII* flour were harder than the WT control but with a similar degree of fracturability, Figure 4.9. It is not possible to confirm whether the differences observed were statistically significant as the raw data of this test was not made available by Campden BRI.

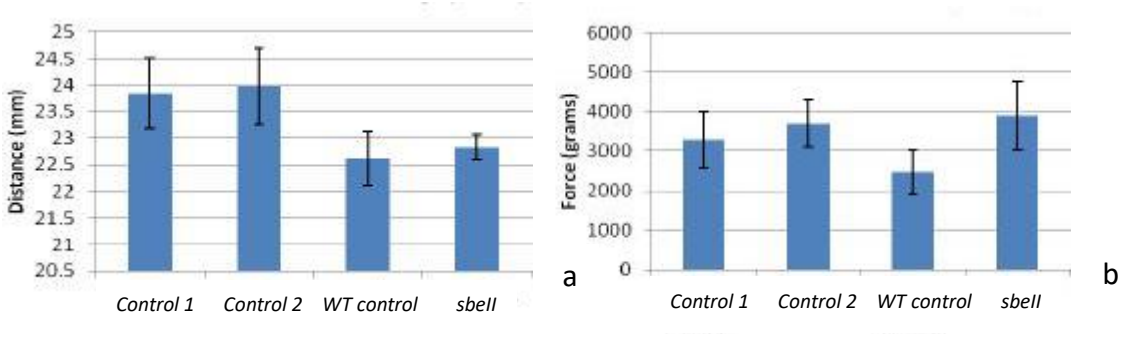


***Figure 4.9 Fracturability (a) and hardness (b) of crackers on day 1 of storage**. Figures were provided by Campden BRI (source data not available. 'Control 1' and 'Control 2' are internal controls. Mean values with error bars = SEMs, n = 3*

4.4.2.3.2 Crackers in vitro *amylolysis*

In vitro amylolysis showed no significant differences in starch hydrolysis between *sbeII* and WT control crackers (C_{60} = 65.0% ± 2.6% and 64.3% ± 1.5%, respectively, unpaired t-test, p-value = 0.83).

The extent of hydrolysis (C_∞) and rate of digestion (k) were calculated from the LOS plot presented in Figure 4.10. Starch digestion progressed at a similar rate for *sbeII* cracker and WT control (k = 0.057 min^{-1}, k = 0.054 min^{-1}, respectively) producing similar concentrations of products of digestion for *sbeII* and WT control (C_∞ = 62.8% and 62.4%, respectively).

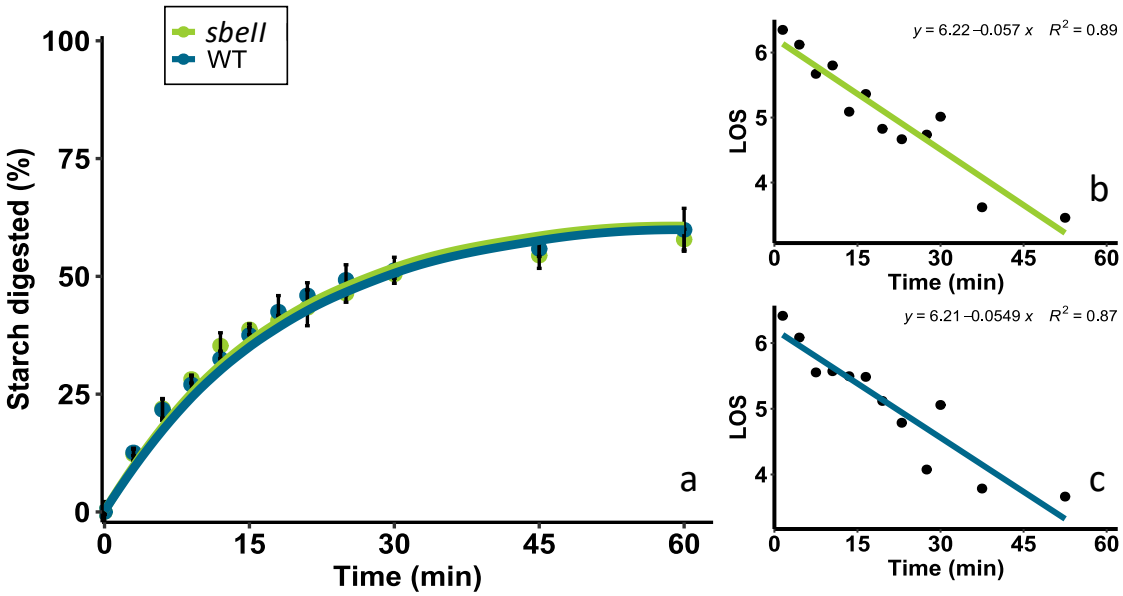


Figure 4.10 a. Starch amylolysis of sbeII and WT control crackers. *Starch amylolysis experimental data are shown by fitting a first-order equation (Equation 2) based on the k and C_∞ values obtained from the LOS analysis. Each experimental data point represents the mean value from the analysis of n = 3 independent samples with error bars = ± SEMs. b. LOS plot obtained for sbeII cracker. c. LOS plot for WT cracker. The linear phase is defined using Equation 3 from which k and C_∞ values can be estimated. Legend applies to all panels*

4.5 Discussion

Intrinsic properties of starch (i.e. physico-chemical characteristics) are determinants of the rate and extent of starch digestion in food matrices but other 'extrinsic' characteristics also play an important role. Wheat grains typically undergo a series of processes aimed improving palatability and increasing starch digestibility. The force and the amount of heat and water involved in the process determine the digestibility and end-use quality of a product. In this chapter, *sbeII* durum and bread wheats underwent different processes after which, starch susceptibility to hydrolysis was measured.

4.5.1 Size distribution of milled wheat fractions

Milling of durum wheat grains resulted in *sbeII* semolina with larger particle size compared to WT semolina, even though the size difference was negligible. No particle size distribution differences were found between *sbeII* and WT control flour obtained from milling bread wheat grains.

The particle size of the wheat fractions obtained from milling depends on the wheat quality, sieve opening, type of rolls, and their adjustment.[277] The milling process was carried out in the same conditions using mill rollers for both WT control and *sbeII* wheats. Since milling of bread

flour using a roller mill produced fractions of similar particle size, it is likely that, in the case of semolina, the milling process was not as efficient in crushing durum wheat kernels. Previous research showed that the *sbeII* wheats are characterised by increased kernel hardness and starch damage upon milling.[151] Conventional milling used may require adjustments when processing *sbeII* durum. Other milling processes such as stone grinding, adjusting the rolls or the grain tempering prior to milling may lead to different results.[255] As particle size is a determinant of end-use quality, it is important to consider the effect of *sbeII* particle size for food formulations.

Particle size can influence starch gelatinisation/retrogradation and susceptibility to hydrolysis. Starch in hard wheats, such as durum wheat, is more prone to physical damage hence, durum wheat is usually milled to a coarser particle size compared to bread wheats.[278] The coarser particle size of milled durum wheat can limit starch gelatinisation and enzymatic hydrolysis of starch; intact granules hydrate more slowly than broken ones and are therefore less susceptible to enzymes.[279] Coarsely milled wheat, such as semolina, may also contain some intact cells where starch is trapped and inaccessible to digestive enzymes.[258] The particle size difference between *sbeII* and WT control raw semolina used in this study was subtle and it seems unlikely to be a contributing mechanism to the difference in RS observed.

Upon hydrothermal processing (hydration and boiling), particle size differences between *sbeII* and WT control semolina were no longer significant. Therefore, it is possible to overcome the size difference with hydrothermal processing, when differences are as subtle as shown in this study.

Particle size is a determinant of semolina and flour functionality and digestibility as the extent of amylolysis is markedly reduced with increasing particle size of flours.[34] Although studies of purified starch provide evidence of the intrinsic factors affecting starch hydrolysis, starch-based foods are usually consumed after a cooking process that involves starch as well as the other components of the endosperm and their interaction with water.

Particle size of the raw material, semolina or flour, determines the rate and extent of gelatinisation and retrogradation of starch. It is therefore important to measure the particle size of the raw materials to provide information regarding this hydrothermal process that ultimately, determines digestibility of starchy foods. It should be considered that semolina is a very heterogeneous material characterised by a broader particle size distribution compared

to finer milled flour. The difference in particle size between *sbeII* and WT control raw semolina reported here could be considered negligible.

4.5.2 Hydrothermal processing effect on *sbeII* starch amylolysis: porridge and pudding

Hydrothermal processing of semolina in excess of water (10:1) and high heat (> 90 °C) for 10 minutes led to similar RS content and susceptibility to hydrolysis of starch in *sbeII* semolina and WT control. Once cooled for 2 hours, some starch retrogradation occurred causing a RS increase in both semolina types. Starch in *sbeII* pudding showed greater RS than the WT control, and lower susceptibility to amylolysis 15 minutes from the start of the incubation however, there were no differences in starch digested at the end of the reaction (60 minutes). Higher RS content in foods is expected to result in a lower starch hydrolysis and lower glycaemic index[263] however, this was not the case for the *sbeII* pudding produced in this study.

Starch retrogradation during cooling greatly affects starch resistance to hydrolysis. High-amylose wheat starches are known to be more prone to gelatinisation and faster retrogradation than conventional wheat starch.[52] Li and colleagues characterised starch physico-chemical properties of a range of wheat lines with different amylose concentration by combining multiple alleles of *SBEII* genes. They reported lower gelatinisation enthalpy (ΔH) and greater gelatinisation range for high amylose mutant starches compared to the corresponding WT control. However, the bread wheat lines analysed by Li *et al*. had considerably higher amylose content, ranging from 36% (WT) to 93% of starch in line RS100.[48] Because of the relatively small difference in amylose concentration between *sbeII* and WT wheat semolina described in this study, a dramatic difference in the degree of gelatinisation seemed unlikely. This is consistent with the starch amylolysis profiles obtained for *sbeII* porridge, similar to those obtained for the WT control.

Retrograded semolina amylolysis resulted in steep increase in hydrolysis products in the first 15 minutes of digestion, reaching a steady plateau phase soon after. This could suggest that the hydrolysis of semolina happened in two consecutive phases: a rapid phase where available starch was hydrolysed (leached linear amylose), followed a second phase of hydrolysis of branched starch components. It is also possible that semolina contained some encapsulated starch, as it is a coarse and heterogeneous material. However, previous research has shown that bi-phasic hydrolysis reactions were observed only for fractions of

much larger particle size in durum wheat.[279] Another reason for the plateau observed could be an imbalance in the enzyme to substrate ratio that limited the reaction. The presence of most data points in the plateau phase introduces bias in the model fit and lowers the confidence in the calculated parameters k and C_∞. While it is not possible to draw clear conclusions on the rate of hydrolysis of processed semolina, the percentage of starch digested after 60 minutes of incubation gives an indication regarding the extent of starch hydrolysis of gelatinised and retrograded *sbeII* semolina. The *sbeII* starch in pudding produced lower amounts of reducing sugars after incubation with α-amylase. Cooked semolina was left to retrograde only for 2 hours so it is likely that a longer cooling period could lead to lower starch susceptibility to hydrolysis.

4.5.3 Hydrothermal processing effect on *sbeII* starch amylolysis: breads and crackers

Bread making requires relatively large amounts of water (~60%) to fully hydrate the flour components. All flour components (particularly proteins, starch and fibres) interact with water in a competitive hydration process. Once gluten is developed, the gluten network can trap starch granules creating a barrier that prevents starch-water interaction and subsequent gelatinisation and therefore starch hydrolysis.[280, 281] Starch in *sbeII* breads was characterised by a lower susceptibility to hydrolysis compared to the WT control. This could be due to the increased amylose content and RS of *sbeII* flour and therefore, to altered gelatinisation and retrogradation of *sbeII* starch. Starch in high moisture doughs that undergo a resting period is prone to gelatinisation during baking and retrogradation during cooling. The cooling temperature also play a role in the degree of starch retrogradation.[282] In this study, breads were stored at room temperature for two days before measuring starch susceptibility to hydrolysis; during this time some retrogradation occurred. Other storage conditions (such as freezing) may lead to a higher degree of retrogradation and resistance to digestion.

The *sbeII* flour used in this study was characterised by increased amylose, water absorption capacity, and a marginal increase in AX, as described in chapter 2 and 3. Other studies have shown that increasing concentration of amylose and/or NSP, can lead to a higher flour water absorption and altered dough viscosity properties.[283-285] Increased water absorption could also be due to increased starch damage; a previous study has shown that *sbeII* wheat is characterised by increased kernel hardness and greater starch damage compared to the WT control.[151] Correcting the water content of *sbeII* bread dough based on the flour water absorption instead of using the same formulation for *sbeII* and WT control flours, allowed for a

correct development of the *sbeII* bread dough that was scored "as sticky" as the WT control dough.

Once baked, the *sbeII* loaves showed a good gluten development and gas retention based on the C-cell analysis but they had higher loaf density and lower loaf volume. Lack of volume generally indicates the use of a weak flour (low in protein content), or one low in amylase activity.[286] A strong (high protein) flour, such as *sbeII* flour, may also produce a small volume loaf, if not left to ferment sufficiently. Short fermentations such as the one of the Chorleywood method may have not allowed the gluten to properly hydrate and become extensible that in turn, does not allow the trapped starch granules to hydrate.[287]

A longer fermentation period could favour gluten hydration and dough expansion.

Unlike bread dough, crackers are made using a process similar to biscuits; cracker dough is normally characterised by a low moisture content, high extensibility and a short baking time at high temperature. The low hydration of the dough may have affected the starch susceptibility to hydrolysis. Doughs with low hydration are characterised by limited starch swelling, gelatinisation and retrogradation. Starch remains partially intact in the final product and it is therefore generally less susceptible to the action of amylolytic enzymes compared to other high moisture doughs, such as bread.[254] There were no differences in starch susceptibility to hydrolysis between *sbeII* and WT control bread, which showed a similar extent of amylolysis (up to ~60% of starch digested) based on the results presented in this chapter.

Cream crackers production usually requires a low-protein soft wheat flour low water absorption to achieve the characteristic flaky texture. In this study, in order to make crackers of reasonable end-use quality using *sbeII* bread flour (high in protein), soda bicarbonate was added to the formulation as to produce a soda cracker. For logistics reasons, the soda crackers were hand-cut rather than perforated and baked as a sheet.

The *sbeII* flour produced a harder cracker but as brittle as the WT control. This could be linked to a low hydration of the *sbeII* dough. Water content of the cracker dough was not adjusted based on the water absorption of the flour; the same water amount was added to *sbeII* and WT control formulation.

The cracker formulation did not involve any added sugars. The amount and type of added sugars can affect starch characteristics because of their ability to bind water reducing the amount of available water for starch gelatinisation.[288] However, *sbeII* cracker dough may have

not reached the optimal level of hydration to allow for some starch gelatinisation and retrogradation to occur. Future studies may require adjusting the water content of doughs based on the flour water absorption to measure changes in the crackers texture and starch susceptibility to hydrolysis.

4.6 Conclusions

Starch susceptibility to hydrolysis depends on several factors. Starch structure and composition investigated in chapter 3 have a great impact of the rate and extent of amylolysis but, the processing influences the starch characteristics in the final food product. This chapter presented the processing performance of *sbeII* wheat.

The starch susceptibility to hydrolysis of *sbeII* flour was comparable to the WT control in a low moisture food system (cracker), while in a high moisture food such as bread, *sbeII* starch was characterised by lower amylolysis compared to the WT control. Starch did not behave the same in all high moisture food systems; starch in porridge showed no differences in amylolysis and RS. In pudding, *sbeII* RS was higher than the WT control and the hydrolysis reaction appeared to be slower, even though the starch digested was similar to that of WT control pudding. This suggests that in a high moisture system, the degree of starch gelatinisation and retrogradation play an important role that future studies should investigate.

Based on the results presented in this chapter, *sbeII* pudding showed potential to study glycaemic response to *sbeII* foods *in vivo*, based on the *in vitro* amylolysis; a longer retrogradation period may be required to achieve higher resistance to hydrolysis.

Bread made from *sbeII* wheat is also a promising food model for *in vivo* testing however, other bread making methods could be investigated to gain insight in the effect of the fermentation step on starch characteristics.

Chapter 5 Glycaemic Index of a semolina pudding made from *sbeII* durum wheat: an *in vivo* pilot study

Abstract

Foods containing resistant forms of starch are expected to result in a lower glycaemic response than those containing mostly available carbohydrates. However, previous results presented in this thesis have shown that starch susceptibility to digestion greatly depends on the structural characteristics of starch and the processing applied to turn starchy materials into foods. This chapter presents the starch susceptibility to hydrolysis and glycaemic index of a *sbeII* semolina pudding containing resistant forms of starch. RS was likely derived from inaccessible starch due to the altered starch structure within the granule (RS2), and from the incomplete gelatinisation and retrogradation of starch, due to the home cooking process applied (RS3). The pudding was developed as a food model, based on the information gathered on starch characteristics in the previous chapters; this was a first approach to investigate the effect of *sbeII* foods on glycaemic response (Figure 5.1).

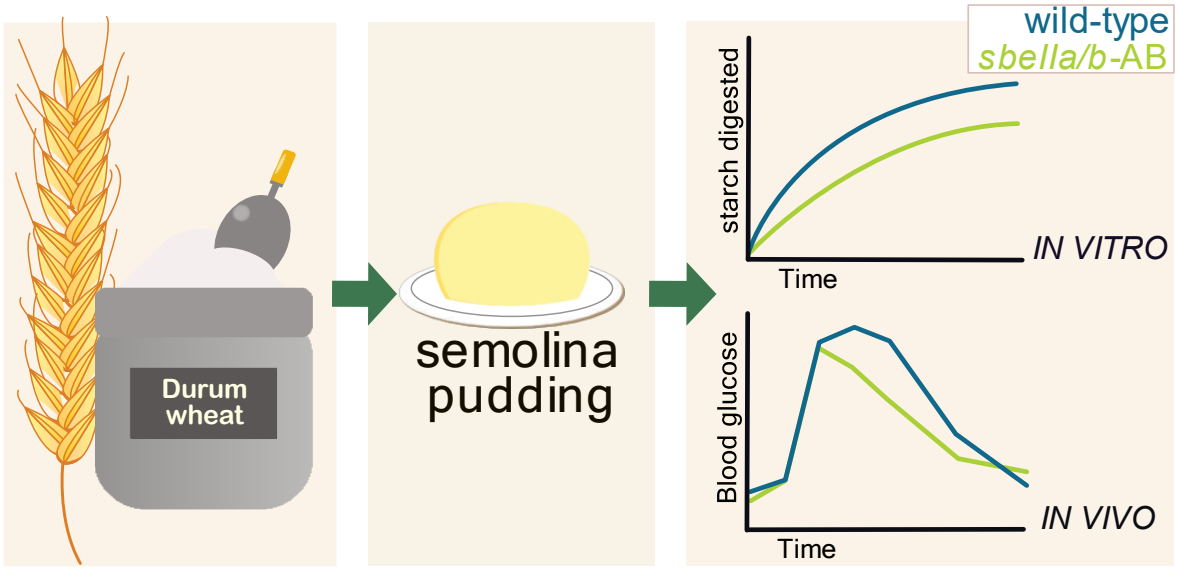


***Figure 5.1 Chapter 5 visual abstract**: development of a semolina food product to determine starch susceptibility to hydrolysis in vitro and glycaemic index in vivo of processed sbeII wheat.*

5.1 Introduction

The aim of the work presented in this chapter was to investigate the effect of a processing on the *sbeII* wheat semolina *in vitro* and *in vivo* digestibility, measured by Glycaemic Index (GI). The *sbeII* durum wheat semolina was used to prepare a semolina pudding to test its feasibility as test food for intervention studies. The starch characteristics of *sbeII* wheat investigated in the previous chapters (the higher amylose content and lower susceptibility to amylolysis of this wheat genotype compared to conventional wheat) were expected to reflect on the blood glucose response when measured in healthy volunteers.

5.1.1 The glycaemic index of foods

The glycaemic impact of carbohydrate-based foods has been related to the rate of digestion of available carbohydrates.[289] In starch-based foods, the starch composition and physicochemical status (degree of gelatinization and retrogradation), depending on the severity of the processing, play a role in the glycemic responses to foods.[290] Cooking starch rich materials in high moisture and heat can cause the starch to gelatinise, increasing its susceptibility to amylolysis. Gelatinised starch will result in large quantities of sugar to be absorbed from the small intestine and entering the systemic circulation.[291] Retrogradation of starch upon cooling can decrease significantly the starch availability to enzymatic hydrolysis, which may affect the GI measured.[292] As discussed in the chapter 4, the extent of starch susceptibility to hydrolysis greatly depends on the starch composition and the severity of the processing applied. Wheat with altered starch properties such as the *sbeII* wheat is characterised by higher amylose content and RS than conventional wheat, when raw. When cooked in a high moisture system like porridge, *sbeII* starch susceptibility to hydrolysis was similar to the WT control as the higher amylose content is prone to gelatinisation. When retrograded sufficiently, *sbeII* starch shows a potentially lower susceptibility to hydrolysis than the WT control, however, no *in vivo* study to date has been conducted to measure GI of foods made from *sbeII* wheat. Consumption of *sbeII* foods could bring nutritional benefits; intakes of elevated unavailable carbohydrates (such as starch characterised by increased resistance to hydrolysis) was shown to lead to improvements in both blood glucose and glycated proteins such as HbA_{1c}, an indicator of glycemic status, particularly in subjects with poor fasting blood glucose control.[293]

The GI is used to rank foods and it often recurs in nutritional recommendation advocating to reduce sugar or starchy-foods consumption.[294] Despite a continued debate on the clinical significance of this index, there is growing evidence that GI values can be used in the management of diabetes. Foods tested in either normo-glycaemic ("healthy") individuals or individuals in the pre-diabetic state (these individuals having glucose levels between the normal and diabetic range, usually 5.5 mmol/L to 6.9 mmol/L for fasting plasma glucose, 42 to 47 mmol/mol for HbA1c) produced GI values often used in diabetes management, as it is thought that a low GI diet may improve management of diabetes by lowering early post-prandial hyperglycaemia and decreasing risk for post-absorptive hypoglycaemia.[295] Wolever *et al.* (1987)[296] and later Foster-Powell *et al.* (2002)[13] reported similarities in GI values of foods obtained from healthy subjects and individuals with diabetes. More recently, Atkinson *et al.* (2008)[297] published a systematic review listing the GI of over 2,480 individual food items, including dairy products, cereal-based products, legumes and fruit, which does not include semolina pudding.

The values derived from studies involving healthy subjects and individuals with impaired glucose metabolism, considered pre-diabetic, were found to be highly correlated making the GI a reliable measurement regardless of the efficiency of the glucose metabolism of the study participants.

Diet induced glycaemic changes have been shown to alter hormonal and metabolic statuses and to directly impact health and disease risk factors for common chronic diseases.[294] Two epidemiological studies by Salmeron *et al.* and Hodge *et al.* showed that low GI foods may also have positive health effects by significantly improving blood glucose control in non-diabetic subjects, and reducing the risk of type 2 diabetes in the longer term (6 – 10 years).[298-300] An extensive Cochrane review compiled data from 11 intervention studies lasting a minimum of four weeks; the review concluded that low GI diets improved greatly the diabetic control and reduced HbA_{1c} in subjects affected by type 2 diabetes, reducing significantly the risk of microvascular complications.[301] Therefore, the use of indeces such as GI was thought to help electing foods that would improve health outcomes. Finally, a recent metanalysis by Livesey et al. (2019)[302] showed that the diet GI plays an important role in contributing to incident type 2 diabetes, whereby GI represent a relevant dietary factor in the prevention of type 2 diabetes development that was found to be more cost-effective in

nutrient based prevention approaches than measurement of dietary fibres or wholegrain intake.

5.1.2 Beyond glycaemic index studies

While the clinical significance of GI studies is often debated, this method does not only allow the categorisation of novel foods based on their glycaemic power; GI studies can be designed as pilot studies to test novel products feasibility and scalability to larger intervention studies.

Pilot intervention studies are defined as small studies carried out in preparation for larger investigations.[303] As pilot studies are often used to explore and generate research questions on a new research topic or a novel food product rather than hypothesis testing, therefore power calculations to determine the sample size are not necessarily a requirement.[304] In conducting a pilot study, researchers can optimize intervention delivery and refine the research methodology, or they can test the feasibility and validity of a study design or a research question. GI studies are often categorised as pilot studies as no power calculation is required and they can be completed with a relatively small sample size of approximately 10 subjects.[305]

5.2 AIM: To measure starch susceptibility to hydrolysis in *sbeII* pudding and explore its potential *in vivo* GI

The objectives of this study were to 1) measure *sbeII* semolina pudding starch susceptibility to hydrolysis *in vitro*, 2) to measure the GI of *sbeII* semolina pudding *in vivo* and *3)* to determine the feasibility of using a semolina pudding as the test food vehicle for larger scale *in vivo* studies on *sbeII* wheat and to obtain preliminary data of the effect size of *sbeII* semolina pudding intake.

5.3 Materials and Methods

5.3.1 Pudding formulation and processing

As described in chapter 4, semolina pudding represents a high moisture food system cooked in high heat where starch is partially gelatinised during cooking and retrograded upon cooling and storage. Semolina pudding formulations were designed to deliver approx. 50 g of total starch with constant solid to liquid ratio consisting of 17.02% semolina, 82.75% water and 0.23% salt (w/w fresh weight basis). The semolina puddings were prepared by boiling and cooling semolina in water. No extra flavourings were added, except salt (~ 1 g). Semolina and salt (pudding mix) were placed in a pot with room temperature water (~ 22 °C). The pudding mix was placed on medium heat for 6 minutes while stirring continuously with a spatula to bring the mix to 80 °C. The mix was left to simmer at ~ 80 °C for 3 min while stirring to encourage a homogeneous starch gelatinisation. Cooked semolina was cooled at room temperature for 10 minutes before storing at + 4 °C overnight (approximately 16 hours) to allow the starch to retrograde.

5.3.2 *In vitro* amylolysis of semolina pudding

Semolina puddings were prepared as described in section 5.3.1. After cooling for 16 hours, they were cut in cubes measuring < 0.5 cm and similar size cubes were used for the analysis. *In vitro* amylolysis was carried out as described in Chapter 2, section 2.2.3.

5.3.3 The GIPIRS Pilot study

The pilot study took place at the Hammersmith Hospital NIHR Wellcome trust Imperial College Clinical Research Facility in collaboration with Professor Gary Frost and his research groups. Blinded pre-weighted single serving semolina pouches were prepared at QIB and provided to Professor Frost's group with to be cooked and served to study participants at the Imperial College Clinical Research Facility by Ms Anna Cherta-Murillo and Dr Edward Chambers, where the study took place in September-November 2017. It was originally agreed that I would assist in the study activities at Imperial College London with meals preparation, participants visits and samples analysis, I was not able to assist because of access issues to the clinical facility at Imperial College. The study was also initially delayed due to internal reasons; when recruitment started, I was already on my Professional Internship Placement for Students at CIMMYT, in Mexico. My role in this study was to design the study foods, prepare and package the semolina in ready-to-cook portions and to blind

the study meals. I also carried out the starch characterisation and amylolysis experiments on the semolina and pudding materials.

Professor Frost's group carried out the clinical study and the preliminary data analysis prior to unblinding the intervention foods. Dr George Savva repeated the data analysis presented in this chapter as independent to the research team.

Study aim: The pilot study aimed to evaluate the use of a pudding as a food to test the effect of *sbeII*-based foods on glycaemic response measured in capillary blood.

5.3.3.1 Ethical considerations

The study was approved by the West London Research Ethics Committee 2 (13/LO/0696). This study was conducted from September to November 2017 according to the Declaration of Helsinki. All participants provided written informed consent before enrolment in the study. Consent to enter the study was sought from each participant only after a full explanation had been given, an information leaflet offered, and time allowed for consideration. Participants informed of their right to withdraw at any time from the study without giving reasons and a signed consent was obtained. The study Chief Investigator ensured confidentiality of participants information in accordance with the Data Protection Act 1988. All data collected was anonymised by the study team at Imperial College London.

5.3.3.2 Study design

The pilot study was conducted using a two-arm double-blind randomised cross-over design. Twelve participants were recruited from the 'Healthy Volunteer Panel' database created by the Imperial College Clinical Research Facility and randomised to four intervention meals: two glucose drinks (The Boots Company PLC, Nottingham, England), one WT semolina pudding and one *sbeII* semolina pudding, each one providing ~50 g of available carbohydrate, given in four different study visits separated by a wash-out period of 2-7 days[305]. All participants on the 'Healthy Volunteer Panel' database signed a consent and agreed to be contacted for research purposes once a study became available. Once they expressed their interest in the study, the potential participant was sent the information sheet and allowed to book a screening visit if they wished to participate in the study. Screening was carried out upon database enrolment. Each study visit was carried out as follows.

Participants arrived at the research facility at 08:30 am following an overnight fast. The weight and the height of the participant was recorded. Two fasting blood samples (-10 and -5 min) were taken followed by administration of the intervention meals (*sbeII* or WT semolina pudding and water drink) or the glucose drink. Test foods were designed to deliver 50 g of total starch. Participants were asked to consume the test meal within 15 minutes at an even pace and capillary blood samples were taken at 15, 30, 45, 60, 90, and 120 minutes after consuming a test meal or glucose drink. Blood samples were self-obtained by using disposable finger-pricks (Unistix needles) and glucose readings were measured using instant glucose analysers (Hemocue, Glucose 201+, Prospect Diagnostics LTD, Dronfield, Derbyshire). Each study visit was of the same nature.

5.3.3.3 Subjects

Twelve healthy adults with a BMI between 18.5-27 kg/m^2, aged 18 to 65 years old were recruited from the 'Healthy volunteer database' of Imperial College London and deemed "healthy" through a screening visit with a health questionnaire. Exclusion criteria were smoking, substance abuse, significant weight change since screening, pre- or diabetic, no family history of diabetes, no food allergy or intolerance to the study ingredients and not having participated in a research trial in the last 6 months. The study flow diagram based on the Consolidated Standards of Reporting Trials (CONSORT) can be found in Figure 5.2. Screening and study visits took place at the National Institute for Health Research (NIHR)/ Wellcome Trust Imperial Clinical Research Facility, London.

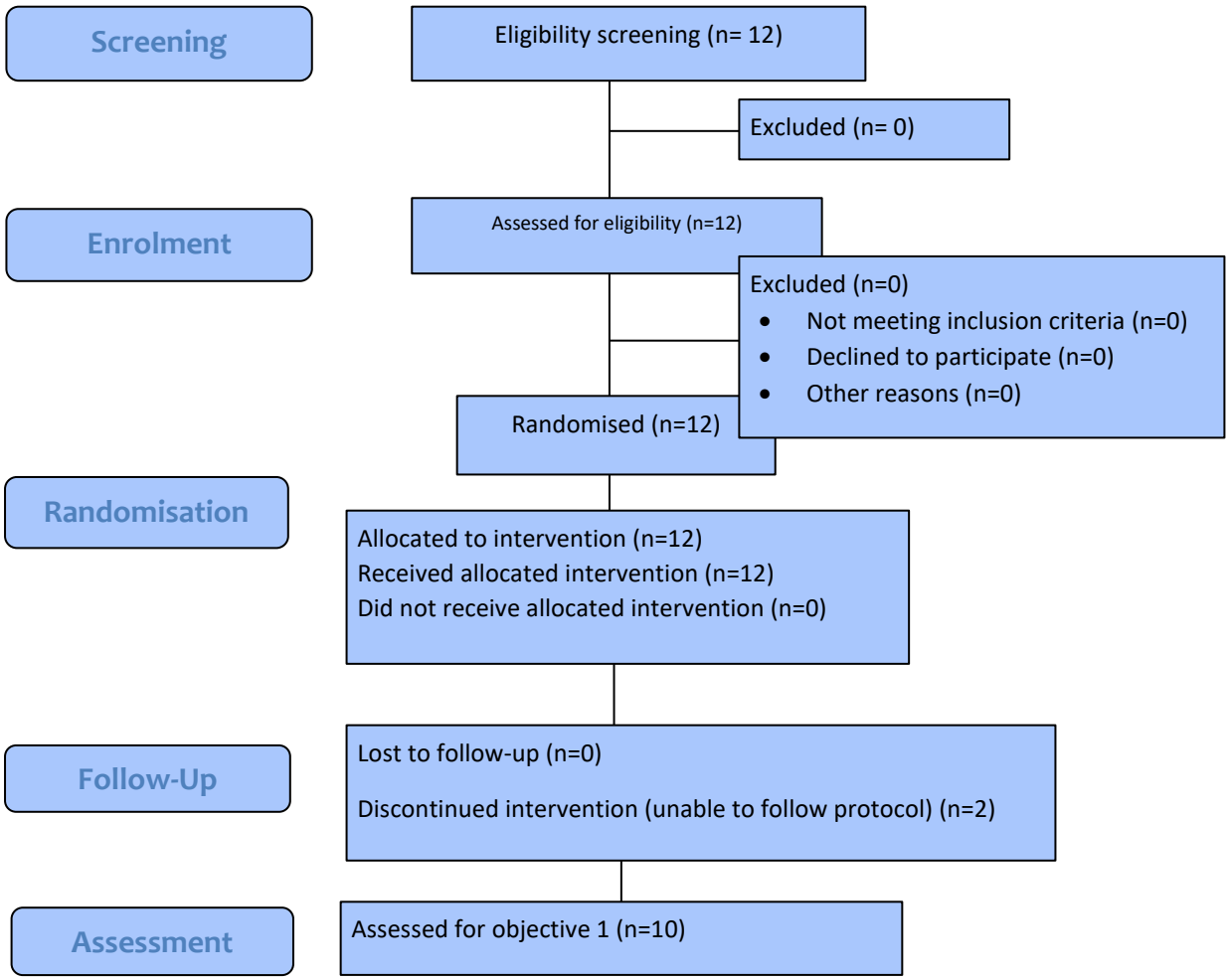


Figure 5.2 CONSORT diagram GIPIRS study

5.3.3.4 Preparation of semolina pudding

Test meals consisted of semolina puddings made from cooked and cooled *sbeII* or WT control wheat semolina. Dry ingredients (*sbeII* or WT semolina and salt) were packed into coded containers at Quadram Institute Bioscience (QIB) and shipped to the NIHR Imperial Clinical Research Facility, London (UK) where the individual portions were cooked prior to serving such that both participants and researchers preparing the pudding and assessing outcomes were blinded. Wheat semolina puddings were prepared as described in section 5.3.1. After a preliminary analysis of the results, test meals were unblinded to complete the statistical analysis.

Each test meal was prepared individually and served with approximately 250 mL of water. The volume of water served with the test meal was adjusted to compensate for the water loss

during the cooking of the semolina pudding and ensured a constant liquid-solid ratio across all meals.

5.3.3.5 Randomisation and blinding

The study team and participants were both blind to the intervention puddings, which were of similar appearance. I blinded the pudding meals by labelling them "A" or "D" prior to shipment to Imperial College London as I was not going to be involved in the preparation or administration of the meals or any study related activities. Participants were randomly allocated to interventions by the study team at Imperial College London, using a simple randomisation obtained from an internet-based computer program such as random.org. The study team at Imperial College London collected the glucose measurements and conducted a preliminary analysis of the results after which, the interventions were unblinded. Dr George Savva (QIB statistician) and I repeated the statistical analysis as described below.

5.3.3.6 Study protocol and sampling

Volunteers were invited to attend a total of four morning visits lasting 2.5 hours, with approximately two days between one visit and the next one. Volunteers were asked to arrive at the Hammersmith Hospital Wellcome Trust NIHR Imperial College Clinical research facility at a fixed time in the morning following a 10 to 12 hour fast. Volunteers were asked to avoid strenuous exercise and alcohol for 24 hours and were asked to consume the same meal prior to their visits. Subjects were asked to sit on a chair and rest for 5 minutes before starting the study. Two fasting blood samples were taken by finger prick (-10, -5) after which, the subjects consumed the test (*sbeII* pudding) or control meal (WT pudding and glucose drink) within 15 minutes. Further blood samples were taken by finger pricks at 15, 30, 31, 32, 45, 60, 90 and 120 min after the beginning of the meal.

5.3.3.7 Statistical analysis

For each participant, glycaemic index (GI) was calculated from blood glucose response values obtained from the incremental Area Under the Curve (iAUC) calculated between 0 to 120 minutes. GI was determined as the ratio of the iAUC of *sbeII* and WT control puddings and the averaged iAUC of two reference glucose drinks, consumed on separate occasions. For food labelling purposes, the GI of food products must be measured using standardised methodology by approved laboratories. To meet the criteria, the standardised method used at Imperial College London requires the mean within-subject CV for two repeated tests of the

reference food (the glucose drink in this case) in 10 subjects to be < 30%. An acceptable standard error around the mean GI of any one food (i.e., SEM) should be within 20% of the mean. Both conditions were met for this pilot study.

To gain insight in the possible effect of sbeII foods on glycaemic response, the post-prandial glucose profiles (iAUC) between 0-120 min and the mean glucose peak (calculated as the maximum glucose concentrations achieved after consuming the meal) of *sbeII* and WT control puddings were compared using a paired t-test. The blood glucose concentrations after consuming *sbeII* pudding and WT pudding were compared using a linear mixed-effects analysis, lme4 package.[228] This work was carried out by Dr George Savva, QIB statistician.

The iAUC was calculated from capillary blood glucose values collected during the two-hour period following intervention. Ignoring the area beneath the baseline, iAUC was calculated geometrically as: $AUC = \sum_{n}^{x=1} A_x$, where A_x = the AUC for the x^{th} time interval (i.e. between t_{x-1} and t_x).

For times t_0, t_1, ... t_n the blood glucose concentrations are G_0, G_1, ... G_n, respectively.

For the first-time interval (i.e. x=1): if G1 > G0, then $A1 = (G1 - G0) \times \frac{t1-t0}{2}$; otherwise, A_1 = 0. For the other time intervals (i.e. x>1)

if $G_x \geq G_0$ *and* $G_{x-1} \geq G_0$, $A_x = \{[(G_x-G_0)/2] + (G_{x-1}-G_0)/2\} \times (t_x-t_{x-1})$

if $G_x > G_0$ *and* $G_{x-1} < G_0$, $A_x = [(G_x-G_0)^2 / (G_x-G_{x-1})] \times (t_x-t_{x-1})/2$

if $G_x < G_0$ *and* $G_{x-1} > G_0$, $A_x = [(G_{x-1}-G_0)^2 / (G_{x-1}-G_x)] \times (t_x-t_{x-1})/2$

if $G_x \leq G_0$ *and* $G_{x-1} \leq G_0$, $A_x = 0$

The linear mixed-effects model included individual responses to the meal as main effect, individual differences over time as random effect and changes over time due to the genotype as fixed effect (with an interaction term). Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality and p-values were obtained by Satterthwaite approximation.

5.4 Results

5.4.1 Formulation and proximate analysis

The intervention meals characteristics are reported in Table 5.1. The average *sbeII* pudding serving was 415.3 g ± 12.9 g (mean ± SEM of 10 puddings served) with 85.1% moisture content while the average WT pudding serving was 384.95 g ± 7.5 g (mean ± SEM of 10 puddings served) with 83.6% moisture content. To ensure that the liquid component of the

meal was standardised, the water drink served as part of the meal was adjusted depending on the water content of each pudding serving. The total water content of *sbeII* and WT control meals was approximately 600 g.

One serving of pudding contained approximately 50 g of total starch; the starch in *sbeII* semolina pudding was characterised by larger portion of resistant starch compared to WT semolina pudding (Welch Two Sample t-test, p= 0.0001). The test meals were consumed within approximately 12 minutes.

Table 5.1. Pudding formulation, starch and nutrient composition per serving.

The proximate determination was carried out by Eurofins Scientific, n = 1.

Pudding formulation	**WT control**	***sbeII***
Semolina (g)	72	80
Water (g)	350	389
Salt (g)	1	1.1
Pudding starch content per serving[5]		
TS (g)	50.4	50.4
RS (g)	1.2	2.6
Intervention meal estimated nutrients per serving		
Protein[6] (g)	10.3	13.7
Total fat (g)	1.08	1.52
Ash	0.6	06
Saturated fat (g)	0.28	0.35
Mono-unsaturated fat (g)	0.13	0.18
Poly-unsaturated fat (g)	0.6	0.9
Carbohydrates[7] (g)	51.7	51.7
Total sugars (g)	4.17	3.12
Fibre AOAC (g)[8]	2.3	3.5
Energy (kcal)	262.6	282.5
Energy (kJ)	1113.5	1196.9

5.4.2 *In vitro* amylolysis of semolina pudding

Starch *in vitro* amylolysis parameters are reported in Table 5.2. Starch in *sbeII* pudding was less susceptible to hydrolysis compared to starch in the WT control pudding (C_{90} mean difference = 14.15 µmol/L unpaired t-test, p = 2.722e-06). Amylolysis profiles of *sbeII* and WT control are shown in Figure 5.3a.

The total breakdown of starch represented by C_∞ and the rate constant (*k)* of starch hydrolysis were determined from the slope and y-intercept of the LOS plot and reported in Table 5.2.

[5] Based of semolina TS and RS content
[6] Nitrogen-to-protein conversion factor = 6.25
[7] Calculated by difference
[8] AOAC method for measurements of fibre includes RS3

Both C_∞ and k were lower for *sbeII* pudding compared to the WT control. The curve fitting to experimental data was deemed acceptable in this case ($R^2 > 60\%$) however, the low R^2 values suggest that the accuracy of the rate of digestion measured by the parameter k could be improved (Figure 5.3, b and c).

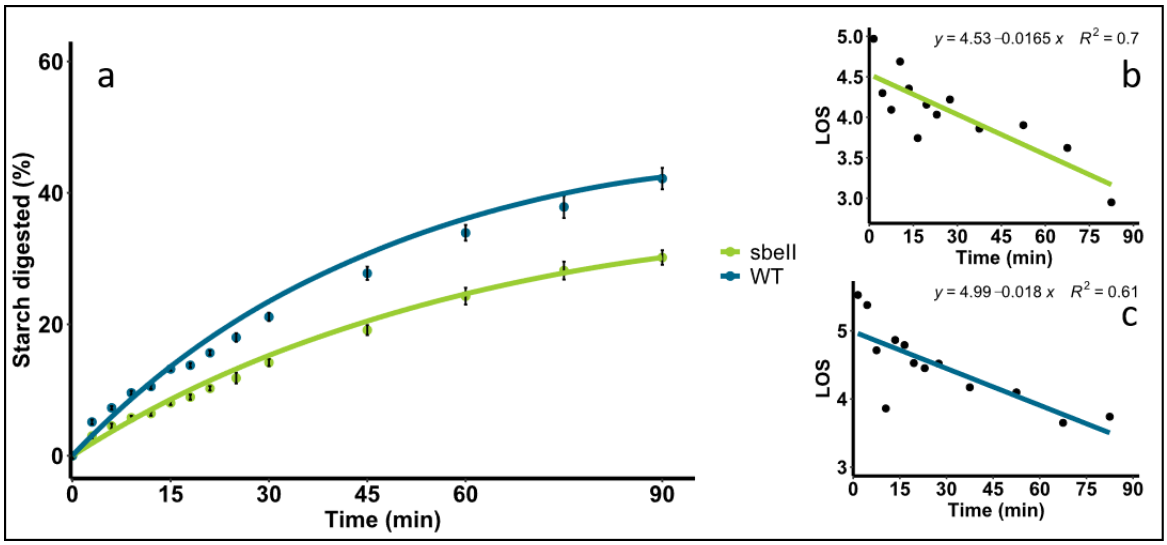


***Figure 5.3 Starch digestibility of sbeII pudding and WT control pudding**, starch amylolysis experimental data are shown fitting a first-order equation (Equation 2) based on the k and C∞ values obtained from the LOS analysis. Each experimental data point represents the mean value from analysis of n = 6 pudding samples prepared independently with error bars = ± SEMs b. LOS plot obtained for WT pudding. c. LOS plot for sbeII pudding. The linear phase is defined by Equation 3 from which values of k and C∞ can be estimated.*

Table 5.2. Variable estimates from digestibility curves and LOS analysis of sbeII pudding compared to WT control pudding

Pudding	**C_{90} (%)**	**C_∞ (%)**	**k(min^{-1})**
WT Control	47.4 ± 0.7	49.9	0.021
sbeII	33.3 ± 1.5	39.0	0.016

Mean ± SEMs of n = 6. C_{90} is the proportion of starch digested by α-amylase after 90 min, this is obtained from the starch digestibility curve. C_∞ is the total extent of starch amylolysis for each digestive phase accounting for Y0 (endogenous baseline sugar), k is the rate constant of the reaction; both parameters are obtained applying the LOS analysis to the digestibility data.

5.4.3 The GIPIRS pilot study

5.4.3.1 Participant characteristics

For this pilot study, 12 participants were recruited from the 'Healthy volunteer database' at Imperial College London. Two participants withdrew from the study for various reasons (unable to complete the meal within 15 minutes and unable to self-finger-prick). The characteristics of the 10 participants completing the study, can be found in Table 5.3. Participants' weight and height were recorded at the beginning of each study visit and did not vary significantly during the study (CV = 0.2% - 2.03%).

Table 5.3. Characteristics of the 10 participants completing the study

	Means ± SEMs
Age (y)	33 ± 2
Height (cm)	154 ± 17
Weight (kg)	69 ± 3
BMI (kg/m^2)	23.5 ± 0.6
Ethnicity (n)	White Caucasian (7) Southwest Asian (3)
Fasted glucose (mmol/L)	4.7 ± 0.07
Time taken to eat test meal (min)	11.8 ± 1.4

N = 10 of which, 2 males and 8 females.

5.4.3.2 Glycaemic Index

Participants' fasting glucose values were within normal range (4.71 ± 0.075 mmol/L/min, mean ± SEM, n = 10) and their fasting glucose values did not vary significantly between visits (paired t-test, p-value = 0.59). Source data, including the baseline concentrations of capillary glucose, are reported in Table A6 2, Table A6 3 and Table A6 4, Appendix A.

The mean GI and iAUCs of *sbeII* and WT control puddings are reported in Table 5.4. We found no evidence of difference in GI (mean difference = -13.9 [95CI -17.00, 44.80], paired t-test, p-value = 0.33). The post-prandial glucose profile after consuming *sbeII* pudding was expected to be lower than the control based on the lower starch susceptibility to hydrolysis observed in vitro however, this did not significantly differ from the WT control, (iAUC0-120, mean difference = -24.019 [95CI -23.46, 71.49], paired t-test, p-value = 0.28). A linear mixed-effects model was used to compare the glucose profiles after *sbeII* pudding consumption to that of the WT control; there were no significant differences in the overall blood glucose

concentrations (p-value = 0.37). Blood glucose concentrations in response to *sbeII* pudding were found to be borderline lower than the response to WT control pudding 60 minutes after the meal intake. This may suggest that the effect of the pudding genotype on blood glucose concentration is dependent on time, an interaction (Time X Genotype) that was significant at 60 minutes (linear mixed-effects model, p-value = 0.042) but not overall (linear mixed-effects model, p-value = 0.07).

All glucose curves showed a distinct peak within the first 60 minutes, followed by a slow decrease of glucose concentration. The blood glucose concentration peak following *sbeII* and WT control puddings was similar (paired t-test, p-value = 0.3). The pooled glucose concentration returned to the fasted baseline within approximately 120 minutes after consuming the WT control pudding, while the response to *sbeII* pudding (and the glucose reference) remained above the fasted baseline, Figure 5.4a. Consumption of *sbeII* pudding elicited a sharp increase in blood glucose concentration that dropped 30 minutes after consumption while glucose concentration in response to WT pudding dropped after 45 minutes. Figure 5.4b shows the individual capillary glucose responses ($iAUC_{0\text{-}120}$): three outliers were identified (participants 4, 5, and 8) but they were not removed from the analysis because of the relatively small sample size.

***Table 5.4. Indicators of blood glucose responses to intervention meals**. GI is calculated as the ratio of food $iAUC_{0\text{-}120}$ (WT control and sbeII) and $iAUC_{0\text{-}120}$ of the glucose reference.*

Pudding	**$iAUC_{0\text{-}30\ min}$ (mmol/L /min)**	**$iAUC_{0\text{-}60\ min}$ (mmol/L /min)**	**$iAUC_{0\text{-}120\ min}$ (mmol/L /min)**	**Glucose peak (mmol/L)**	**Glycaemic Index**
WT control	18.4 ± 2.6	78.1 ± 7.2	135.0 ± 17.1	7.2 ± 0.2	73.8 ± 12.0
sbeII	19.2 ± 4.2	68.3 ± 10.0	111.0 ± 14.0	6.8 ± 0.3	59.8 ± 7.6
Glucose reference[9]	35.7 ± 4.5	115.5 ± 12.7	198.2 ± 19.8	7.9 ± 0.3	100

Values are means ± SEM, n = 10 individuals for sbeII and WT control puddings, n=20 for the glucose reference.

[9] Average of two visits, the glucose drink reference was repeated twice.

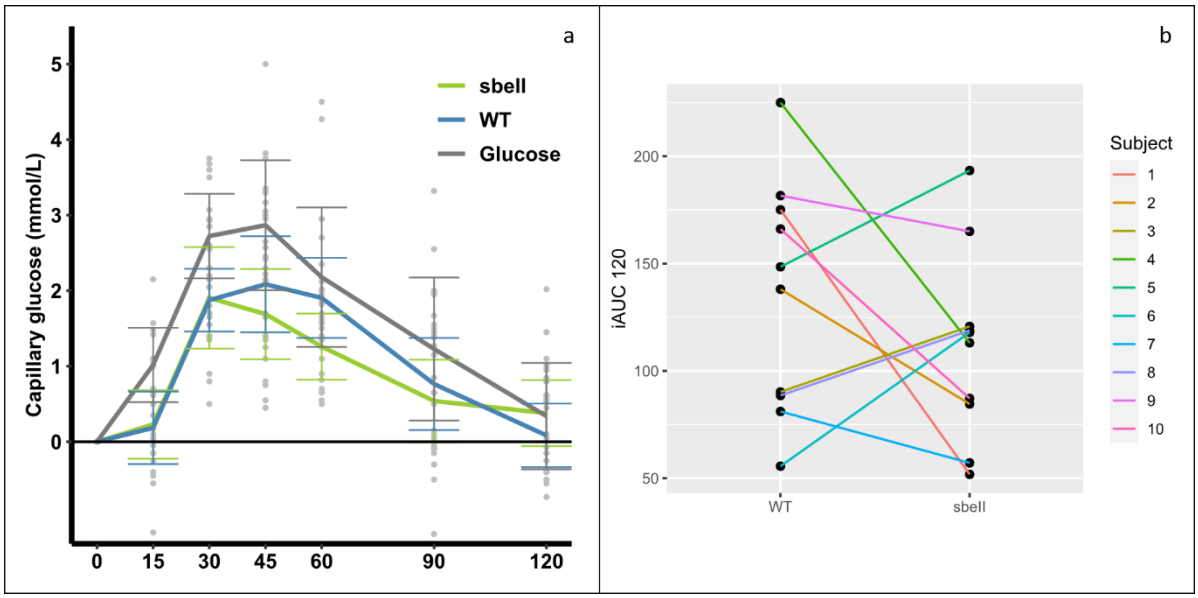


Figure 5.4 . Post-prandial capillary glucose concentrations 0 to 120 minutes*; datapoints represent individual glucose values, curves are pooled glucose profile n = 10 and error bars are 95% CI. b. Individual incremental capillary glucose ($iAUC_{0\text{-}120}$) showing variable response to the test (sbeII) and control (WT) puddings.*

5.5 Discussion

This chapter presents the results of a glycaemic index pilot study aimed to measure *sbeII* pudding GI and to test a pudding as food type for larger intervention studies. The food development and characterisation were carried out at QIB, in Norwich, while the clinical study was carried out at Imperial College London by Professor Gary Frost.

The pudding meals were developed to deliver 50 g of total starch; while the starch content of puddings was similar, starch in *sbeII* wheat had different intrinsic and extrinsic properties, as discussed in the previous chapters, which may reflect on the glycaemic responses measured *in vivo*. To achieve an equivalent total starch between *sbeII* and WT control puddings, the portion size of the *sbeII* pudding had to be larger than the control. The water to semolina ratio was kept constant between pudding types (*sbeII* and WT control), without adjusting the water added during cooking based on semolina water absorption. As reported in chapter 2, the *sbeII* semolina was characterised by a higher water absorption compared to the WT control; failure to adjust the water content of a food based on the semolina water absorption may affect the product's texture characteristics and its starch properties. However, water was added in excess compared to the WT and *sbeII* semolina weights, thus, this seems an unlikely confounder in the analysis.

Semolina and salt were pre-weighted and stored at -20 °C to ensure that the product was safe for consumption. The puddings were cooked and then stored overnight to be served cold the morning of intervention. This protocol was chosen to allow starch to retrograde. Starch susceptibility to hydrolysis in gelatinised foods decreases with cooling due to the retrogradation of the amylose chains. As reported in chapter 4, cooling cooked semolina per few hours did not result in large differences in starch amylolysis between *sbeII* and WT control foods; a longer cooling period (overnight), was expected to lead to larger differences in amylolysis as the amylose in *sbeII* starch retrograded. The retrogradation rate of *sbeII* starch was not studied in this project however, previous research by Patel *et al.* (2017)[249] showed that the catalytic efficiency of amylolytic enzymes decreases with storage time, as starch retrogradation progresses. For instance, the catalytic efficiency of wheat starch stored at 4 °C for seven days decreases to approximately 60% compared to the freshly gelatinised starch. The catalytic activity for high amylose starches was shown to decrease up to 20-25% within 20 hours of gelatinisation but the rate of change greatly depends on the on the starch composition (i.e. botanical origin, chain length distribution).[249]
Furthermore, overnight storage allowed flexibility in the organisation of the intervention visits. The semolina was cooked in single portions the night before each intervention visit; this allowed for the study team (at Imperial College London) to be flexible in organising the visits, while delivering a product with consistent starch characteristics as no extended storage was required. However, pudding preparation in single batches may have contributed to variation and in water losses during cooking.

Durum wheat is normally used to produce pasta that can require extrusion. The extrusion process involves the use of high shear and pressure to generate the pasta shape desired, altering starch properties.[306] Pasta production also requires specialised equipment for the drying process, necessary to produce a product with extended shelf-life. Extruders and dryers are also often made on industrial scale and usually they are not suitable for the relatively small quantities of semolina available for the GIPIRS study. Producing pasta for a clinical study was not possible for this study. Production of semolina pudding was instead a straightforward and cost-efficient method to generate a food product while controlling starch characteristics. Nevertheless, the *sbeII* semolina pudding used in this study showed similar starch characteristics to the *sbeII* pasta described by Sisson et *al.* (2020).[146] They reported on the starch susceptibility to hydrolysis in pasta made from *starch branching enzymes IIa* semolina

(SBEIIa). Pasta was cooked and cooled so gelatinised starch may have retrograded partially. The degree of starch susceptibility to hydrolysis in SBEIIa pasta was found to be close to that of starch in *sbeII* pudding measured in this study (24% and 33% of the starch digested within one hour of incubation with amylolytic enzymes, respectively). However, the SBEIIa pasta was characterised by higher RS (7.36 g ± 0.10 g) and amylose content (57.8% ± 1.5%) compared to the *sbeII* pudding. Pasta is normally characterised by a complex matrix as a result of the extrusion process. The matrix is a major rate-limiting factor in the digestion of pasta, whereby a strong gluten network can hinder starch granules limiting gelatinisation, retrogradation and enzymatic degradation.[307] On the other hand, semolina pudding rate-limiting factor would be primarily the degree of starch gelatinisation and retrogradation. Puddings were cooked in excess of water favouring gelatinisation of starch, however, the relatively short cooking time and temperature (<100 °C) may have not been sufficient to completely gelatinise the starch in semolina, particularly in *sbeII* semolina, as starch with higher amylose content is known to be more thermally stable.

Therefore, the higher RS of SBEIIa pasta reported by Sisson *et al*. (2020),[146] could be explained by the different processing applied to *sbeII* semolina. It should be considered that amylose content of the raw wheat material is known to be affected by environmental factors.[52] Apart from the effect of the extrusion process to produce pasta on starch characteristics, the mutant SBEIIa wheat grains used by Sisson et *al*.[146] were characterised by the presence of mutations only in genes encoding SBEIIa, while the durum wheat used in this study carried mutations in *sbeII a* and *b* genes.

No other intervention study to date as reported on the effect of *sbeII* food products on glycaemic response, except the study by Sisson *et al*. (2020)[146] mentioned above and another study by Belobrajdic *et al.* (2019)[147] on bread made from *sbeII* wheat grains (discussed in the next chapter). In this study, starch in *sbeII* pudding was found to be less susceptible to hydrolysis compared to the WT control pudding. This was investigated by measuring RS content and starch rate and extent of hydrolysis using the amylolysis model described in chapter 2. The small difference measured in RS content between *sbeII* and WT control puddings did not reflect the changes in starch digestibility due to the formation of resistant forms of starch. This was likely due to limitation of the analytical method to measure RS that requires sample homogenisation, destroying the matrix of the food, which may reduce the accessibility of the substrate to amylolytic enzymes.

The C_{90} obtained *in vitro* is known to correlate well with *in vivo* GI measurements and it represents a better indicator of GI, as shown by Edwards et *al.* (2019),[174] even though *in vivo* measurements are known to be characterised by a greater variation compared to *in vitro* ones. This was reflected in this study; the extent of starch hydrolysis within 90 minutes of incubation with amylase (C_{90}) of *sbeII* pudding was 14 units lower than that of the WT control. While starch hydrolysis was found to be significantly lower in *sbeII* pudding (compared to the WT control), there was no significant difference in GI between puddings and the GI of *sbeII* pudding was moderately high. It should be noted that pilot studies are not usually powered for hypothesis testing but refer to a standard sample size (~10 participants) to test feasibility of an intervention.[305] The ancillary aim of the GIPIRS study was to explore the feasibility and efficacy of the type of food, the portion size, and the logistics of the intervention study for a larger scale intervention trial aimed at assessing the effect of *sbeII* foods on glycaemic response of healthy volunteers.

Overall, the pilot study demonstrated that some improvements to the protocol and test food could be beneficial. Based on the results, it became clear that a standardised batch processing of semolina could have led to more consistent pudding foods. Pudding texture was not analysed in this pilot study, but it could have provided important information regarding the product consistency and uniformity. It was also noted that other variable such as background diet and a standard meal the night before may have helped explaining some of the unexpected responses observed in glucose response (iAUC).

One participant found the pudding portion difficult to consume and eventually dropped out before completing the study. Other food types of different consistency and/or portion size may be better suited for this type of intervention study where participants have a limited amount of time to consume them. Finger prick testing can also be challenging; one participant dropped out because unable to prick their fingers. Finger prick test can also be limiting in the number of samples that can be collected per session as repeated pricking can be painful for participants. Other methods of sampling and/or measuring glucose were not available at this time but were explored in the REST study, reported in chapter 6.

5.6 Conclusions

The use of *sbeII* wheat in foods may not change dramatically the available carbohydrate fraction to result in foods of low GI (below 50). The relatively small changes in starch structure and properties may result in more subtle glucose concentration differences that require a larger intervention study to be detected or more accurate detection method. The use of glycaemic parameters other than GI may provide a more detailed and complete picture of the overall response, including iAUC, glucose peak, time to peak and number of peaks.

Chapter 6 Glycaemic response to *sbeII* bread: from *in vitro* amylolysis to *in vivo* glucose response

Abstract

It is well known that foods containing equal amounts of starch can be metabolised to a different rate and extent producing a range of glycaemic responses. This chapter presents the results of a double-blind cross-over study designed to measure the effect of *sbeII* white bread on postprandial glycaemic response, compared to an isoglucidic WT control white bread. This study builds on the knowledge acquired from previous studies described in chapter 3 to 5 of this thesis on *sbeII* starch characteristics to generate a novel *sbeII* bread with lower starch susceptibility to hydrolysis *in vitro*. The lower susceptibility to amylolysis of *sbeII* bread compared to the control bread was expected to elicit a lower postprandial glycaemic response measured *in vivo*, in capillary glucose. The study also investigates the use of an alternative method to measure glycaemic response in individuals by using a continuous glucose monitoring device (CGM) to measure glucose fluctuations in interstitial fluid (Figure 6.1). This is one of the first studies using *sbeII* bread and CGMs in an intervention study to investigate the effect of starchy foods on postprandial glycaemia.

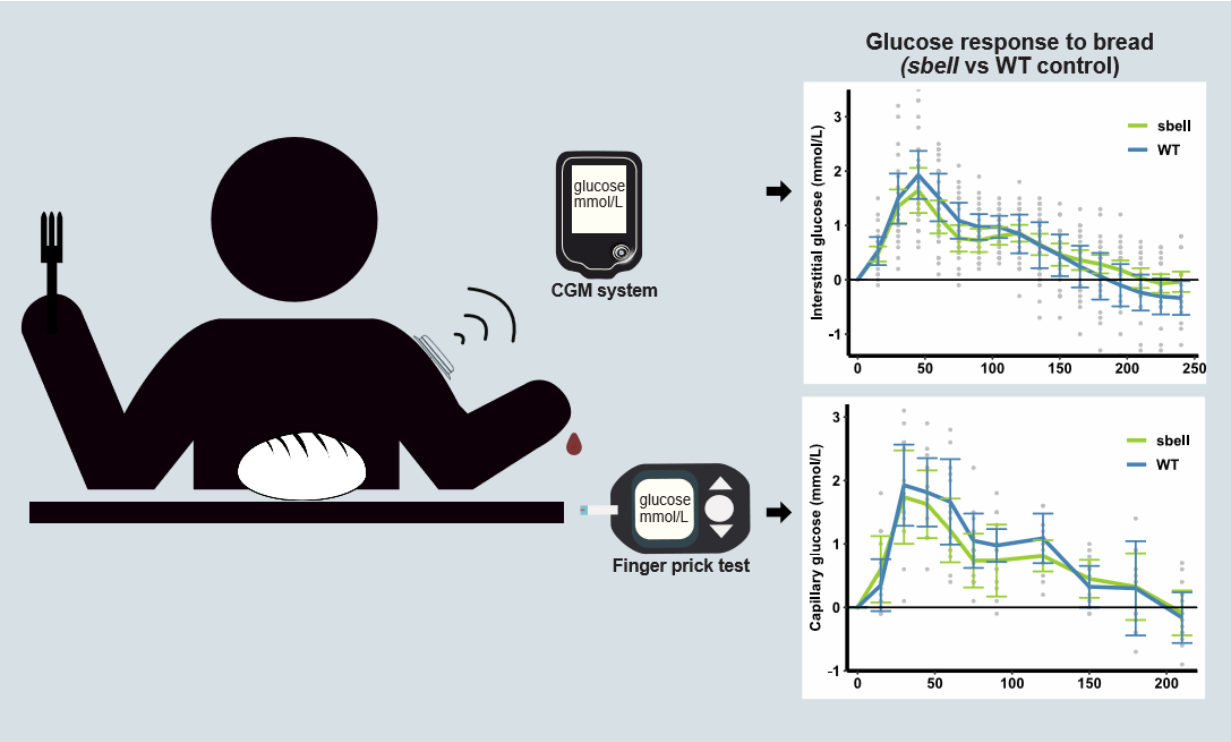


Figure 6.1 Chapter 6 visual abstract: *to determine the effect of sbeII white bread on the glycaemic response of healthy individuals, compared to that of a conventional white bread, post-prandial glucose concentrations were measured in capillary blood (by finger prick test) and in interstitial fluid (by CGM system), in an acute double-blind cross-over intervention study.*

6.1 Introduction

High amylose foods have shown great potential to attenuate glycaemia[308, 309] even though the results of studies were not always consistent.[310, 311] For example, Heijnen *et al.*(1995)[312] showed that increasing the amylose content of starchy drinks and puddings, but not rolls, lowered postprandial glucose and insulin responses. Van Amelsvoort *et al.* (1992)[310] showed that high amylose meals resulted in altered glucose response in healthy volunteers. They observed lower glucose level after consuming the high amylose meal, compared to low amylose one. Weststrate *et al.* (1993)[311] found no differences in healthy volunteers glucose responses after consuming a high amylose breakfast but significantly smaller glucose responses when consuming a high amylose lunch.

The effect of a high amylose intake on glycaemia may depend on the nature and the concentration of amylose added,[313] the technological process applied to food and the physico-chemical characteristics of the starch.[312] A diet based on highly digestible carbohydrate-based foods, mostly made of starch, can be associated with high blood glucose levels that, with time, can lead to altered glucose homeostasis and impaired glucose metabolism.[96] Adopting a nutritional approach to improve blood glucose control can help to reduce the risk of cardiovascular events or type 2 diabetes mellitus, especially when coupled with other lifestyle changes.[290] To implement a successful nutritional strategy, it is important to fully understand how to manipulate specific food characteristics that could help reduce the risk of developing certain metabolic conditions. Starch properties can be manipulated within the grain, and through processing to generate novel foods aimed to adjust the glucose intake and absorption to individuals' needs.

The rate of starch hydrolysis *in vitro* is a key determinant of glycaemic responses to most products, particularly when comparing the final rate of starch hydrolysis of high amylose white bread to conventional white wheat bread.[50] In chapter 4, starch in foods made from *sbeII* flour were shown to have lower susceptibility to amylolysis than WT wheat flour-based foods, indicating potential to elicit lower glucose responses compared to conventional wheat-based foods made from refined flour. The *in vitro* amylolysis assay used within this thesis has been shown to provide reasonable indication of glycaemic responses to a range of food products. [10]

This chapter presents the results of a randomised double-blind cross-over intervention human study (the REST study) to measure the glycaemic, satiety and sensory responses of healthy individuals after consuming *sbeII* bread. REST is an acronym for resistant starch, and the study

followed on from the work presented in Chapter 4, where *sbeII* bread made from high amylose *sbeII* flour was characterised by higher RS and lower susceptibility to amylolysis compared to a WT wheat bread (control). The aim of the human study was to determine if bread made from *sbeII* wheat would lead to lower glycaemic responses than bread made from the WT wheat. Bread as a type of food is widely consumed across the UK population and represent a good food type to test efficacy of a nutritional intervention. Bread was selected as the most suitable food type to be used in intervention studies (chapter 4) compared with the other wheat-based food products tested (chapter 5) since it is a widely consumed food[314] that can be produced in batches and stored frozen, extending its shelf-life considerably. Because of logistic reasons a semolina pudding was used in the pilot study in chapter 5. The semolina was characterised by a coarse particle size, unsuitable for bread making. Furthermore, the pilot study in chapter 5 was carried out while the Quadram Institute Bioscience building was located in Colney lane where a food grade kitchen equipped for bread making was not available. Therefore, it was not possible to produce *sbeII* bread and to ship it to Imperial College London, where the pilot study took place. The use of a 'pudding' as test food allowed to measure GI of retrograded *sbeII* starch; this work underpins the work presented in this chapter. After QIB relocation in the new building on Rosalind Franklin road, a fully equipped food grade kitchen became available for on-site bread production allowing the REST study to use bread as intervention food product.

The first part of this chapter reports the formulation and technological processes used to achieve the final bread formulation with low *in vitro* amylolysis, together with the bread end-use quality characteristics, while the second part of the chapter describes the use of these breads in the human intervention study.

6.1.1 Development of a *sbeII* bread

6.1.1.1 Bread making – from novel wheat to novel bread

The technological processes applied to raw flour during bread-making alter the native starch structure to increase palatability and digestibility; This can lead to a larger blood glucose response. Amylose's linear helical chain conformation can form compact structures that resist to enzymatic access.[315] By increasing the amylose content of foods, it may be possible to reduce the rate and extent of starch digestion and therefore reduce the rate of glucose absorption and increase the rate of glucose removal from blood.[316] An increase in the amylose content of white wheat flour may help to produce foods that lead to lower glucose responses

after ingestion; this can be achieved only if starch maintains its crystallinity, either by maintaining the native starch structure or re-crystallising during processing.[317] However, the end-use characteristics of a flour greatly affect the property of the foods produced, particularly bread. Factors such as bread density and structure are known to alter the glycaemic responses to bread,[266] and so an important objective was to develop a formulation to achieve bread of similar quality and appearance to that of conventional wheat white bread (the control). This was also necessary to ensure that the participants were 'blinded' to the study intervention. Different processing methods (straight-dough or Chorleywood and sponge methods) were tested to obtain breads of acceptable and matched quality, and with specific starch characteristics using *sbeII* and a WT control flour.

The straight dough method requires high mixing speed and a relatively short fermentation while the sponge method requires a shorter mixing and a longer fermentation, often achieved by splitting the yeast to be added in two subsequent steps or by using a sourdough starter. The fermentation and mixing rate not only affect the end-use quality of bread but also the extent of starch gelatinisation and retrogradation and therefore susceptibility to hydrolysis, enhancing or nullifying the higher amylose and RS content of the formulation used.[318] Starch characteristics (RS and susceptibility to hydrolysis) were measured in bread obtained with these two baking processes as described in this chapter.

6.1.2 Identifying a target dose

In 2011 the European Food Safety Authority (EFSA) established that "sufficient evidence exists to establish a cause and effect relationship between the consumption of foods/beverages containing non-digestible carbohydrates (including RS) and a reduction in postprandial glycaemic responses, as compared with foods/beverages containing glycaemic carbohydrates".[319] The claim's condition of use include a 14% RS replacement of TS to reduce the postprandial response of individuals. However, more work is needed to establish whether the RS is expected to act because of a reduction in the total available carbohydrates content; if the increase in RS would affect glycaemic response regardless of the food type and regardless of the 'type' and origin of RS; whether the same effect of decrease in glycaemic response should be expected in normo-glycaemic subjects and individuals with diabetes.[141]

Lockyer and colleagues (2017)[141] have reviewed a series of studies published after the EFSA opinion and compared the results presented. In studies where the available carbohydrate content was not matched between intervention foods and regardless of the food used or RS

type involved, RS consumption was generally associated with improved glycaemic outcomes (e.g. reduced fasting blood glucose concentrations, reduced postprandial responses (iAUC) and enhanced skeletal muscle uptake of glucose).[320, 321] In studies where the available carbohydrate content was matched across interventions, the findings were less consistent.[322-326] In all studies considered by Lockyer *et al.* (2017), the RS supplemented was lower than the 14% replacement indicated by EFSA. While the increased consumption of high RS foods is said to improve markers of glucose metabolism, the extent and direction of the effect remains unclear.

When higher amylose amounts are used, other considerations are to be made. Behall *et al.* (2002)[309] have reported lower glycaemic responses in healthy volunteers (for glucose peak and Area Under the Curve) after consuming one serving of high amylose breads, particularly for breads where at least 50% of the starch is amylose or at least 8 g is RS. In this study, the authors report a lower available carbohydrate content for bread with higher amylose content. Therefore, it is likely that any effects measured were due to the difference in carbohydrates intake. Furthermore, some subjects in this study reported mild side effects following the high amylose intake including bloating and flatulence. Thus, research is needed to identify the correct amount of amylose/RS to obtain an attenuating effect on glycaemia but limiting the side effects observed.

Despite the great number of studies conducted on this topic, it remains challenging to establish the effects of RS on glycaemia. There is still a need to understand the influence of the food matrix and subsequent interactions between the RS types and other fibres in foods. A major limitation of many previous studies is the very limited attention paid to characterisation of the food matrix, which may play a role glycaemic response. A recent study by Berry *et al.* (2020)[327] has shown that the meal composition is in fact one of the main determinants of the post-prandial glucose response, altering the $iAUC_{0\text{-}120}$ by up to 16%. The REST study has addressed this by providing a good starch characterisation of the intervention meals and by standardising the nutritional composition of all study meals, thus controlling for possible confounders of the results. There is also a need to identify a suitable dose to provide glycaemic benefits without side-effects. In this study, the *sbeII* flour used in the intervention meal had higher amylose (39%) than conventional white flour (25%, chapter 3) and greater RS (6% of TS and 0.6% of TS, respectively).

6.1.3 The REST study

6.1.3.1 Glycaemic response to bread in healthy individuals

Typically, the glycaemic response to carbohydrate-based foods can be assessed by measuring glucose concentration in capillary blood collected through a 'finger-prick' test, as in the pilot study described in Chapter 5. However, new technologies for continuous glucose monitoring allow for accurate measurement of glucose fluctuations over time. Continuous Glucose Monitoring systems (CGMs) are a minimally invasive method to measure glucose levels in interstitial fluid and are currently used in the clinical management of diabetes. CGMs have recently been used in research to supplement information gained from repeated capillary blood glucose measurements obtained from finger-prick test.[328]

The REST study presented in this chapter investigated the use of a continuous monitoring system device (CGM) as a potentially reliable method to assess the impact of novel foods on post-prandial glucose response. To this purpose, the study aimed to measure glucose response of healthy volunteers in capillary blood (by finger-prick test) and interstitial fluid (by CGM), after consumption of bread with different starch characteristics and to assess the reliability of IF glucose measured using a CGM system.

6.1.3.2 Resistant starch and appetite regulation

Appetite is a complex mechanism where several aspects of food intake regulate the so called 'satiety cascade' through different signals. Choosing to initiate a meal (food choice), to end a meal because of decline in hunger and increase in fullness (satiation) and the extent of hunger suppression between meals (satiety) are controlled by a cascade of cognitive, sensory, post-ingestive and post-absorptive signals.[329, 330] While appetite is mostly regulated by metabolic signals (hormones), other external factors can also influence the amount of food consumed including portion size, the environment where food is consumed, food palatability, appearance and eating memory. Controlling appetite and reducing the feeling of hunger can determine the success of a weight management programme so, research has focused on identifying foods or ingredients that can promote fullness or decrease hunger.[331] Based on the current research available, there is some evidence that intake of RS may increase satiety and decrease energy intake in the short-term, compared to available carbohydrates. Willis *et al.* (2009)[332] found a highly satiating effect of muffin enriched with 8 g of RS type 2 and reported that hunger and prospective food intake were also lower after the RS muffin compared to the control muffin.

However, there is no clear agreement on the mechanism of action, the dose and type of RS required to observe an effect. de Roos *et al.* (1995)[333] reported that 30 g of RS type 2 or RS type 3 (high-amylose corn starch or extruded and retrograded high-amylose corn starch) had little effect on appetite and satiety compared to glucose; Bodinham *et al.* (2010)[334] found no differences in appetite after consuming 48 g of RS intake and a placebo, but they reported a lower *ad libitum* intake following the RS meal and in the 24-hour after the RS meal. Two published reviews concluded that the main effect of high RS intake can be observed on subsequent energy intake rather than on satiety ratings.[335, 336]

The effect of RS on satiety remains unclear also because of inconsistencies between studies. A study published by Nilsson *et al*.[337] in 2008 showed that high-amylose barley bread containing 22 g of RS did not affect satiety significantly, compared to a control white bread (1.33 g of RS) but the results were difficult to interpret as the other fibre components of the breads, particularly β-glucans, were variable.

Studies reporting a mechanistic effect suggested that RS may delay gastric emptying causing sustained blood glucose levels[338, 339] however, gastric emptying is thought to be determined by the ingestion of viscous/soluble fibre and this mechanisms, which is established for NSPs, may not apply to RS.[340, 341] Colonic fermentation signals, short chain fatty acids (SCFA), and prolonged release of gut hormones may play a role in regulating appetite responses, and it has been suggested that RS giving a slow sustained glucose response and production of SCFAs by fermentation, could be beneficial in promoting satiety. However, it is not yet clear if this is the case; studies on SCFA production following RS intake are often acute studies or of a few weeks' time or their sample size is limited to clearly identify the mechanism of action, as the results are often affected by a large individual variability.[323, 342] Sustained levels of blood glucose can trigger the prolonged release of gut hormones, which in turn, prolongs satiety.[334, 343] It remains unclear whether the increased satiety is linked to a lower energy density for low-GI foods or to a direct effect on the level of circulating gut hormones. Furthermore, most studies use RS in combination with other fibres so, it is difficult to relate the effects observed directly to high-amylose or RS only. Studies comparing foods with similar fibre content are needed to clarify the effect of these novel fibres.

Satiety and satiation are also linked to the food texture and sensory properties such as palatability, acceptability, taste and flavour; these will be explored in the following section.

6.1.3.3 Sensory analysis of sbeII bread

One of the first quality judgement made by a consumer regards the food organoleptic properties. The colour, taste, odour and texture are attributes used to determine the sensory quality of a food. Abnormal colours or taste can be associated with altered or deteriorated quality and may discourage consumers.[344] Texture is an important determinant of end-use quality; texture abnormalities may not only discourage consumers from choosing a certain type of food but they could indicate that a product is more or less suitable for handling and packaging or that it has a longer/shorter shelf life than others. Texture evaluation is therefore a key step in developing novel food products, particularly when the novel food is used in an intervention study. Texture consistency between intervention meals is important in intervention studies as dramatic differences in texture can act as a confounder. Palatability, for instance, as well as meal volume can have an indirect effect on food intake in glycaemia studies.[345]

Measuring food texture instrumentally and by sensory analysis can provide an accurate insight of the end-use quality of a certain food. Sensory evaluation of foods is also important because the food texture can play a role in satiation and satiety changes.[346] The satiation is thought to be linked to a product's perceived characteristics, particularly the product's texture. In fact, certain instrumental texture parameters such as hardness and springiness were shown by previous studies[178] to be highly correlated with sensory hardness and springiness, as discussed in chapter 2.

6.2 <u>AIM:</u> Evaluate the effect of *sbeII* bread with low starch susceptibility on the post-prandial glycaemic response of healthy individuals

The aim of the REST study was to evaluate the postprandial glucose responses of normo-glycaemic (healthy) individuals to *sbeII* white bread, characterised by greater RS with lower starch susceptibility to hydrolysis than a WT control white bread.

The *sbeII* bread was made from high amylose *sbeII* wheat flour (as described in chapter 3 and 4) using the processing method that was found to be best suited for this type of flour. Different formulation and methods for bread making were tested to achieve breads matched by macronutrient composition and energy, which delivered the same amount of total starch (~75 g). Processing was aimed to produce breads of similar appearance allowing both researchers and study participants to remain blind to the interventions.

The glucose responses to *sbeII* bread measured in capillary blood and interstitial fluid are reported in this chapter together with the evaluation of satiety responses and energy intake following *sbeII* bread intake, compared to WT control bread.
This chapter also includes the sensory evaluation of *sbeII* bread compared to WT control bread.

I was the chief investigator and principal investigator of the REST study; I designed the study, wrote the study protocol and prepared the study ethics application to the Health Research Authority, submitted for review to the South Cambridge Research Ethics Committee. As principal investigator, I conducted most study visits and face-to-face activities with participants, from recruitment to study follow-up. I also carried out the statistical analysis. The blinding/unblinding procedures were carried out by Dr Natalia Perez-Moral; Dr George Savva designed the allocation concealment method and contributed to the statistical analysis; Dr Jennifer Ahn-Jarvis was involved in bread formulation and preparation method development as well as study activities (intervention visits and *ad libitum* lunch, primarily); Dr Brittany Hazard and Mr Brendan Fahy were involved in study activities (breakfast after screening and *ad libitum* lunch). The QI research nurses and Mrs Clare Ferns carried out the consent and screening visits.

6.3 Methods

6.3.1 Bread development: formulation and process

Bread recipe and processing method optimisation were carried out using the bread flour produced from the 'Autumn' field trial previously described (chapter 3). Due to the limited amount of flour available, the flour produced from the 'Spring' field trial (described in chapter 3) was used to make bread with the optimised method for the REST intervention study. The two batches of *sbeII* flour had a slightly different TS and RS content due to seasonal differences, as previously described. These are also reported in the results section of this chapter. Breads were produced and stored at QIB by Dr Jennifer Ahn-Jarvis (QIB Research scientist).

The flour obtained from milling the 8 plots of 'Spring' trial were blended after milling and homogenised using a Hobart planetary mixer with paddle attachment located at the Debut Restaurant of the City College Norwich. After blending, flour was packed in food grade sealed vacuum bags and stored at -20 °C at the Quadram Institute Clinical Research Facility.

All ingredients were weighed using OHaus (Model Explorer) scale to 0.05 g precision.

6.3.1.1 Straight-dough method (one stage fermentation)

The method and the formulation were tested and scaled up to produce four rolls per batch, for a total of 50 bread rolls delivering 75 g of TS. Bread rolls were preferred to loaves as they did not require further portioning before storage, each roll represented one serving.

Bread rolls were produced using the straight-dough method (AACC Method 10-10.03); the ingredients used to make *sbeII* and WT control rolls are reported in Table 6.1. In brief, flour, yeast, salt, shortening and water (34.6℃ - 35.3℃) were combined into the mixing bowl of a commercial planetary mixer (KitchenAid Heavy Duty Stand Mixer 5KSM7591XBSM) with a dough hook attachment. Ingredients were mixed at low speed (level 2) for three minutes; the mixing speed was gradually increased over five minutes (level 4 and then 7) for a total mixing time of eight minutes. Each batch of dough was placed in a sealable bag to ferment for two hours at room temperature (~23℃).

At the end of the 'resting' period, the dough was beaten down using a dough hook attachment for 2.5 minutes at level 10 (max speed) and then portioned and shaped into rolls. Bread rolls were placed on the baking tray and their position in the oven was recorded as part of the bread code (Figure 6.2). Each bread was assigned a unique bread code containing, the flour type (WT or *sbeII*), the position in the oven, the batch number and day of bake. The rolls were proofed (15 minutes at 38°C with 100% relative humidity) and then baked for 15 minutes at 185°C (40% humidity for 10 minutes and 10% humidity for the last 5 minutes) using a combination oven (SelfCookingCenter® model SCCWE61 - RATIONAL AG, USA). The rolls were considered "baked" when they reached a core temperature > 95°C. Baked rolls were placed on a metal cooling rack and cooled for two hours; after cooling they were packaged into resealable polyethylene bags for storage at -20°C.

Table 6.1 Straight-dough method bread rolls. *Ingredients are reported as mass (g), as a percentage on wet basis and as a percentage on dry basis* for WT control and sbeII flour. Dry basis percentage corresponds to the baker's percentage shown in chapter 4.*

	WT control			*sbeII*		
Ingredients	**Mass (g)**	**% wet basis**	**% dry basis**	**Mass (g)**	**% wet basis**	**% dry basis**
Water	369.3	36.9%	N/A	389.2	38.9%	N/A
Bread Flour	566.3	56.6%	100.0%	533.1	53.3%	100.0%
Sugar	20.0	2.0%	3.5%	20.0	2.0%	3.7%
Shortening	18.3	1.8%	3.2%	18.3	1.8%	3.4%
Yeast	16.0	1.6%	2.8%	16.0	1.6%	3%
Salt	10.0	1.0%	1.7%	10.0	1.0%	1.9%

** dry basis percentage corresponds to the baker's percentage where the ingredients are referenced against the amount of flour.*

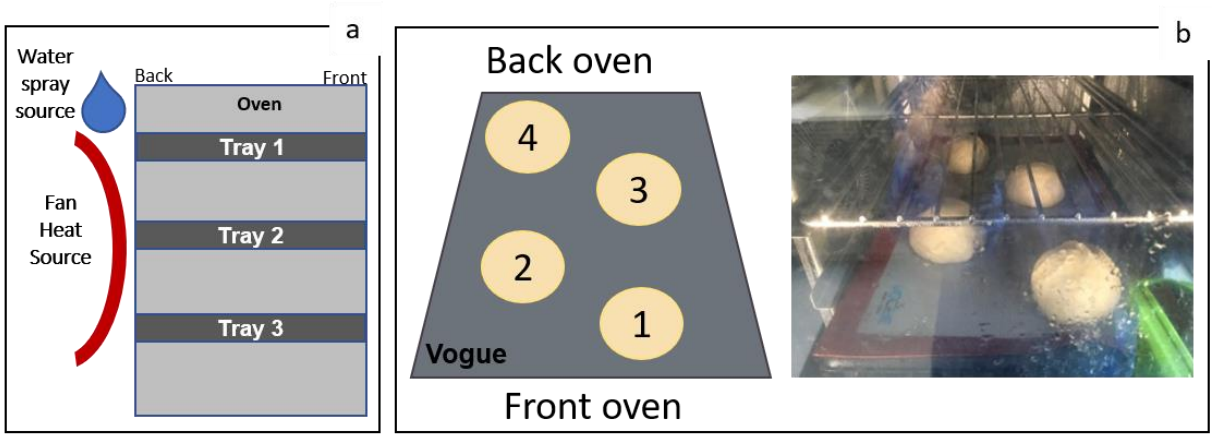


Figure 6.2 a. Self-Cooking Center combination oven layout. b. Rolls' position in the oven*. Rolls were matched for their position in the oven to ensure that baking was consistent across batches.*

6.3.1.2 Sponge method (two-stage fermentation)

The sponge method is a two-stage process requiring the formation of a 'sponge' followed by a 'dough'. The ingredients used to produce the sponge and the dough are show in Table 6.2. To form the sponge, dry ingredients (flour, yeast, salt) were mixed with tap water (34.6 to 35.3°C) in the mixing bowl of a commercial planetary mixer (KitchenAid Heavy Duty Stand Mixer 5KSM7591XBSM) with a dough hook attachment. Ingredients were mixed at low speed (level 2) for 3 minutes; the mixing speed was gradually increased over five minutes (level 4 and then 7) for a total mixing time of 8 minutes. After mixing, the sponge was placed in a resealable bag to ferment for two hours at room temperature (~21℃). After 1 hour 55 minutes, the remaining 'dough' ingredients (bread flour, sugar, shortening, yeast, and water) were mixed using a

dough hook attachment for three minutes at level two and then sponge was introduced into the mixture and mixed for two minutes at level four and then at level eight for an additional three minutes. The dough was portioned and shaped into rolls; these were placed on the baking tray and their position in the oven was recorded as part of the bread code (Figure 6.2). Each bread was assigned a unique bread code containing, the flour type (WT or *sbeII*), the position in the oven, the batch number and day of bake. Rolls were proofed for 30 minutes at 38°C with 100% relative humidity and then baked for 15 minutes at 185°C (20% humidity for 10 minutes and 5% humidity for the last 5 minutes) using a combination oven (Self-Cooking Center® model SCCWE61 - RATIONAL AG, USA). The rolls were considered "baked" when they reached a temperature > 95°C. Baked rolls were placed on a metal cooling rack and cooled for two hours; after cooling they were packaged into resealable polyethylene bags for storage at -20°C.

***Table 6.2 Sponge method bread rolls**. Ingredients are reported as mass (g) and as a percentage on wet basis. The 'Total mass' refers to the total weight of each ingredient. The 'Sponge' and 'Dough' weights refer to the weight of the ingredient added at each stage of the process.*

	WT			*sbeII*		
Ingredients	**Total mass (g)**	**Sponge (g)**	**Dough (g)**	**Total mass (g)**	**Sponge (g)**	**Dough (g)**
Water	289.6	153 (~53%)	131 (~46%)	296.3	152 (~52%)	141 (~48%)
Bread Flour	445.2	267.1 (60%)	178.1 (30%)	416.0	249.6 (60%)	166.4 (30%)
Sugar	15.7	0	15.7	15.2	0	15.2
Shortening	14.4	0	14.4	13.9	0	13.9
Yeast	12.6	6.3 (50%)	6.3 (50%)	12.2	6.1 (50%)	6.1 (50%)
Salt	7.9	7.9	0	7.6	7.6	0

6.3.2 Starch properties in bread: sample preparation

For starch characterisation and *in vitro* amylolysis analysis, samples were prepared as shown in Figure 6.3. Bread rolls were left to thaw at room temperature (~21°C) for 16 hours. On the morning of analysis, the edges of the roll or the slice were removed, leaving top and bottom crust. The rolls were blended in a food processer for 50 seconds at full speed and then sieved with a 1 mm and a 500 µm sieve. The sieved fractions were used for the analysis: the 1 mm fraction was used for the *in vitro* amylolysis assay and the 500 µm fraction was used for the TS and RS starch measurements.

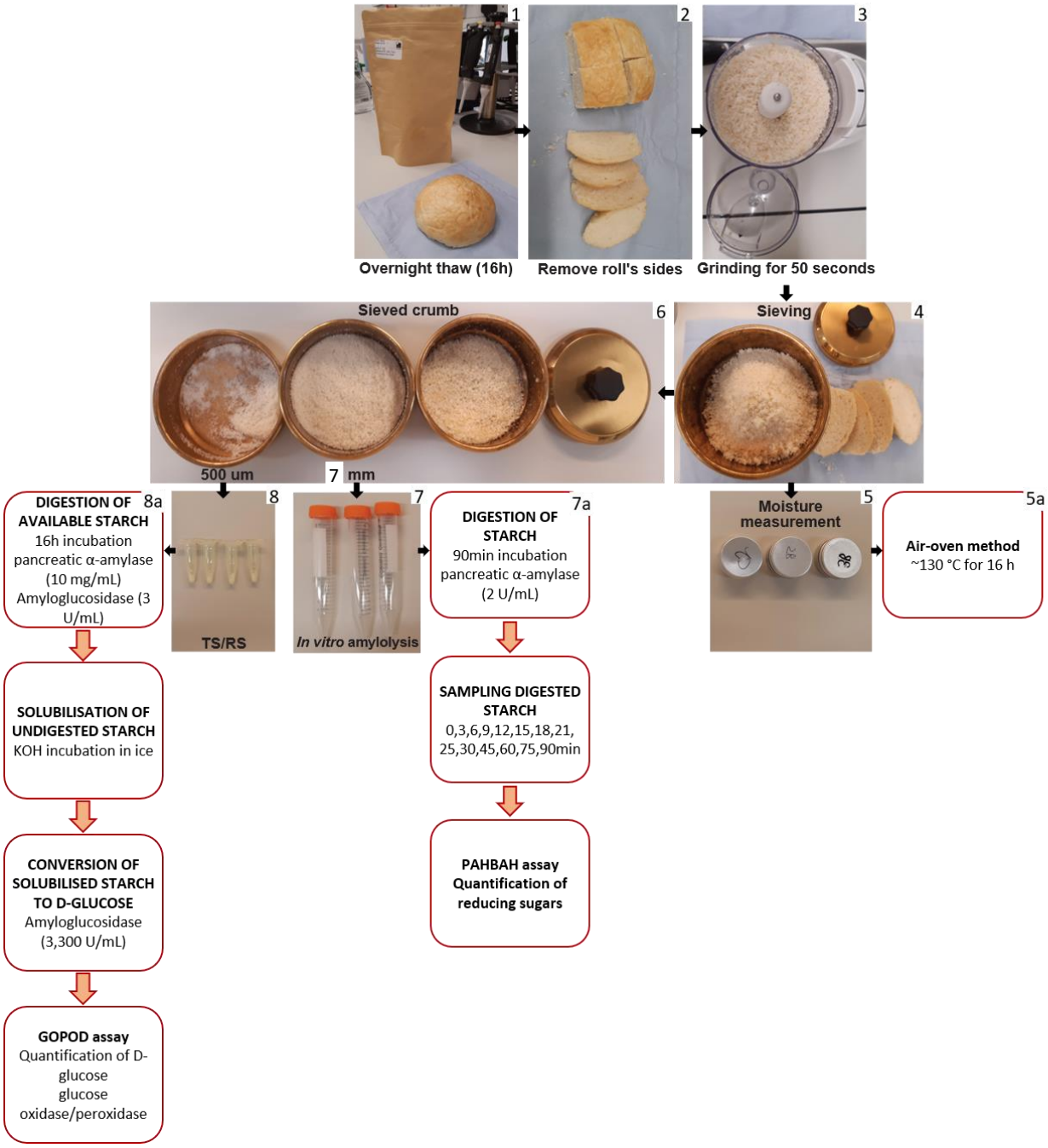


Figure 6.3 Starch characterisation methods diagram*. 1) Breads were leaft to thaw overnight (16h) at room temperature in the sealed bag. Prior to start the analysis, the sides of the rolls were removed (2) and the roll was cut in four pieces. 3) The rolls were reduced to a crumb using a food processer and the crumb was sieved through a 1mm and a 500 µm sieves (4, 6). Before seiving, samples for moisture analysis were weighted out into metal containers with lids (5). Moisture analysis was started on the same day of sample preparation, using an AACC air-oven method (44-15) described in Chapter 2. Samples for in vitro amylolysis (7) and TS/RS analysis (8) were taken from the sieved fractions. In vitro amylolysis (7a) and TS/RS analyses were started on the same day of sample preapration.*

6.3.2.1 Total and resistant starch in bread

A bread serving was designed to deliver either 50 g or 75 g of TS. Total and resistant starch content of the flour was measured using the Megazyme assay kit described in Chapter 2. TS and RS content of bread rolls were measured using the 'small scale TS and RS method' described in chapter 2.

6.3.3 *In vitro* amylolysis of bread

In vitro amylolysis of bread was carried out as described in the method section (chapter 2). Sample preparation is described in section 6.3.2. The plate reader used to measure colour formation was a VersaMax Microplate Reader (Molecular Devices, LLC., CA, USA). This was the plate reader available after moving to the new QI building on Rosalind Franklin Road.

All samples were tested in triplicates, details of the number of independent replicates used can be found in the results section as this varied depending on the sample availability. Amylolysis assay usually included 3 technical replicates of each sample and two technical replicates on the internal standard (data not shown). The internal standard used was a commercially processed rice starch (Remypure, BENEO GmbH, Germany).

6.3.4 Bread quality analysis

Bread quality characteristics were measured by Dr Jennifer Ahn-Jarvis (QIB Research Scientist) on bread rolls as described below. Figure 6.4 shows the different methods used to determine bread "quality".

Texture, colour, and specific volume of breads produced at the QI CRF were measured on independent replicates of bread rolls derived from four separate batches produced on two consecutive days. Breads were paired by the baking locations.

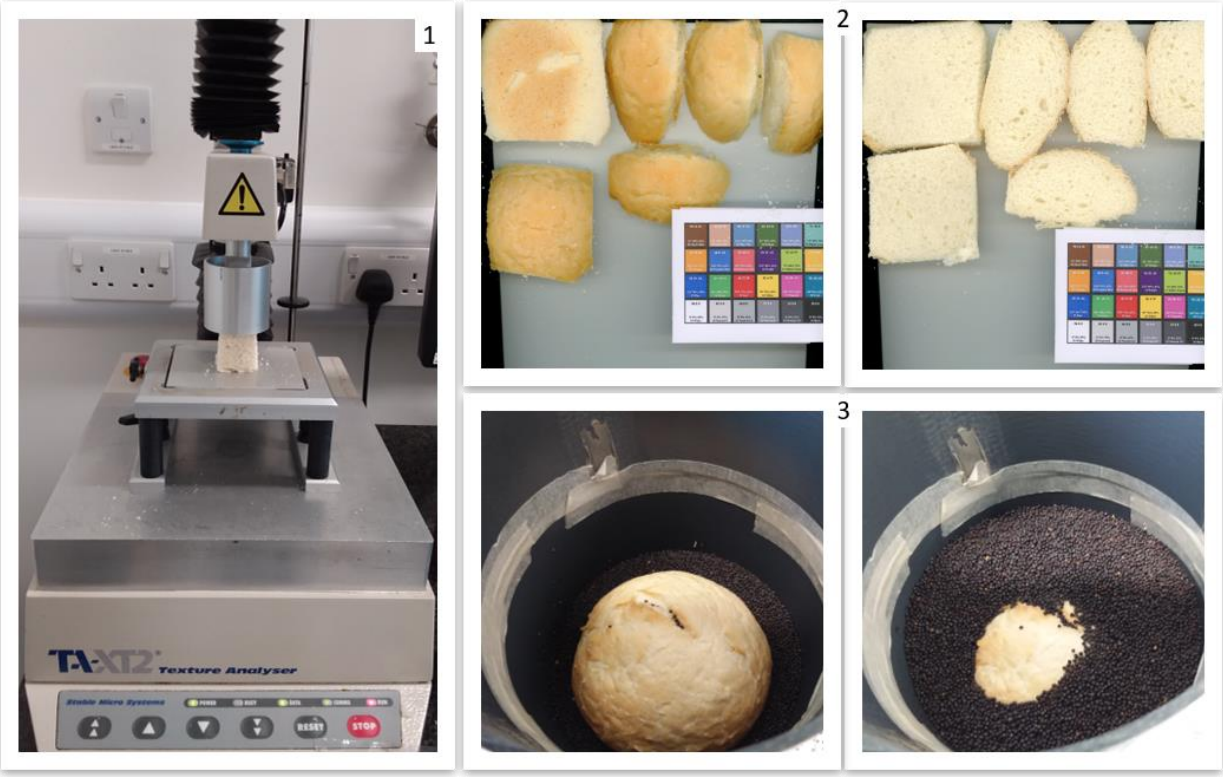


***Figure 6.4 Bread quality methods diagram**. 1) Compression test using a Texture Analyser TA-XT2. 2) Colour of crust (left) and crumb (right) using an Epson bed scanner. 3) Bread specific volume using a rapeseed displacement method: on the left, bread roll before adding the rapeseed, on the right bread after adding the rapeseed.*

6.3.4.1 Specific volume of bread rolls

The specific bread volume (cm^3/g) was determined as the volume/weight ratio of baked breads. The bread mass was measured on an OHaus (Model Explorer) scale to 0.05 g precision after two hours of cooling. The bread volume was measured using the AACC 10-05.01 rapeseeds displacement method.

To measure the volume, an empty container was filled with rapeseeds. The rapeseeds required to fill the container were transferred to a graduated cylinder to measure the volume of the container (V_1). The bread sample was placed in the container and covered with rapeseeds; the amount of rapeseed required to cover the bread sample was measured in the graduated cylinder and recorded as V_2. The bread volume was calculated as described in Equation 5.

$$\text{Bread volume (ml)} = V_1 - V_2$$

Equation 5: Bread volume calculation

The specific volume was calculated as described in Equation 6.

$$\text{Specific volume}\left(\frac{\text{cm}^3}{\text{g}}\right) = \frac{\text{Bread volume}}{\text{Bread weight}}$$

Equation 6: Bread specific volume calculation

A metal block (14 x 6.3 x 5 cm) was measured with a ruler and then placed in the same container and covered with rapeseeds. The volume of seeds recorded to cover the block was used as a reference for method calibration.

The bulk density of each bread roll and loaf was measured in duplicate as in Equation 7.

$$\text{Bread mass (g)} \;/\; \text{Bread volume (ml)}$$

Equation 7: Bread bulk density calculation

6.3.4.2 Crumb and crust colour

Crumb and crust colour were measured in an Epson bed scanner (Epson Perfection V850 Pro, Seiko Epson Corporation, Suwa, JP) using the CIE 1976 L*, a*, b* (CIELAB) scale, where L* indicates 'brightness/darkness', a* indicates redness/greenness and b* indicates yellowness/darkness. The colour values were measured on crust and crumb from the top, bottom, and two sides of each bread. Four independent replicate measurements were taken for each bread roll type, using a 'Gretag Macbeth' twenty-four patch Colour Checker as colour reference.[347] *Hue angle*, *Chroma*, and *Browning Index* were calculated as from the L*, a*, b* parameters as described by Maskan *et al*.[344] The *Hue angle* is a qualitative attribute used to define the difference of a colour with reference to a grey colour with the same lightness; *Chroma* indicates the degree of saturation (strength) of colour; the *Browning Index* represents the 'purity' of brown colour and it is linked to enzymatic and non-enzymatic browning such as Maillard reactions.[348]

6.3.4.3 Bread texture analysis

Bread roll crumb texture was characterised using a 'two-bite test' on a TA-XT2 Texture Analyser (Stable Micro Systems, Godalming, UK) equipped with a five kg load cell using a modified AACC method 74-09. The Texture Analyser was equipped with a 50 mm diameter compression plate (P50); a uniaxial compression with crosshead speed of 100 mm/min was applied to 25 x 25 x 25 mm samples to mimic mastication, with crumb hardness corresponding to the force (N) required for 40% compression. Exponent (version 6.0, Stable Micro Systems, Godalming, UK) software for texture profile analysis was used to assess the following texture parameters:

hardness, springiness, cohesiveness, gumminess, chewiness and resilience. A detailed description of these parameters and their calculation can be found in chapter 2, section 2.2.4.

6.3.5 The REST study: a double-blind cross-over intervention study

6.3.5.1 Ethical considerations

The study was approved by the Health Research Authority England (South Cambridge Ethics Committee, REC reference 19/EE/0260, IRAS 262271) and registered with the ClinicalTrial.gov registry (Identifier: NCT04197726). The study was also approved by the Human Research Governance Committee of the Quadram Institute and by the Department of Research and Development (R&D) of the Norfolk and Norwich University Hospitals NHS Foundation Trust (NNUH, reference 125-07-19). The study was conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

Consent to enter the study was sought from each participant only after a full explanation of the study had been given, an information leaflet offered, and time allowed for consideration. Signed participant consent was obtained. Participants were informed of their right to withdraw at any time from the study without giving reasons. Participants were assigned a unique identification code number (ID) at screening. Confidential information was kept in a secure locker and on a password protected encrypted local network, accessible only by the authorised researchers working on the trial (in accordance with the Data Protection Act 1988). All samples and data were labelled with an identification number to ensure anonymity. The samples collected during the study were handled, transported and disposed of in accordance with the Human Tissue Act (2011). Participants were reimbursed for their time and travel expenses upon completion of the study. The study was conducted January 2020 to March 2020 and all study visits took place at the Clinical Research Facility (CRF) of the Quadram Institute, Norwich.

6.3.5.2 Hypothesis

Based on the increase in RS and decreased starch susceptibility to hydrolysis of *sbeII* bread *in vitro*, *sbeII* bread was expected to elicit a lower glycaemic response ($iAUC_{0\text{-}210}$) than the WT control bread obtained with the same processing, when measured in capillary blood. It was also hypothesised that the glycaemic response measured in interstitial glucose, after consuming *sbeII* bread would be lower than the WT control bread, as measured in capillary blood.

6.3.5.3 Study aims

6.3.5.3.1 Primary objective

To determine the effect of *sbeII* bread on the post-prandial glucose rise measured in capillary blood ($iAUC_{0\text{-}210}$).

6.3.5.3.2 Secondary objectives

a. To determine the effect of *sbeII* bread on the post-prandial glucose rise measured in IF ($iAUC_{0\text{-}240}$).
b. To explore satiety (VAS scores) and energy intake (kcal) changes following the intake of *sbeII* bread, compared to the WT control.
c. To evaluate sensory differences between *sbeII* white bread at and WT control bread (Acceptability and JAR scores).

6.3.5.3.3 Ancillary objective

To examine the relationship between glucose concentrations measured in interstitial fluid with those measured in capillary blood and the reliability of the interstitial fluid measurements obtained with CGM sensors.

6.3.5.4 Study design

A randomised, double-blind, cross-over dietary intervention study was conducted to test the effect of *sbeII* white bread (test bread) consumption on postprandial capillary and interstitial glucose responses compared to WT white bread (control bread), in healthy subjects. Twenty-one healthy participants, male and female, aged between 18 and 65 years, were recruited onto this study. The sample size calculation was based on previous studies of glycaemic responses in healthy subjects that aimed to improve insulin response by increasing fibre intake with similar test breads (see power calculation, section 6.3.5.6). Volunteers' eligibility to take part in the study was assessed during a screening visit. A complete list of inclusion/exclusion criteria is provided in Appendix B1 (eligibility assessment). *Intervention 1* was followed by a washout phase of at least four days, when participants were free to return to their typical diet. During the washout phase participants completed a non-consecutive three-day food diary, on two weekdays and one weekend day, to provide us with information on their background diet. After the washout phase, *Intervention 2* took place: participants that initially consumed the test bread were given the control bread, and vice versa. For both groups, a portion of bread

providing 75 g of total starch, was administered at breakfast. A summarised diagram of the study design is in Figure 6.5.

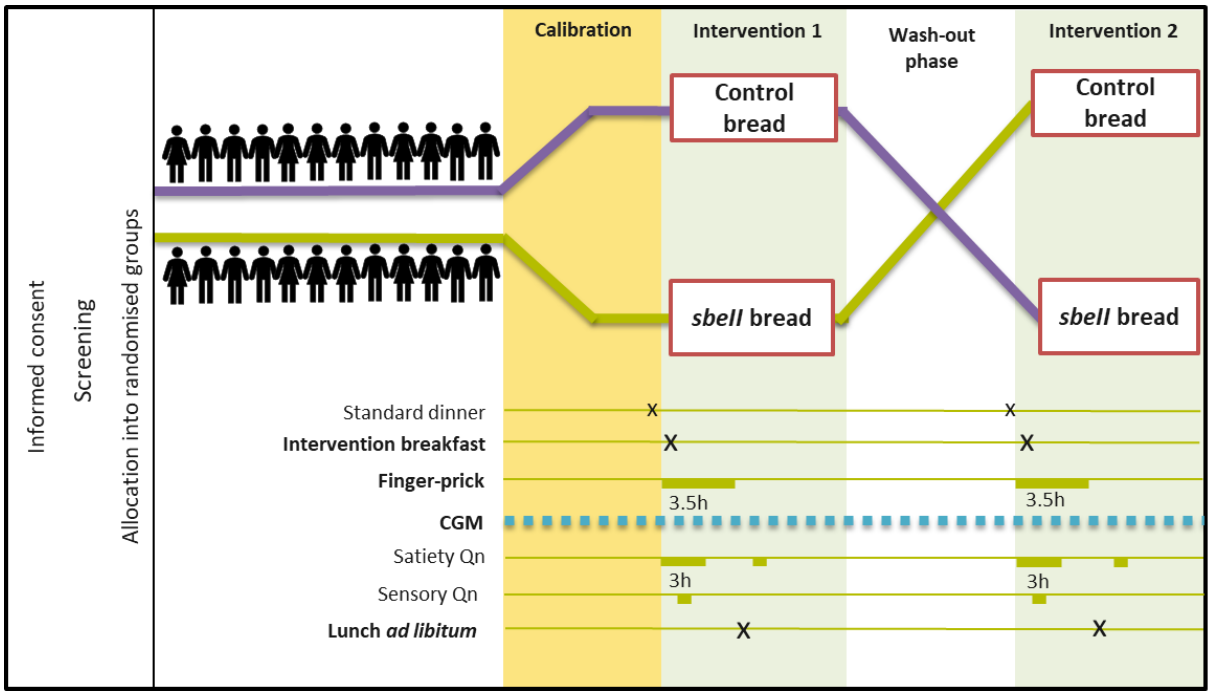


Figure 6.5 REST study diagram

6.3.5.5 Subjects

The study cohort consisted of healthy men and women, aged between 18 and 65 years with a BMI between 18-25 kg/m^2, non-smokers, that lived within a 40-mile radius of Norwich. Participants were recruited through the QIB volunteer database as well though approved posters and flyers posted across the Norwich Research Park and social media. Volunteers who expressed an interest in the study were sent a participant information sheet (Appendix B3) (approved by South Cambridge Ethical Committee) and, those likely to be eligible to participate in the study were invited to attend a screening visit at the QI Clinical Research Facility (CRF) with a member of the QI CRF team. Following consent, eligibility to participate in the study was assessed with a health questionnaire to confirm that participants were healthy, with no history of diabetes or diagnosed gastro-intestinal conditions, a physical exam (BMI and blood pressure) and a blood test (a HbA1c, fasting plasma glucose, full blood count, liver and renal function test, lipids profile). Exclusion criteria included fasting glucose > 6.1 mmol/L and/or HbA1c > 42 mmol/mol, as assessed by a fasting blood test; a complete list of criteria can be found in Appendix B2 (eligibility criteria).

6.3.5.6 Power calculation

A power calculation was carried out by Dr George Savva (QIB statistician) to estimate the number of study participants required to complete the study. The likely effect size was calculated as in Equation 8.

$$Effect\ size = \frac{[Mean\ of\ treatment\ group] - [Mean\ of\ control\ group]}{Standard\ deviation}$$

Equation 8: Calculation of effect size

The mean difference between *sbeII* and WT breads was estimated from the Hydrolysis Index (HI) calibrated with an expected change in Glycemic Index (GI). The HI is the iAUC ratio of starch digested after 90 minutes of hydrolysis of *sbeII* bread compared to the WT control bread, obtained from the *in vitro* amylolysis assay. The GI is the iAUC glucose response ratio of test and reference breads measured in capillary blood glucose. The HI of starch-based foods was shown to correlate with Glycaemic Index (GI) measured *in vivo.*[174]

The HI of *sbeII* bread was 81 while the WT control bread HI was 100 (reference). The calculation was carried out with a standard deviation (SD) of 28% based on similar types of studies that reported similar or slightly lower SD.[322, 349, 350]

Using a paired t-test with a difference within pairs of 19 units (100-81) and standard deviation between pairs for this difference of 28.7, at p-value < 0.05, it was estimated that a total of 19 participants were required to detect a relative difference of 20% in blood glucose response over time (iAUC) between *sbeII* white bread and WT white bread, with 80% power. The study aimed to recruit 25 participants in total, allowing for a 20% dropout rate.

6.3.5.7 Study meals

6.3.5.7.1 Standard dinner meal

Food intake before intervention visits was standardised by providing participants with a ready meal. Participants were asked to consume the same meal before each intervention visit, which they could choose from a list of ready meals. The pooled average nutrients composition of the ready meals is reported in **Error! Reference source not found.**.

Table 6.3 Standard meal consumed the evening before the study visit.

Average nutrients composition of each ready meal consumed by the study participants, presented as the mean, standard deviation (SD) and SE of the mean (SEM), n=5 types of meals consumed by the 8 participants who completed the study.

Per serving	Energy intake (kJ)	Energy intake (kcal)	Fat (g)	Saturated fat (g)	Carbohydrates (g)	Sugars (g)	Fibre (g)	Protein (g)	Salt (g)
Mean	1525.4	364	13.93	5.22	38.8	8.06	7.80	16.92	1.5
SD	114	27.5	4.37	2.24	6.7	2.40	4.08	3.72	0.2
SEM	43.1	10.4	1.7	0.8	2.5	0.9	1.5	1.4	0.1

6.3.5.7.2 Breakfast test meals

The breakfast test and control meals consisted of a bread roll with 10 g of Flora dairy free spread and a drink of water prepared in the QI CRF kitchen.

Both *sbeII* and WT control bread rolls contained the same ingredients and were matched by macronutrient composition and energy to ensure delivery of the same amount of total starch (~ 75 g); breads were similar in appearance allowing both researchers and study participants to remain blind to the interventions. From in-house laboratory analysis (AOAC ref 2002.02) it was expected that at least 4.35% and 1.39% of the starch was RS, in the *sbeII* and WT white breads respectively. This concentration of RS was within a normal serving of starch-rich food; for example, starch in a conventional white wheat bread can contain 1% to 2% RS. [351]

Blinded bread rolls were stored in the QI CRF (-20 °C) freezers. Sixteen hours before an intervention visit, the bread roll allocated to the participant was thawed at room temperature in the QI CRF kitchen. The morning of the visit, the roll was sliced horizontally and placed crust side down, the top crumb was covered evenly with Flora spread and it was administered according to the participant accompanied by approximately 250 mL water. The liquid component of the meal was standardised at 317 mL using a look-up table that specified the volume of drinking water required to account for differences in evaporative losses. Subjects were instructed to consume the breakfast meal within the allocated 15 minutes timeframe.

6.3.5.7.3 Lunch meals

A carbohydrate-based lunch *ad libitum* was served four hours after the consumption of the test or reference bread. Participants could choose between two options of comparable nutritional composition for the lunch meal; each participant consumed the same meal option during both visits to enable within subject comparisons. The lunch meal was made of white rice with either A) Tomato sauce and parmesan cheese or B) Beef Bolognese sauce and water for drink; the pooled average nutrient content (calculated from the nutrient declaration on food packaging) of the meals is shown in Table 6.4. Participants were instructed to eat until ‘comfortably satisfied’.

Table 6.4. Ad libitum lunch nutrients intake per serving. *Option A was rice with tomato sauce and parmesan cheese, option B was rice with Bolognese sauce.*

	Per serving (A)	**Per serving (B)**
Energy (kcal)	177.8	176.5
Energy (kj)	747.4	744.4
Fat (g)	4.24	3.96
Sat fat (g)	1.62	1.34
Carbohydrate (g)	27.67	26.9
of which sugars (g)	5.5	3.73
Fibre (g)	1.41	1.01
Protein (g)	6.04	7.74
Salt (g)	0.36	0.55

Average composition estimated from the ingredients' label, n = 3 ingredients in option A and n = 2 ingredients in option B.

6.3.5.8 Randomisation and blinding

A double-blind approach was used in this study where the study team and participants were both blind to the different breads thanks to the similar appearance of the bread rolls and the use of an allocation concealment for blinding. Test and reference breads were assigned a unique code by Dr Natalia Perez Moral (QIB Research Scientist, independent from the study team); the study team and participants were blind to the type of bread consumed.

Block randomisation was used to ensure groups were balanced in number. Participants were randomly allocated to the two treatment arms in blocks of four. Blocks were stratified by gender and ordered at random to prevent a possible order effect of the treatments. The blocks allocation sequence was generated by the R package 'randomizeR' by an independent researcher. Following randomisation to blocks, two participants per block (dependent on randomisation results) consumed a portion of *sbeII* white bread (test bread) with 10 g of low-fat spread (Flora Dairy Free) providing ~75 g of total starch, once, at the first intervention visit. The remaining two participants from the same block consumed a portion of WT white reference bread with 10 g of low-fat spread (Flora Dairy Free) providing ~75 g of total starch, once, at the first intervention visit. At the second intervention visit, participants that received the test bread on their first visit were given the control bread and vice versa.

Study breads were assigned a unique numeric code at blinding known only to Dr Natalia Perez Moral, who blinded the study. At the end of all the study visits, breads were grouped as 'treatment A' or 'treatment B' to allow grouping for the data analysis, without unblinding the interventions. Breads remained blinded to the study team until the analysis was completed.

6.3.5.9 Study protocol and sampling

An overview of the study activities is provided in Figure 6.6 , details of the study Standard Operating Procedure of the study can be found in Appendix B4

6.3.5.9.1 Finger prick test for capillary blood glucose

A Unistik needle was used to collect one drop of capillary blood at different timepoints throughout the intervention visits (13 pricks in total). A blood drop was placed on a disposable glucose strip (Accu-Check, Roche Holding AG) and inserted into a glucose meter (Accu-Check Performa nano-meter calibrated daily according to manufacturer instructions, Roche Holding AG) to measure capillary blood glucose. The strip was then be discarded, and the glucose value recorded.

6.3.5.9.2 Continuous Glucose Monitoring readings

Glucose levels in the interstitial fluid were measured every 15 minutes before, during, and after the interventions, using a factory calibrated CGM sensor (Freestyle Libre System, Abbot Laboratories). Glucose measurements were recorded automatically every 15 minutes for 14 consecutive days, including interventions 1 and 2. Participants were provided with two sensors and two readers. The reader was required to scan and download the glucose recordings from the sensors every 8 hours. At the end of the study, the glucose concentration collected over 14 days were downloaded by the research team through the *FreeStyle LibreView* web-based programme and the sensors were discarded.

6.3.5.9.3 Satiety VAS and Energy intake

A validated visual analogue scale (VAS) questionnaire was used to rate the degrees of hunger, fullness, desire to eat, satisfaction, thirst and nausea. The scales consisted of horizontal lines 100 mm long, anchored by "not at all" and "extremely" at opposite ends. Participants were asked to place a vertical line to indicate their subjective ratings.

VAS questionnaires were completed before and after the intervention breakfast every 30 minutes between breakfast and lunch, during study day. The last questionnaire (270 min) was completed after an *ad libitum* lunch. The questionnaire was developed and validated by collaborators at Imperial College London.[352]

Satiety changes were estimated based on the *total energy intake,* measured as the 'kcal', consumed during the lunch *ad libitum* and the VAS responses.

6.3.5.9.4 Sensory Analysis

Sensory analysis questionnaires were developed and analysed by Dr Jennifer Ahn-Jarvis.

Paper surveys designed with a 9-point hedonic scale (1 = dislike extremely to 9 = like extremely) were administered to assess acceptability of *sbeII* and WT control bread rolls, overall likability, aroma, flavour, moisture, texture. A 'Just-About-Right' (JAR) scale with five anchor points (JAR; 1 = much too weak to 5 = much too strong) was used to measure the appropriateness of the level of specific attributes: aroma (nutty, malty, yeasty), flavour (salty, sweet, bitter) and texture (hardness, dryness, smoothness) and quantity (serving size). Participants were asked to give their opinion on the intensity of specified product attributes in relation to their JAR level. The attributes intensities (or levels) were expressed using a target lexicon forcing the participants to focus a specific attribute (e.g., flavour) in the context of a particular flavour (e.g., salty). The study participants were not trained in the use of the sensory lexicon in order to obtain their unbiased perception of the bread attributes.

A 'penalty analysis' determined where the acceptability had decreased because of attributes that were not optimal or JAR, identifying attributes that were most penalising to the product performance. To carry out the penalty analysis, overall acceptability scores (from the 9-point hedonic scale) and JAR scale responses were combined. For attributes that received at least 20% responses (Pareto principle: "80% of effects occur from 20% of causes" or the 80-20 rule), mean decrease in liking was calculated by subtracting the liking values obtained from the hedonic scale (acceptability test) of the participants in the not-JAR category from the JAR category (i.e., mean decrease = JAR liking − not-JAR liking).[353] The penalty score was calculated as in Equation 9, where 'JAR' is the number of participants that indicated Just-About-Right and 'not-JAR' is the

number of participants that indicated either too much or too little for the attribute on the JAR scale.

$$Penalty\ score = \frac{not - JAR}{JAR} \times mean\ decrease\ in\ liking$$

Equation 9: Calculation of penalty score

6.3.5.9.5 Follow up questionnaire

A follow up questionnaire was designed to capture participants experiences wearing the CGMs sensors. The questionnaire was designed as a list of open questions where participants were asked to indicate three terms to describe the experience of applying the sensors and wearing the sensors; the questionnaire was developed with support from Dr Gene Rowe, expert in consumer risk perception, public engagement and participation.

Study Talk – By phone
A member of the study team will discuss the PIS and answer any questions
Eligibility Screening – Visit 1, CRF, ~3 hours
A member of the study team will explain the study. The potential participant can ask questions
Study team member to show participants the CGM and fingerprick collection devices
Participant to sign consent for study participation and a medical declaration form
A screening questionnaire will be completed by QI CRF research nurse with the participant
Pulse, blood pressure, height and weight will be measured, and BMI calculated
Fasted blood samples collected for analysis followed by breakfast
Potential study appointment dates will be identified and provisionally scheduled
Participant enrolment
Eligible participant will be contacted to confirm or arrange date and time for all study appointments
Calibration – Visit 2, CRF, ~3 hours
QI CRF nurse will complete general health assessment (CR Form)
Participant will be given two sensors and two independent readers – to scan at least every 8h
Member of study team will go through preparation to intervention day and finger-prick test
Participant will be trained on how to use Nutritics app/paper to record dietary intake
Participant will be provided with ready meal to be consumed the night before intervention 1
Preparation for intervention day - Diet and exercise restrictions apply
Participant to avoid strenuous exercise, caffeinated drinks or alcohol
One ready meal to be consumed for dinner. No food or drink after that except for water
Visit 3 – Dietary Intervention Visit, CRF, ~5 hours
Participant will be asked to arrive at the QI CRF between 7.30 and 8.30 am, fasted (10h)
Study team member to complete the case report form and go through "Study bundle" with participant
Before consuming bread: scan sensors, complete satiety questionnaire and fingerprick baseline
Participant to complete fingerprick test at 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 min
Participant to fill in satiety questionnaires at 32, 92, and 182 min
Participant to fill in a sensory questionnaire after (35 and 50 min) consuming the test bread
Lunch will be served *ad libitum* (270 min)
Record any other food intake for 4h after lunch
Washout phase
Three non-consecutive days food diary (2-week days, 1 weekend day)
Normal diet while wearing the continuous glucose monitoring sensors scanned at least every 8h
Preparation to intervention day – Diet and exercise restrictions apply
Participant is reminded to avoid strenuous exercise, caffeinated drinks and alcohol
One ready meal to be consumed for dinner
Visit 4 – Dietary Intervention Visit, CRF, ~5 hours
As described in phase 1 – but the alternative bread will be served
Follow up
Participant scans CGM sensors before removing them
Participant will fill in a follow-up questionnaire
End of study

Figure 6.6 Schedule of activities REST study

6.3.5.10 Statistical analysis

The power calculation was carried out using an R core function, *power.t.test.* [179]

The iAUC was calculated geometrically for values above the baseline between 0 and 210 minutes by applying the trapezoid rule to the area above the glucose fasting concentration as described by Brouns *et al*. [305] Further details of the iAUC calculations were reported in chapter 5. Mean glucose peak and mean glucose dip were calculated as the maximum and minimum glucose concentrations achieved after consuming the meal; for capillary glucose this was calculated between 15-210 minutes, for interstitial glucose, between 15- 240 minutes. Time to peak was calculated as the time to reach the maximum glucose concentration, on average.

It was hypothesised that *sbeII* bread, with lower susceptibility to hydrolysis *in vitro* than the WT control, would give rise to a lower glucose response compared to the WT control *in vivo* (iAUC). A paired t-test was used to identify statistical differences between calculated capillary and interstitial glucose iAUCs. Other indicators of glucose responses were *maximum peak* and *maximum dip* as the maximum and minimum glucose concentrations from start of the meal consumption of the meal and *time to peak*, as the time to the reach the maximum glucose concentration.

Datasets were curated using package *reshape,* graphs and plots were made using *ggplot2* and *ggpmisc.*[180, 182, 230] Data interpolation was obtained using *dplyr, tidyr* and *lubridate* packages,[354-356]

The relationship between glucose concentrations measured from the interstitial fluid and capillary fluids were examined by calculating the Kendall concordance coefficient (*W*) and by Bland-Altman plot. This objective was added to determine the reliability of CGM systems used in intervention studies involving healthy participants, which to date has not been fully investigated. Capillary and interstitial glucose values were measured every 15 minutes although, because of logistics reasons, the measurements did not match exactly. Data was calibrated on a minute-by-minute basis, with time *zero* as the start of consumption of each meal. An interpolation of capillary and interstitial data was performed using R software version 3.6.3[179], using a locally weighted smoothing (*loess*) as described by Dye *et al*.[120] and a smoothing span of 0.2 for interstitial data and 0.4 for FP data. The span was selected to be as small as possible while providing a good fit to the observed values. Bland–Altman plots were produced for the paired of measures (capillary and interstitial) using *BlandAltmanLeh*

package[357], and Kendall's *W*, a non-parametric measure of concordance, was calculated. More details of the statistical tests used can be found with the relevant results, in the results section.

6.4 Results

6.4.1 REST bread development

6.4.1.1 Starch characteristics of bread

Flour from the 'Autumn' field trial was used to make breads using the sponge and straight-dough methods. The TS content of *sbeII* plot number seven was 72.13 g / 100 g of flour and the WT plot number five TS content was 67.39 g / 100 g of flour, expressed on a dry weight basis. Of the TS, 3.75% was RS in *sbeII* flour and 0.49% was RS in the WT control flour, expressed on a dry weight basis. The mean moisture content of the 'Autumn' flour was 12.6% ± 0.2% for *sbeII* flour and 11.9% ± 0.19% for WT control flour. Bread rolls made with the sponge method were designed to deliver approximately 75 g of TS; part of the optimisation process was to standardise TS content of the bread serving to 75 g for all bread types. Each roll represented one serving of bread and no further portioning was required. The serving size and TS content of each bread roll are reported in Table 6.5.

Table 6.5 Serving size represents the weight of one roll served as one portion (g), TS per serving is the TS content of one bread roll (g).

	Bread making method	Serving size (g)	TS per serving (g)
sbeII	Straight-dough	161.31 ± 0.17	72.13 ± 0.03
WT control		162.94 ± 0.04	71.81 ± 0.01
sbeII	Sponge	160.84 ± 0.03	73.16 ± 0.01
WT control		164.97 ± 0.02	71.80 ± 0.02

Values are reported as means ± SEM, n = 3 independent replicates of bread (3 replicates per batch, 1 batch).

6.4.1.2 In vitro amylolysis of bread

6.4.1.2.1 Straight-dough bread method

The *sbeII* bread rolls showed a significantly lower starch hydrolysis compared to WT control rolls (C_{90} mean difference = 16.0 µmol L^{-1} min^{-1}, [95CI 1.33, 30.75], unpaired t-test, p-value = 0.04). The total breakdown of starch represented by C_∞ and the rate constant (k) of starch digestion were determined from the slope and y-intercept of the LOS plot (Figure 6.7) and are reported in Table 6.6.

6.4.1.2.2 Sponge bread method

In vitro amylolysis showed a significant difference in starch hydrolysis between *sbeII* sponge bread rolls and the WT control rolls (C_{90} mean difference = 13.4 µmol L^{-1} min^{-1}, [95CI 3.74, 23.11], unpaired t-test, p-value = 0.018, Table 6.6) and after 90 minutes of hydrolysis, more than 50% of the starch from *sbeII* and WT control breads was digested (Figure 6.7). Sponge breads were characterised by a higher concentration of endogenous sugars compared to straight-dough breads, detected at the beginning of the incubation with α-amylase. The presence of greater reducing sugars concentrations resulted in a faster enzymatic reaction, compared to straight dough breads, particularly for the WT control (*k*).

It should be noted that the straight-dough breads were proofed for 15 minutes while the sponge breads were proofed for 30 minutes. Breads with longer proofing time were more consistent in shape and colour so it was decided to apply a longer proofing time to the straight-dough method. A test baking was repeated to confirm that straight-dough method with the extended proofing would result in breads of consistent shape and colour (Appendix A, Figure A6 1). This modified straight dough method was then selected to produce breads for the REST *in vivo* study; this was preferred to the sponge method as all ingredients were added at once reducing possible variability between breads due to moisture and flour losses during dough formation.

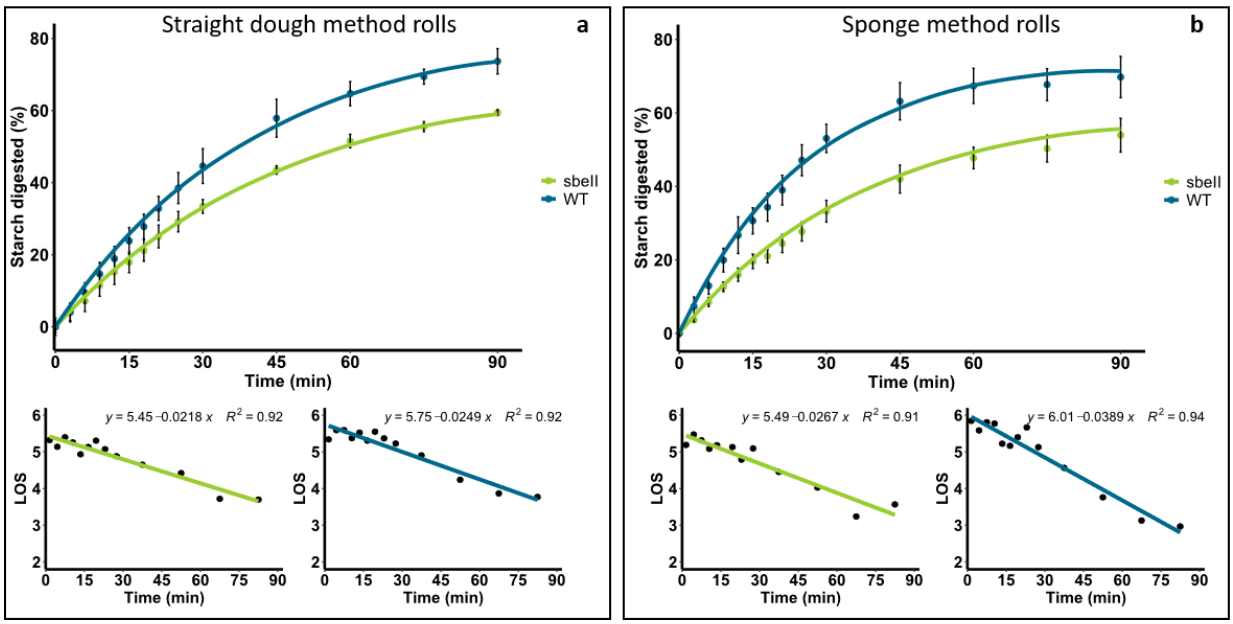


***Figure 6.7 In vitro amylolysis of sbeII (green) and WT (blue) bread**. a. Starch hydrolysis (top) and LOS plots (bottom) of bread rolls produced with the straight-dough method, n = 3. b. Starch hydrolysis (top) and LOS plots (bottom) of bread rolls produced with the sponge method, n =3. Starch amylolysis experimental data are shown by fitting a first-order equation (Equation 2) based on the k and C_∞ values obtained from the LOS analysis. Each experimental data point represents the mean value from the analysis of independent samples with error bars = ± SEMs. LOS plot was obtained from sbeII and WT bread experimental data, the linear phase is defined using Equation 3 from which k and C_∞ values can be estimated. Legend applies to all panels.*

Table 6.6 Variables estimates from the amylolysis curves and LOS analysis of sbeII and WT control breads.

Genotype	*Bread making method*	*C_{90} (%)*	*C_∞ (%)*	*k (min^{-1})*
sbeII	Straight-dough	60.4 ± 0.7	68.8	0.022
WT		76.5 ± 3.6	82.5	0.025
sbeII	Sponge	69.3 ± 2.6	61.5	0.026
WT		82.7 ± 2.3	74.0	0.039

Experimental values (C_{90}) are presented as means ± SEMs of 3 independent replicates for the bread loaves and five independent replicates for the bread rolls. C_{90} represent the proportion of starch digested by α-amylase after 90 min obtained from the starch hydrolysis curves, reported as means ± SE of three independent replicates of the bread rolls. C_∞ is the total extent of starch amylolysis accounting for Y_0 (endogenous baseline sugar) and *k* is the rate constant of the reaction; both parameters were obtained applying the LOS analysis to the starch hydrolysis data.

6.4.2 The REST study *in vitro*

6.4.3 Characterisation of bread rolls

6.4.3.1 Formulation and proximate analysis

The breakfast meal characteristics and nutritional composition are reported in Table 6.7. The breakfast meal consisted of one serving of bread (roll), a vegetable spread and a drink of water. The breads were designed to deliver ~ 75 g of TS based on the TS content of flour from the 'Spring' field trial (73.9 ± 1.21 g / 100 g of *sbeII* flour and 74.9 ± 0.83 g / 100 g of WT flour). The breads were produced by straight-dough method as described in the methods section (6.3.1.1) with a proofing time of 30 minutes that produced breads of consistent colour and shape.

The bread water content was adjusted based on the flour water absorption in the formulation. While the mass of the baked rolls mass was highly consistent across baking batches (the Coefficient of Variation (CV) was 1% for the WT bread and 2.7% for *sbeII* bread), the moisture loss during baking was higher in *sbeII* bread rolls (CV = 20.5%) than the WT control breads (CV = 8.6%).

To ensure that the liquid component of the meal was standardised, the water drink served as part of the meal was adjusted depending on the water content of each bread roll. The total water content of *sbeII* and WT control meals was approximately 317 g, the average serving size of *sbeII* breads was 153.19 g ± 0.49 g while the average size of WT control breads was 147.28 g ± 0.73 g.

Table 6.7. Bread formulation and breakfast meal ingredients and nutrients composition per serving. *Bread proximate analysis was carried by ALS Laboratories (UK) Limited, Sands Mill, Huddersfield Road, Mirfield, West Yorkshire, WF14 9DQ*

	WT control	***sbeII***
Bread ingredients per serving		
Water	34.76	39.82
Bread flour (g)	100.11	101.47
Caster sugar (g)	3.53	3.40
Vegetable fat (g)	3.23	3.71
Baker's yeast (g)	2.82	2.97
Salt (g)	1.76	1.85
Vegetable spread (g)[j]	9.5	9.5
Water drink (g)[b]	256.2	251.4
Breakfast serving (g)[k]	411.9	414.3
Breakfast meal estimated nutrients per serving		
Protein[l] (g)	12.15	12.96
Total fat (g)	11.00	11.54
Ash	2.19	2.29
Saturated fat (g)	2.92	3.07
Mono-unsaturated fat (g)	4.24	4.38
Poly-unsaturated fat (g)	3.54	3.77
Available Carbohydrates[m] (g)	70.9	65.1
Total sugars (g)	2.92	3.06
Fibre AOAC (g)[n]	4.53	7.50
Energy (kcal)	438.7	430.8
Energy (kJ)	1854.9	1815.7

[j] Vegetable spread and water drink accompanied the bread in the "breakfast meal"
[k] One bread roll, Flora dairy free spread and a drink of water
[l] Nitrogen-to-protein conversion factor = 6.25
[m] Calculated by difference
[n] AOAC method for measurements of fibre includes RS3

The study bread rolls were prepared according to the formulation reported in Table 6.7 using the straight-dough method previously described. They were stored at -20 °C for and starch susceptibility to hydrolysis was measured at the end of the recruitment phase of the REST study, after five months storage. One serving of bread contained 74.9 g of TS: of this, 5.40% ± 0.81% was RS in *sbeII* bread while 1.94% ± 0.31% was RS in WT bread. Starch *in vitro* amylolysis of bread and bread quality parameters are reported in Table 6.8. Starch in *sbeII* bread resulted in a lower starch hydrolysis compared to the WT control after five months of storage (C_{90} mean difference = 12.99 µmol L^{-1} min^{-1}, [95CI 2.963, 23.023], unpaired t-test, p-value = 0.01), consistent with the extent of starch amylolysis measured in the bread development phase of this study. The *sbeII* bread was characterised by a lower starch breakdown after 90 minutes of hydrolysis (Figure 6.8a) and the lower digestion rate (k). The kinetic parameters obtained from the LOS plot analysis provided a good fit to the experimental data (R^2 ~ 0.9), Figure 6.8 b and c.

Table 6.8. Bread and starch characteristics measured 5 months after production and storage at -20 °C.

Bread characteristics	**WT control**	***sbeII***
Bread roll (g)	146.2 ± 2.5	153.2 ±0.4
Moisture (%)	38.6 ± 0.2	42.1 ± 1.0
***In vitro* amylolysis**		
C_{90} (%)	88.9 ± 2.2	76.9 ± 3.9 *
C_∞ (%)	88.9	78.6
k (min^{-1})	0.036	0.024

Experimental values are reported as the mean of six independent bread samples ± SEMs. Parameter values that are significantly different between the two bread types are indicated with an asterisk: *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired Students t-test).

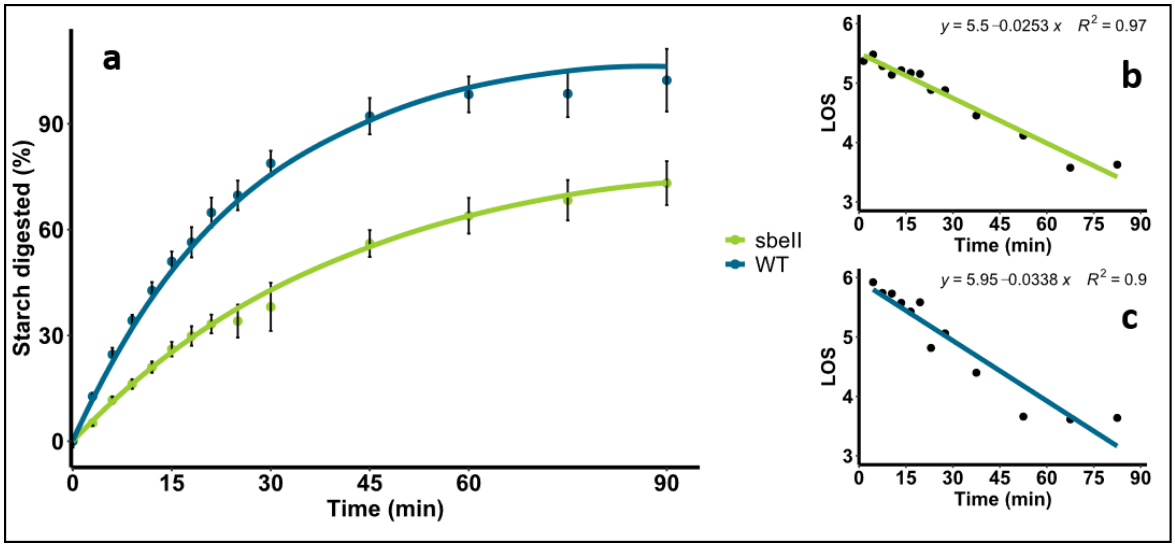


***Figure 6.8 In vitro amylolysis of sbeII (green) and WT (blue) bread**. a. Starch hydrolysis. b,c. LOS plots of bread rolls after 5 months of storage at -20 °C, n = 6. Starch amylolysis experimental data are shown by fitting a first-order equation (Equation 2) based on the k and C_∞ values obtained from the LOS analysis. Each experimental data point represents the mean value from the analysis of independent samples with error bars = ± SEMs. LOS plot was obtained from sbeII and WT bread experimental data, the linear phase is defined using Equation 3 from which k and C_∞ values can be estimated.*

6.4.3.2 Bread quality analysis

Bread quality analysis results are reported in Table A6 1 (Appendix A). Overall, there were significant quality differences between *sbeII* breads and WT control breads.

The specific volume of *sbeII* bread was marginally lower than the WT control and the bulk density of *sbeII* bread was slightly greater than the WT control.

The colour analysis revealed significant differences between *sbeII* and WT breads however, only the crust colour of *sbeII* breads appeared to be significantly different from the WT control, showing a darker colour and a matte finish of the crust, compared to the WT control. There was also a small but significant difference in Browning Index between the two types of bread.

The crumb texture analysis indicated that *hardness* and *chewiness* were significantly higher in *sbeII* bread rolls than WT control breads while there were no differences in *cohesiveness* (Table A6 1, Appendix A). There were no significant differences in *springiness* and in *resilience* between bread types. Bread hardness is expected to increase with storage as staling progresses; the higher hardness measured in the *sbeII* bread crumb texture analysis could suggest structural crumb differences or differences in bread staling and moisture loss. Cohesiveness is inversely related to crumb breakdown (in the mouth or in hand); it is usually higher on stale bread than fresh bread as the moisture loss makes the bread crumbly. There

were no differences in springiness despite the long storage period, which suggests that *sbeII* bread staling was similar to that of the WT control.

6.4.4 The REST study *in vivo*

6.4.4.1 Power calculation

Only 8 participants completed the study due to the COVID-19 outbreak and consequent lock-down. The effect size in the obtained sample (18%) was close to the effect size estimated (20%), based on the target sample size (n = 19). The estimates of effects of WT bread consumption on glucose response was $iAUC_{0\text{-}120}$ = 160.51 mmol/L/min, 95% CI [131.79, 189.03], and *sbeII* bread $iAUC_{0\text{-}120}$ = 131.63 mmol/L/min, 95% CI [81.88, 181.38].

6.4.4.2 Participant characteristics

A total of 76 expression of interest forms were returned to the study team between January and March 2020. Participants that successfully completed the study talk over the phone, which included an assessment of the basic inclusion and exclusion criteria, were invited to complete a screening visit. Twenty-two participants were screened and 19 were enrolled in the study, seven males and 12 females. Of the participants enrolled, eight completed all study visits before the COVID-19 lockdown (CONSORT diagram, Figure 6.9). All other visits booked after the 20th March were cancelled as the study entered a phase of temporary halt because of COVID-19. In June 2020, it was decided to suspend indefinitely the study due to the uncertainty of the current situation and timescales with the Coronavirus pandemic. A preliminary data analysis was carried out after which, the study meals were unblinded.

The characteristics and average dietary intake of the participants that completed the study before the study halt are reported in Table 6.9.

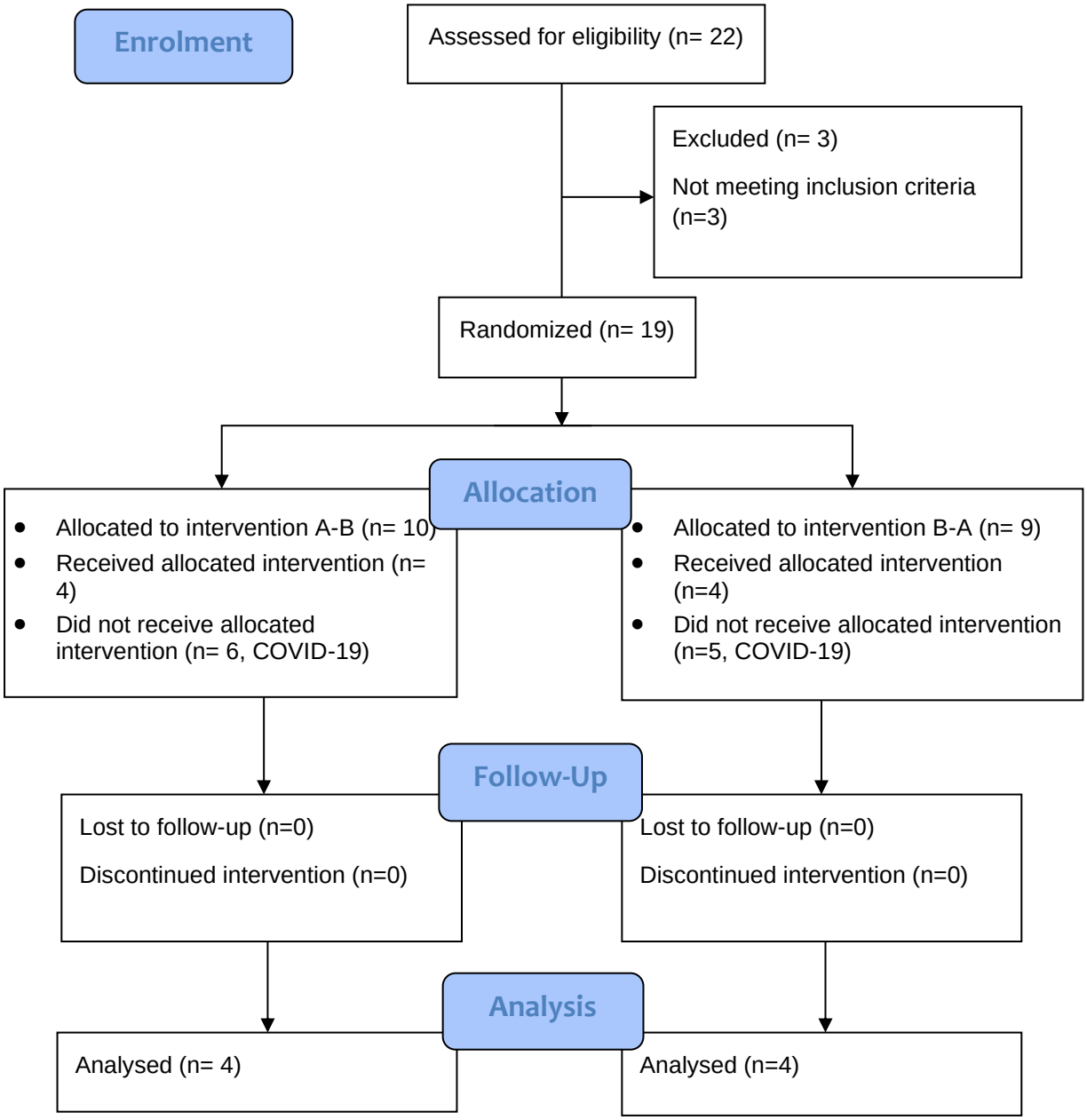


Figure 6.9 CONSORT diagram of the REST study. *Interventions were blinded to the participants and the study team using an allocation concealment as explained in Chapter 6, section 6.7.1.4. At the end of recruitment, breads were grouped as 'A' or 'B' to allow the study team to complete the data analysis. Bread were unblinded once the data analysis was completed.*

Table 6.9 REST study participant characteristics obtained at screening

	Mean	SEM	Min	Max
Age (y)	33	5	23	58
Height (m)	1.73	0.03	1.60	1.90
Weight (kg)	69.8	3.9	58.1	91.9
BMI (kg/m^2)	23.2	0.6	20.7	25.4
Systolic blood pressure (mmHg)	124	4	108	137
Diastolic blood pressure (mmHg)	81	2	69	88
Venous fasting glucose (mmol/L)	4.4	0.1	4.1	4.7
HbA1c (mmol/mol)	34.4	0.8	31.0	36.0
Cholesterol (mmol/L)	4.8	0.3	3.1	6.2
HDLC (mmol/L)	1.5	0.1	0.8	2.1
LDLC (mmol/L)	2.9	0.3	1.6	3.8
Triacylglycerol (mmol/L)	0.8	0.1	0.6	1.5
Total bilirubin (mmol/L)	19.4	2.9	9.0	30.0
Total protein (g/L)	76.0	1.3	70.0	79.0
Albumin (g/L)	44.0	1.1	41.0	51.0
Globulin (g/L)	32.0	1.3	28.0	35.0
Alkaline Phosphatase (U/L)	66.3	4.4	50.0	90.0
Alanine aminotransferase (U/L)	21.8	4.0	11.0	46.0
Sodium (mmol/L)	138.1	0.4	137.0	140.0
Potassium (mmol/L)	4.6	0.1	4.2	5.1
Urea (mmol/L)	4.3	0.4	2.6	6.7
Creatinine (µmol/L)	74.5	5.2	50.0	96.0
Average of three days dietary intake (per day)				
Energy (kJ)	9996.8	1142.6	5819.9	15221.1
Energy (kcal)	2384.7	272.6	1390.75	3631.11
Protein (g)	94.3	10.4	56.5	138.2
Carbohydrates (g)	250.9	33.1	118	365.5
of which sugars (g	86.9	15.0	28.3	145.5
Fat (g)	104.1	11.1	69.1	163.2
of which saturates (g)	32.2	4.5	16.4	48.7

N = 8 of which 4 males and 4 females. Dietary intake was recorded using the *Libro, 2019* mobile application software. Intake data was analysed using the *Nutritics (2019)* Computer Software, Research Edition (v5.09)

6.4.5 REST post-prandial glycaemic responses

Post-prandial blood glucose fluctuations are linked to the digestion rate of carbohydrate-based foods and the release and absorption of digestion products (i.e., glucose). In the REST study, the post-prandial glucose responses of eight subjects were measured after consuming one *sbeII* bread roll, with lower susceptibility to hydrolysis *in vitro* than the WT control, and one WT control bread roll.

6.4.5.1 Capillary glucose

Participants' fasting glucose values were within normal range (5.075 ± 0.08 mmol/L/min, mean ± SEM, n = 8) and their fasting glucose values did not vary significantly between the two visits (paired t-test, p-value = 0.5). The source data (including the baseline values) of capillary and interstitial glucose concentrations are reported in

Table A6 **2**, Table A6 3 and Table A6 4, Appendix A.

There were no significant differences between the postprandial glucose response after consuming *sbeII* bread and WT control, the glucose response $iAUC_{0\text{-}210}$ was 151.51 mmol/L/min ± 22.08 mmol/L/min after consuming *sbeII* bread and 181.48 ± 14.42 after the WT control (mean and SEMs, n = 8, paired t-test = 1.38 mmol/L/min, p-value = 0.2). Figure 6.10b shows the individual capillary glucose responses ($iAUC_{0\text{-}120}$). Other postprandial capillary glucose indicators are reported in Table 6.10, even though no significant differences were found.

Post-prandial glucose followed a similar pattern after the consumption of *sbeII* bread and WT control (Figure 6.10a); there was a similar number of glucose peaks of decreasing height after 30, 120- and 180-minutes. After an initial sharp increase in capillary glucose, the glucose concentration in response to *sbeII* bread dropped earlier than the WT control. The glucose concentrations returned to the baseline within 200 minutes from the consumption of both bread types.

6.4.5.2 Interstitial fluid glucose

The fasting glucose values measured in interstitial fluid were within normal range and did not vary significantly between visits (4.70 ± 0.09 mmol/L/min, mean ± SEM, n = 8, paired t-test, p-value = 0.47). There were no significant differences between the postprandial glucose response after consuming *sbeII* bread and WT control ($iAUC_{0\text{-}240}$, paired t-test = 2.05 mmol/L/min, p-

value = 0.0783) however, there was a tendency to a lower interstitial glucose response within 120 minutes of consuming *sbeII* bread, compared to the WT control bread, ($iAUC_{0\text{-}120}$, paired t-test = 2.33 mmol/L/min, p-value = 0.0519). Figure 6.10d shows the individual interstitial glucose responses ($iAUC_{0\text{-}120}$). Other postprandial capillary glucose indicators are reported in Table 6.10; values are reported as the average of two CGM sensors readings per participant or as pooled mean of the eight participants that completed the study. The glucose response measure in interstitial fluid followed a similar pattern to the response measured in capillary blood glucose (Figure 6.10c). Both glucose curves showed two distinct peaks within the first 120 minutes, followed by a slow decrease of glucose level. The pooled glucose concentration returned to the fasted baseline within approximately 180 minutes, after consuming the WT control bread, while *sbeII* bread appeared to elicit a late rise in interstitial glucose concentration at 180 minutes, before returning to the baseline value. There was no difference in dip value between *sbeII* and WT control bread.

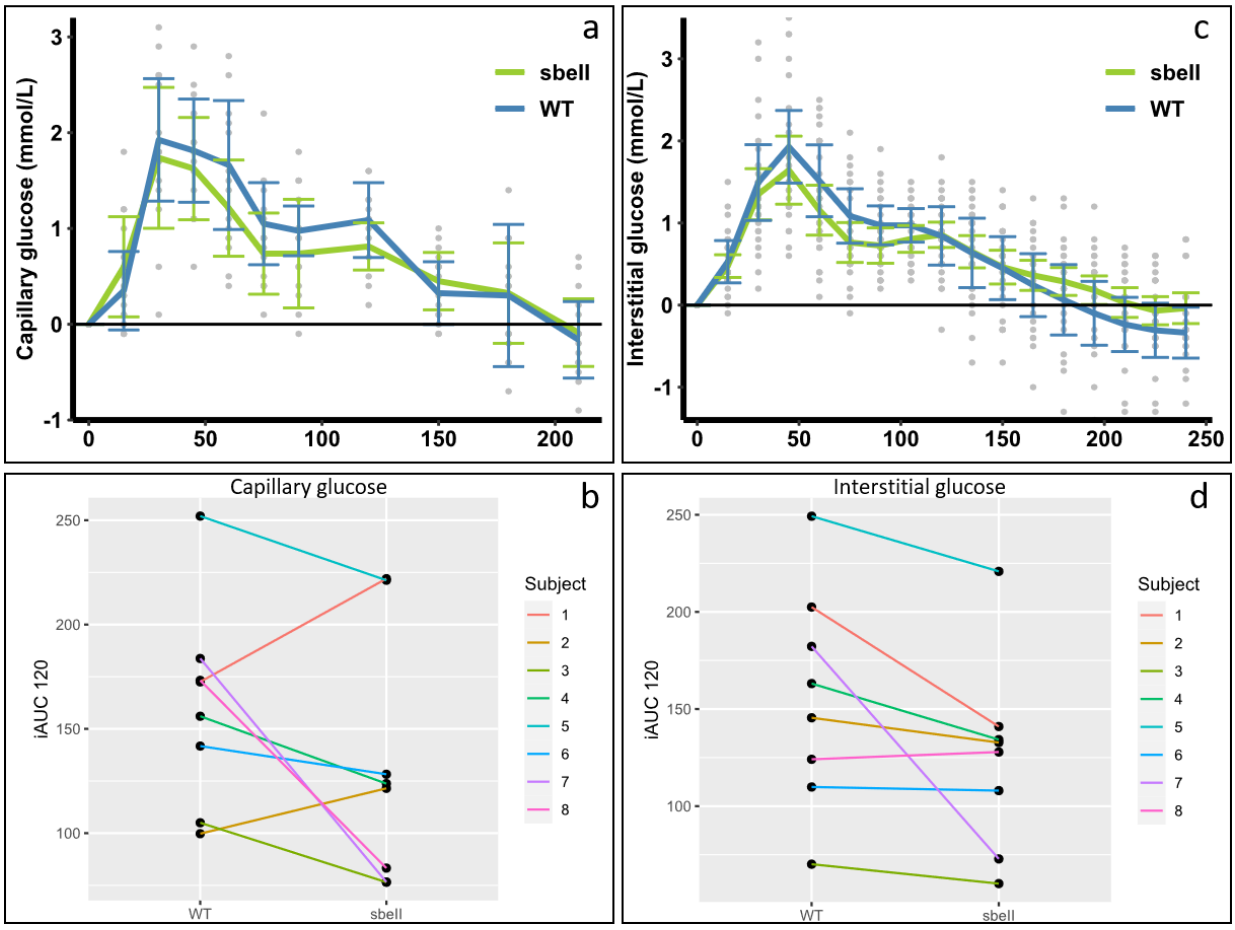


***Figure 6.10 a. Post-prandial capillary glucose concentrations 0 to 210 minutes**; datapoints represent individual glucose values, curves are pooled glucose profile n = 8 and error bars are 95% CI. b. Individual incremental capillary glucose ($iAUC_{0\text{-}120}$). c. Post-prandial interstitial glucose concentrations 0 to 240 minutes; datapoints represent individual glucose values, curves are pooled*

glucose profile of 2 CGM sensors reading from n = 8 and error bars are 95% CI. d. Individual incremental interstitial glucose ($iAUC_{0-120}$).

Table 6.10 Indicators of glycaemic response to intervention meals measured for capillary and interstitial glucose.

Meals	Method	$iAUC_{0-120}$ (mmol/L /min)	Glucose Peak (mmol/L)	Glucose Dip (mmol/L)	Time to peak (min)
sbeII bread	Capillary glucose	131. 63 ± 21.0	7.10 ± 0.2	4.93 ± 0.1	39.3 ± 3.9
	Interstitial fluid	124.71 ± 17.3	6.43 ± 0.3	4.48 ± 0.1	53.0 ± 2.8
WT control bread	Capillary glucose	160.51 ± 17.1	7.30 ± 0.2	4.81 ± 0.1	40.0 ± 6.3
	Interstitial fluid	155.84 ± 19.9	6.68 ± 0.4	4.19 ± 0.2	49.3 ± 1.9

Values are reported as Mean ± SEM, n = 8. 'Peak' and 'Dip' are the maximum and minimum glucose concentration achieved after consuming the meal (capillary glucose 15-210 minutes, interstitial glucose 15- 240 minutes). Time to peak of capillary response to WT bread n=6, 2 were excluded.

6.4.5.3 CGMs: reliability of measurement for intervention studies

Time to peak measure showed that the interstitial fluid measurements lagged for approximately nine minutes for responses to WT control bread and 13 minutes for responses to *sbeII* bread, Table 6.10. Two participants (participant 2 and 3) had their capillary glucose peak (maximum glucose concentration) at 180 and 120 minutes so they were excluded from the time to peak calculation.

Glucose responses in interstitial fluid were measured using two CGM sensors per participant. Each participant wore the two sensors for all intervention visits on the same arm; all sensors were the same model, manufactured by Abbot. Two participants lost one sensor after intervention 1 and completed intervention 2 with one sensor only. Other two participants lost one sensor before intervention 1 and had it replaced. A Bland-Altman plot obtained from the interstitial glucose values showed agreement between measurements obtained with sensor A and B, for each participant (n = 6), Figure 6.11a. The plot shows an increasing difference between the measurements with increasing level of circulating glucose, particularly after consuming WT bread, Figure 6.11 b,c. Concordance between measurements from sensor A and B was strong (Kendall's *W* = 0.813) regardless of the intervention meal consumed. Concordance between sensors varied when comparing measurements between intervention visits: sensors measurements after consuming WT bread were in greater concordance than after consuming *sbeII* bread (Kendall's *W* = 0.85 and *W* = 0.78, respectively), particularly for lower glucose concentrations. Concordance (Bland-Altman and Kendall's *W* coefficient) was determined using interpolated values to overcome any lag in glucose response between the measures.

The Bland-Altman plot (Figure 6.11d) and the Kendall's coefficient showed a positive relationship between glucose concentrations measured in interstitial and capillary glucose however, Kendall's correlation coefficient indicated a moderate concordance (*W* = 0.58), possibly due to the limited number of participants in the study or some background noise in capillary glucose measurements. Agreement between measurements was tested after excluding the measurements of one participant (participant 4) with outlying values (Figure A6 3, Appendix A). Visual assessment of the interpolated capillary and interstitial glucose values showed glucose curves mostly comparable in shape but not matching absolute values. Curves appeared shifted because of different baseline glucose values (Figure A6 2, Appendix A).

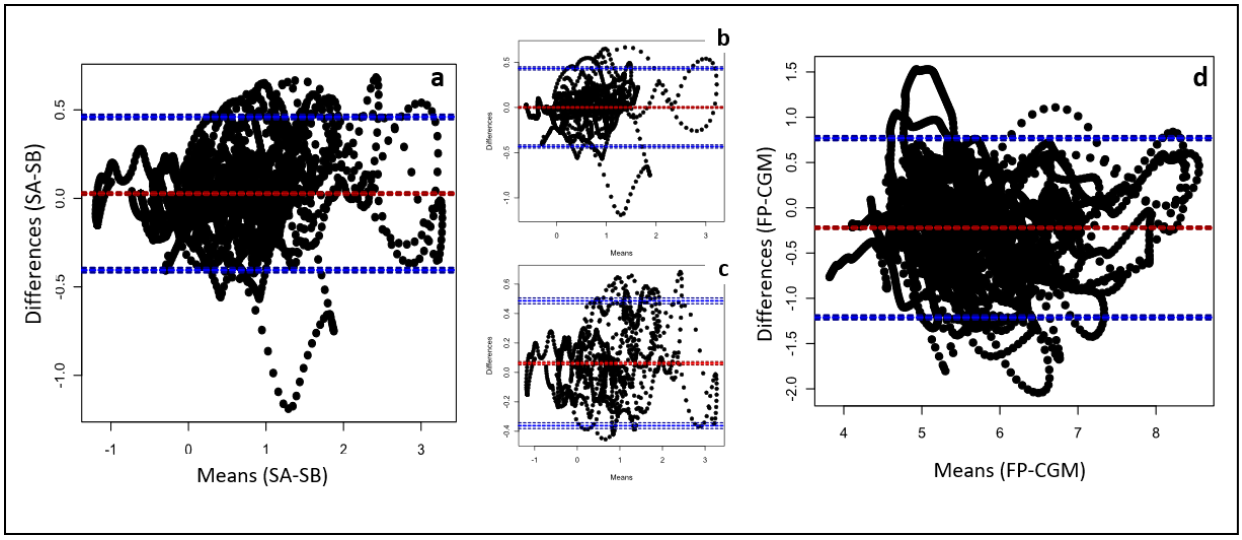


Figure 6.11 Bland-Altman plots *showing the difference between the two measures (y-axis) against the mean of the two measures (x-axis). Plots show the mean difference (dotted red line) and the 95% CI (dotted blue lines) a. Glucose concentrations differences measured in interstitial fluid by sensor A and sensor B across intervention visits. b,c. Glucose concentrations differences measured in interstitial fluid consumption of sbeII bread (b) and WT bread (c). Glucose concentrations differences measured in interstitial fluid by CGM and in capillary glucose measured by Finger-prick test (FP).*

6.4.6 REST Satiety and energy intake

Four hours after the intervention breakfast, participants were administered an *ad libitum* lunch. Energy (kcal) and water (ml) intakes were measured, to compare the intake after consuming *sbeII* bread to the intake after consuming WT control bread. There were no differences in intake between the two intervention visits. Energy intake was 1131.04 ± 154 kcal after consuming *sbeII* bread and 1076.72 ± 130 kcal after consuming WT control bread (mean ± SEM, n=8, paired t-test= -0.084, p-value = 0.4). Water intake did not differ during the *ad libitum* lunch following the two intervention meals (paired t-test= -0.025, p-value = 0.9).

Satiety VAS questionnaires were used to measure satiety changes after each intervention meal. Participants consumed the test intervention with *sbeII* bread in approximately 10 minutes while it took them approximately eight minutes to consume the WT control bread meal.

There were no significant differences in 'desire to eat' or 'hunger' after consuming the *sbeII* bread compared to the WT control bread, when comparing individual timepoints. There is some evidence of increased 'fullness' up to 30 minutes after consuming *sbeII* bread (paired t-test = -3.4016, p-value = 0.01). The increased 'fullness' appeared to be temporary and did not seem to affect the following meal (lunch) consumed based on the energy intake measured at lunch. Participants indicated similar hunger, desire to eat and feeling full at lunch time, Figure 6.12.

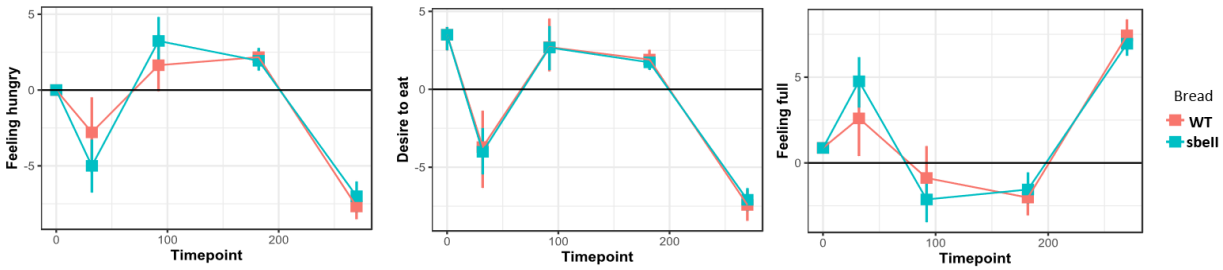


Figure 6.12 VAS scores for satiety indicators; *Feeling hungry, Desire to eat, Feeling full. Mean and CI, n= 8.*

6.4.7 REST sensory analysis

The acceptability test did not reveal major differences in bread liking. The *sbeII* bread scores for overall liking, aroma, flavour, sweetness, moistness and size were above neutral, as the WT control. For texture, *sbeII* bread scored lower than the WT control but 'acceptable' (Figure A6 4, Appendix A).

JAR assessment of the *sbeII* bread suggested that moistness and texture (dryness, doughy) were not JAR as the penalty scores for these levels were above 1 according to more than 50% of the study participants. This may require an adjustment of the formulation for future studies.

6.5 Discussion

The REST study was designed to study the effect of *sbeII* bread consumption on post-prandial glucose response in healthy individuals and to explore precursory effects of the novel *sbeII* bread on satiety and energy intake. The test breads were designed to deliver a similar amount of TS but with different starch properties, which were investigated in this study providing a good starch characterisation. In order to deliver similar TS content, *sbeII* breads were approximately 6 g larger than the WT control (a portion of bread was approximately 150 g).

6.5.1 Starch characteristics

The TS and RS content of breads was measured using a modified version of the Megazyme TS assay kit. This method was modified to allow more samples to be tested per assay run, with less sample material and less reagents. It also required an added step, compared to the Megazyme kit, that usually results in more starch to be solubilised and detected than when measured using the Megazyme kit. While this approach appeared to be more appropriate to measure the TS of the bread rolls produced, when used to measure RS on flour, the results were not always comparable with measurements from the Megazyme assay kit (Figure A6 5, Appendix A), nor with the RS flour content measured by other research groups.[11, 12] Measurement of RS presents several challenges. The definition of RS reflects resistance to

digestion in physiological conditions that are not easily replicated *in vitro*. The use of purified porcine pancreatic α-amylase in a prolonged digestion period (16 hours) may not accurately mimic conditions of human digestion.[358] This is a lengthy method with several opportunities for human error (such as when decanting supernatants), which may be more reliable if used in combination with other measurements of starch susceptibility to hydrolysis based on the Englyst method[158] described in chapter 2 (amylolysis assay).

6.5.2 REST bread development

Starch in *sbeII* bread rolls produced with the straight-dough method were characterised by a greater RS and lower susceptibility to amylolysis *in vitro* than the WT control. The breads (*sbeII* and WT control) were similar in size and appearance making them suitable for a double-blind cross-over trial. The *sbeII* breads were characterised by higher browning index, usually linked to high concentration of sugars and proteins contributing to the Maillard reaction.[271] The reducing sugars detected by *in vitro* amylolysis and the sugar content of bread measured by proximate analysis in *sbeII* and WT control breads were comparable. The increase in browning could also be linked to increased water absorption and damaged starch as these characteristics are known to affect dough stickiness and colour development during baking.[267] Future experiments could include texture analysis of bread dough as well as starch damage to explore other factors contributing to end-use quality characteristics of *sbeII* breads.

Bread rolls produced by sponge method resulted in *sbeII* breads with lower starch susceptibility to amylolysis compared to the WT control, where the extent of *sbeII* starch hydrolysis was comparable to that of starch in *sbeII* breads made with straight-dough method. Sponge bread rolls were characterised by a greater concentration of endogenous sugar (Y0) compared to straight-dough rolls. In both *sbeII* and WT control sponge breads, 15% of the total reducing sugars was detected at the beginning of the incubation compared to only 5% detected in straight-dough method breads. This is consistent with the relatively higher reaction rate observed in sponge breads compared to straight-dough breads. It is possible that the extended fermentation of the sponge dough may have resulted in a larger concentration of reducing sugars.

Typically, the sponge dough is subjected to double mixing and extended fermentation resulting in bread with lighter crumb texture, common in sourdough fermentation. It is possible that the extensive mixing led to higher starch gelatinisation or the extended fermentation favoured starch degradation and higher endogenous sugars concentrations in sponge breads. It should

be noted that the TS per serving in *sbeII* and WT control bread did not match exactly in breads prepared during the development phase. This could have biased the *in vitro* analyses of starch susceptibility to amylolysis. The straight-dough method initially produced breads of irregular colour and shape, so the method was modified extending the proofing time. This resulted in well-shaped and uniformly coloured breads. This was an important achievement as a regular crust helps protect the crumb from excessive moisture losses during storage as well as allowing for double blinding of the intervention and control breads.[351] The straight-dough method was considered suitable to produce breads for the REST *in vivo* study. The extent of starch hydrolysis in straight-dough breads was comparable with amylolysis measured on breads produced by Campden BRI using the Chorleywood method (chapter 4). The straight-dough and Chorleywood method rely on the same process. In this study, the only difference with the method described in chapter 4 was the degree of manual handling. Breads produced at QIB (described in this chapter) were shaped by hand and baked as rolls (no mould) while at Campden BRI, breads were shaped using a conical moulder and baked in tins.

The bread formulation was based on the flour TS content measured with the Megazyme assay kit, described in chapter 2, to match *sbeII* and WT control breads on their starch content. It is estimated that both bread types delivered approximately 74.9 g of TS. The available carbohydrates measured by difference by ALS (reported in Table 6.7) showed a ~5 g difference between *sbeII* and WT control breads. This is consistent with the starch and sugar content once RS is excluded (measured as a "fibre" by the AOAC fibre method used). The total starch content of the breads was similar but 5% of the starch in *sbeII* bread was RS while only 1.9% of the starch in the WT control bread was RS. Since the aim of this study was to measure changes in glucose response due to the starch characteristics of *sbeII* breads, it was decided to match breads on the TS content rather than the available carbohydrates.

6.5.3 Starch amylolysis *in vitro*

Starch susceptibility to hydrolysis was measured on breads after one freeze-thaw cycle. Breads were baked and left to cool for 2h before storage -20 °C; during this process some gelatinisation and then some retrogradation occurred. Breads were analysed at the end of the REST study recruitment phase, after five months of storage. It is known that temperature and length of storage can affect the extent of starch retrogradation and its susceptibility to hydrolysis by digestive enzymes.[359] After five months storage, the difference in starch amylolysis between *sbeII* and WT control breads was consistent with that of breads analysed

during the bread development phase. Overall, the C_{90} values for both breads were higher after storage however, this could be due to a number of factors, including variability in batch baking or water loss during prolong storage. As long-term storage can have detrimental effect on bread quality[360] and on starch characteristics, it was decided to store bread for a maximum of six months to be used in the REST study.

6.5.4 Glucose response to *sbeII* bread

Based on the results collected from 8 participants, the REST study did not show significant differences in glucose response following consumption of *sbeII* bread, compared to the WT control bread. Based on the interstitial fluid measurements, there is some indication that the *sbeII* bread could elicit a lower glucose response than the WT control. The mean glucose response (iAUC) to *sbeII* and WT control breads is comparable to the mean glucose response (iAUC) to *sbeII* an WT control pudding tested in the GIPIRS pilot study (chapter 5) and effect size measured in the REST study matches the 20% difference estimated with the power calculation.

Measuring glucose in IF by CGM was found to be an effective and minimally invasive method to carry out intervention studies. Based on the follow up questionnaire completed by the study participants, the experience of wearing two sensors was described as “quick” and “easy”, some participants found the application slightly painful “like a jab” however, most described them “comfortable” to wear and “interesting”.

Measurements from the two sensors applied returned comparable glucose readings. When comparing sensors within participants, it was noticed that most glucose responses aligned even though values had an offset of 0.5-2 mmol/L. This difference was adjusted by correcting for the fasted glucose baseline. When comparing participants sensor readings within intervention days, it was noticed that the glucose measurements concordance between sensors was marginally lower after participants consumed *sbeII* bread, compared to the measurement’s concordance after consuming WT control bread. A number of factors could have affected the readings including glucose day-to-day variation, malfunction or damage to one of the two sensors or local inflammation at the insertion site. The accuracy of the readings could also depend on the extent of the glucose response and the type of meal.

In individuals with diabetes, especially type 1 diabetes, the differences in glucose concentrations between plasma and IF are increased as glucose concentrations become larger: the faster the blood glucose changes, the longer the lagging time is, leading to increased

differences and worse correlations between blood glucose and IF glucose. [124, 361] This seemed to be the case for normo-glycaemic subjects too. The differences in glucose concentrations between interstitial fluid glucose and capillary blood glucose were small particularly with small to moderate glucose fluctuations. Measurement of insulin concentrations could provide useful information regarding the primary cause of the lag time, which could be device related as well as tissue-specific (insulin dependent).

When comparing the glucose values obtained from the finger prick test to those measured with CGM sensors, there was a trend for a positive difference between the measures, particularly in the presence of greater glucose concentrations, possibly because of individual differences in the peak response to foods. The measurements were moderately in agreement, as shown in a study by Dye *et al.* (2010)[120]. The Kendall coefficient calculated based on the REST study measurements was lower than when measured by Dye and colleagues however, this is one of the first food intervention studies using CGMs sensors to measure glucose response in healthy volunteers. CGM sensors were developed to measure glucose levels in individuals with diabetes, normally characterised by rapid fluctuations between low and high values. Little is known on how sensitive CGM sensors are to measure small glycaemic excursions experienced by healthy subjects. The study by Dye *et al.* (2010)[120] used a glucose drink to test the capillary and IF glucose measurements agreement that likely to elicit a much different response compared to white bread, possibly higher.

The use of CGMs sensors in clinical trials certainly has some advantages: sensors measurements are reliably taken every 15 minutes (this may vary depending on the type of sensor), allowing studies to collect larger glucose datasets with higher resolution, compared to capillary glucose measured by finger-prick. A finger-prick test allows for a limited number of data points to be collected and requires changing the pricking site; this may reduce consistency of the values measured. CGM systems measure IF glucose continuously and consistently since glucose is measured repeatedly at the same site.

As small as the REST study cohort was, a borderline difference in IF glucose levels after consumption of *sbeII* and WT control breads was measured, possibly because of the higher accuracy of the measurements collected using CGM sensors. By observing the individuals' glucose profiles, a different number of post-prandial glucose peaks and dips was noted. The peaks and particularly the dips did not seem to depend on the type of bread consumed as,

when present, they were present for both bread types, often of different height or depth. This could suggest that the number of glucose peaks and dips could be person specific rather than meal specific. The number and extent of these fluctuations could be an important outcome for future intervention studies. Results from a recently published intervention study (PREDICT) by Berry *et al*. (2020) showed that glucose dips below the fasted baseline are correlated to an increase in hunger and energy intake.[362] They report that, after a standardised breakfast, a large glucose dip was usually followed by greater hunger, a high energy intake at the following meal and a subsequent high glucose peak. Based on their observations, they concluded that the glucose dips occur in approximately 40% of healthy individuals. Individuals prone to 'dip' could respond differently to foods rich in available carbohydrates compared to individuals who do not regularly experience glucose dips, contributing to the large variation measured in most glycaemic response studies. Dips are also important to consider when choosing the outcome measure for glycaemic intervention studies. Normally, glycaemic responses to food are compared based on the iAUC, calculated based on the values above the fasted baseline; this calculation excludes the dip. The presence of dips should instead be considered as this may explain some of the inter-individual variation in glucose response observed when comparing the iAUC. In light of this, other outcome measures could be considered for future glycaemic studies, such as peaks and dips or time to peak and time to dip, to provide a good characterisation of the response. Besides the dietary intake of participants, degree of exercise and sleep patterns seem to be also important factors defining the metabolic response to foods;[327] these were not investigated in the REST study but may be incorporated in future study protocols. In the REST study, exercise was controlled by asking participants to avoid any intense exercise the day before a study visit and prior the morning before a study visit.

A secondary aim of the REST study was to investigate the effect of *sbeII* bread on satiety. Participants indicated a stronger feeling of fullness after consuming *sbeII* bread, even though this remains a speculation as the study sample size was very limited. Most satiety related-research studies usually recruit a minimum of 20–25 subjects to capture a 10% difference in appetite ratings between foods.[363] It should be noted that the sensory analysis and *in vitro* texture analysis suggest an increased hardness of *sbeII* bread; participants required longer time to consume *sbeII* bread compared to the WT control. A harder bread that requires to be chewed for longer may have a strong effect on satiation and early satiety. [364] The need to masticate hard or viscous foods increases the time spent in the oral cavity (oro-sensory

exposure time), prompting sensory signalling and therefore, satiation[365]. An extended oro-sensory exposure may also trigger satiety responses as more saliva will be swallowed during consumption, leading to greater volumes entering the stomach. Furthermore, it is thought that "chewy" or "thick/dry" foods may generate expectations that these foods will be filling, which may also suppress hunger after consumption.[366, 367] Participants did not report changes in hunger after consuming *sbeII* bread compared to the WT control. While the *sbeII* and WT control breads differed in some of the texture attributes observed and experimentally measured, these appeared to be subtle differences and are not likely to be a confounder in the study results.

6.6 Conclusions

Obtaining a glucose response reduction after consumption of high-fibre or high-amylose bread has been the objective of many research studies. Poor glycaemic control over time can lead to an impaired glucose metabolism and increased risk of developing diabetes. For this reason, it is of interest to produce commonly consumed wheat-based food products, such as bread, incorporating novel starches and fibres in white flour. It was hypothesised that the use of *sbeII* wheat flour with higher amylose content and RS compared to conventional (WT) white flour in foods could reduce the post-prandial glucose levels of healthy subjects, based on *in vitro* data. The REST study was designed as a highly controlled acute intervention study aimed to measure changes in glucose response after consuming *sbeII* bread with lower starch susceptibility to hydrolysis than the WT control. Unfortunately, due to the COVID-19 outbreak, the study was terminated early with only 8 subjects completing all study visits, and did not find a significant difference between breads, possibly due to lack of statistical power. Successful outcomes of the study were the comparison of CGM and fingerprick glucose values that showed good agreement between measurements and the comparison of measurements from multiple *Freestyle* CGM sensors, which showed good reproducibility of the data obtained from different sensors of the same manufacturing company.

The REST study also provided a characterisation of the starch in *sbeII* flour and bread together with a controlled nutrient composition of the breads used in the intervention study, which is not always reported. This is one of the first studies using *sbeII* wheat-based products to measure the effect of *sbeII* starch on glycaemic response in healthy individuals. Only one other study (published to date) reported on the post-prandial response to *sbeII* bread consumption measured *in vivo* but the lack of a detailed bread characterisation does not allow for a direct comparison.[147]

Future *in vivo* studies should aim to achieve a sufficient power to answer the research questions. Based on the results obtained from this partially completed study, research should also focus on identifying and clustering volunteers based on their metabolic type. This could allow future studies to be more specific in identifying the beneficial effects of different formulations of carbohydrates-based foods, as a personalised approach to glycaemic response.

Chapter 7 General conclusions and future directions

The project presented in this thesis aimed to study the factors contributing to the resistance of wheat starch to hydrolysis, and the impact of starchy foods with low susceptibility to hydrolysis on postprandial glucose metabolism. The approach used was a combination of *in vitro* and *in vivo* studies to investigate how starch susceptibility to hydrolysis is modulated by intrinsic and extrinsic factors in wheat-based foods. These included studies of the starch characteristics on isolated starch and starch within different food structures. To study the intrinsic factors contributing to susceptibility to enzymatic hydrolysis, starch from *sbeII* wheat was used because of its altered starch structure, characterised by an increase in amylose content in the grain compared to a WT control wheat. The same wheat materials were used to study the effect of extrinsic factors related to processing on the formation of resistant forms of starch in high and low amylose wheat.

Instead of increasing the amylose content of starch-based foods by post-extraction chemical or physical starch modifications, the high amylose content that characterised *sbeII* wheat was achieved by previous research through modulation of starch synthesis during grain development, as described in previous studies.[11, 12] The increased amylose proportion in *sbeII* starch was previously achieved by non-transgenic downregulation of genes encoding SBEII, required for the formation of starch branched structures that form the crystalline regions of the starch granule. Foods containing starch with a higher proportion of amylose are thought to elicit lower glycaemic responses, yet the extent of starch digestibility greatly depends on the processing applied to the raw starch and its physicochemical properties. It was hypothesised that the susceptibility to amylolysis of *sbeII* starch would be lower than the WT control starch when processed into foods. It was anticipated that the lower rate and extent of *in vitro* starch amylolysis of *sbeII* wheat-based foods would translate to a lower rate and/or extent of starch digested and absorbed post-meal, measured *in vivo*.

Overall, the results from these studies have provided evidence that small increments in the amylose proportion of starch can result in foods of lower digestibility, depending on the type of processing used. The comparison of different processing methods provided additional understanding of the mechanisms affecting starch digestibility and their relation to starch intrinsic properties.

7.1 Factors influencing starch digestibility in wheat

7.1.1 The role of intrinsic starch characteristics on starch digestibility

The *sbeII* wheat used in this project is known to be characterised by starch with an increase in amylose and RS content. The presence of combined mutations in *sbeII* genes was shown by other authors to cause structural changes in starch branching patterns, resulting in altered starch properties.[48] The *sbeII* starch is characterised by an increased proportion of linear structures that can be measured analytically as 'amylose'; this contributes to the altered crystallinity of *sbeII* starch granules, as visible in the irregular granule morphology, loss of birefringence pattern and ultimately, increase in RS.

A characteristic of *sbeII* starch that had not been previously investigated is the proportion of A and B-type starch granules. These are formed at different stages of grain development;[14] they differ in size and in molecular composition which could confer different properties to the starch. While it is generally accepted that A and B starch granules are digested at a different rate, it is unclear whether their susceptibility to hydrolysis is due primarily to the different surface-area exposed to digestive enzymes or their composition. Nevertheless, a change in the ratio of granules type could contribute to altering starch digestibility.

The presence of a lower proportion of the smaller B granules in *sbeII* starch compared to the WT control starch became evident during the analysis of starch particle size by laser diffraction. The *sbeII* starch showed a lower proportion of B granules compared to the WT control. This result was consistent in isolated starch from two consecutive bread wheat field trials. This suggested that a low B-granule content is a characteristic of *sbeII* wheat and that it could potentially contribute to the altered *sbeII* starch digestibility measured *in vitro*. To investigate this further, the proportion of A and B granules was measured using the electro sensing zone method on a Coulter Counter Multisizer4e. This analysis showed that *sbeII* starch was indeed characterised by a lower B granule proportions compared to the WT control. However, the proportion of B granules in *sbeII* starch was still within the range indicated for other wheat starches.[224] This suggested that the altered ratio of A and B granules in *sbeII* starch may be a contributing factor to starch resistance to digestion but not the main determinant.

Based on this analysis, it was of interest to understand whether the A and B granules of *sbeII* wheat starch differed in starch physicochemical properties. In WT starches, A and B granule

differ in the architecture of the glucans chains and B granules are likely to be more susceptible to the activity of amylolytic enzymes because of the larger surface-area to volume ratio available for enzyme binding.[192, 198] Measuring granule properties can be challenging as granules need to be separated before proceeding with any further analyses. An outcome of this study was the development of an optimised Percoll separation method based on a method previously published by Peng *et al.* (1999).[194] The method was aimed at separating the two fractions to allow the characterisation of A and B granule properties and susceptibility to hydrolysis. This method was successful in separating the two fractions, although the purity of the fractions could be improved by removing the Percoll silica particles that may interfere with other starch analyses.

Overall, these studies provided a deeper understanding of the starch characteristics of *sbeII* wheat.

As starch is cooked, other factors that influence starch susceptibility to digestion come into play; the processing techniques can alter the starch structure and thereby have major effects on digestibility. Therefore, it was of interest to understand how the starch structural changes would translate when hydrothermally processed, and which resistant form of starch would be predominant in *sbeII* foods. Two main mechanisms were investigated: 1) the effect of temperature change on starch in hydrated milled wheat and 2) the role of water during thermal processing of milled wheat. Evidence supporting each of these two mechanisms is reported in the two sections below.

7.1.2 The effect of thermal processes on *sbeII* starch susceptibility to hydrolysis

To explore the effect of heat process on *sbeII* starch digestibility, a range of *sbeII* wheat-based products were developed with different hydrothermal processes. A semolina porridge and a semolina pudding were used to study starch characteristics in excess of water following heating and cooling.

Semolina porridge was a high moisture food where starch was cooked briefly at 90 °C. Similarly, semolina pudding was obtained by cooking briefly semolina in excess of water followed by a cooling period. These two products contained the same ratio of starch and water but resulted in very different products after thermal processing; semolina porridge had a paste-like consistency while semolina pudding had a thick gel texture.

The *sbeII* starch in semolina porridge was hydrolysed by amylolytic enzymes at a similar rate and to a similar extent as the starch in WT porridge, it and contained similar levels of RS. Because of the high moisture content of the porridge but short cooking time, part of the starch in porridge gelatinised resulting in up to 60% being digested *in vitro*. Because of the relatively small difference in amylose concentration between *sbeII* and WT wheat semolina described in this study, there were no differences in the extent of starch amylolysis between *sbeII* porridge and WT control porridge, likely due to a similar degree of gelatinisation.

On the other hand, when porridge was left to cool for several hours, starch susceptibility to amylolysis drastically decreased compared to that of starch in semolina porridge suggesting that some retrogradation occurred for both *sbeII* and WT control starches. The importance of starch retrogradation became evident during the initial *in vitro* amylolysis experiments on cooked semolina. Here, no significant differences were observed between *sbeII* and WT control semolina porridge whereas *sbeII* semolina pudding, cooled for two hours, showed a lower rate of amylolysis relative to WT semolina pudding but no difference in the overall extent of starch amylolysis. In the following experiment, the semolina pudding was left to retrograde for 16 hours, as previous studies have shown that this time can reduce the rate of hydrolysis by amylolytic enzymes of 20% due to amylose retrogradation.[249] After 16 hours, the starch in *sbeII* pudding showed a significantly lower susceptibility to hydrolysis than the WT control.

Therefore, it was concluded that the processing temperature and duration have a great influence on the starch susceptibility to hydrolysis when starch is in excess of water. In the case of *sbeII* wheat, starch susceptibility to hydrolysis was greatly reduced, compared to the WT control, with starch retrogradation during cooling/storage.

7.1.3 The importance of water in the formation of resistant forms of starch during thermal processing

To further understand the processing performance of *sbeII* wheat starch, other food products with varied water content were produced to study the effect of baking on starch susceptibility to hydrolysis.

Bread wheat was used to produce white bread and crackers, baked at a high temperature (>200 °C). The baking time was based on the size of the products and their water content,

smaller crackers baked for approximately half the time (eight minutes) compared to larger bread loaves (20 minutes).

Bread is made from a high moisture dough (> 50% water) that is thermally processed at high temperature favouring starch gelatinisation. While starch hydrolysis of freshly baked bread was not investigated in this study, based on the experiments involving porridge and pudding, it was expected that *sbeII* hydrated starch granules within the bread dough would gelatinise with the high baking temperature. Freshly baked bread is characterised by a very limited shelf-life that does not suit the logistics of an experimental or clinical setting, therefore all bread was tested following a period of storage during which, some staling occurred as starch retrograded.

Like semolina pudding, *sbeII* bread showed a consistently lower starch susceptibility to amylolysis upon cooling, particularly after storage at -20 °C. Breads were stored for several days before analysis during which time, not only amylose but also amylopectin may have retrograded. While both bread types, *sbeII* and WT control, retrograded during the long storage, *sbeII* bread maintained a lower starch susceptibility to hydrolysis compared to the WT control bread.

On the contrary, in the case of the crackers, when cooked in low moisture at high temperatures, *sbeII* starch amylolysis did not appear to differ from the WT control. Similarly to semolina porridge, even though crackers are low moisture as a dough, starch only partially gelatinised and retrograded due to the limited water available in the cracker dough. Despite the high baking temperatures, there were no differences in starch susceptibility to hydrolysis between *sbeII* crackers and the WT control.

Other factors that could contribute to hydrolysis differences could include the fine structure of *sbeII* starch within a food matrix, the amount of protein and NSP in the dough, the digestive activity of the yeast during dough fermentation (for fermented doughs), and the amount of water bound within the matrix.

Overall, these studies indicated that the amylolysis rate-limiting factor in *sbeII* foods may be starch retrogradation rather than inaccessible or native granular starch, as expected from extruded foods such as pasta, extruded breakfast cereals and extruded snacks. Thus, the lower susceptibility to amylolysis of retrograded *sbeII* starch compared to the WT control measured *in vitro* suggested that a similar behaviour would be observed *in vivo*, translating

into physiologically relevant differences in glycaemic response. The small increase in amylose content observed in raw *sbeII* wheats used in these studies may not result in a high content of resistant forms of starch in freshly consumed foods or low moisture foods. Nonetheless, *sbeII* wheat could be used in the production of low starch amylolysis products that require a storage period, without a major impact on the organoleptic properties, usually associated with high increases in RS.[368]

7.2 Other factors influencing starch digestibility

It has been suggested that under certain conditions (such as high molecular weight and high content) some NSP components, especially the soluble fraction, may influence the viscosity of a food matrix and therefore alter the interaction between digestive enzymes and substrates. The NSP content of wheat used in these studies was within the normal range reported for other milled wheats[205, 231, 242] even though the *sbeII* flour and semolina used in this project had a relatively higher content of NSP than the WT controls. The higher concentration of NSP may contribute to some of the features observed in *sbeII* materials, particularly the increase in water absorption capacity in flour that plays a role in food viscosity.[369] The *sbeII* semolina and flour were characterised by a higher bran percentage than in WT flour, which could explain the marginal increase in NSP content. Observing NSP differences in flour with an equal amount of bran could clarify if the differences are a pleiotropic effect of the *sbeII* mutations or the result of the milling process. The standardisation of the NSP content of *sbeII* and WT control wheats could provide further insight into the role of NSP in the reduction in starch amylolysis of certain *sbeII* foods.

The *sbeII* wheat also resulted in milled semolina and flour of slightly larger particle size compared to the WT control wheat. The *sbeII* wheat grains are known to be characterised by increased hardness[151] that can lead to increased starch damage and larger particle size fractions after milling. Particle size is also a critical factor in digestibility studies[34, 279] therefore, controlling milling efficiency to produce milled fractions of consistent particle size contributes to robust experimental results. Using fractions with different particle sizes can result in biased results, however, the size differences observed in the semolina and flour used in these studies are marginal and unlikely to be a major factor in starch gelatinisation and amylolysis.

7.3 Effect of resistant forms of starch on the glycaemic response: GI and postprandial glucose response to retrograded *sbeII* foods

The dietary intervention studies described in chapter 5 and 6 were designed to measure GI of *sbeII* wheat-based foods and to examine how starchy foods with different susceptibility to hydrolysis would impact the glycaemic response. The glycaemic response represents the amount of starch-hydrolysis products absorbed following digestion in the upper gastrointestinal tract. This can be measured using a standardised methodology and used to calculate an index of glycaemic power (GI) to compare the glycaemic impact of different foods or, from a physiological perspective, to measure the rate and extent of dietary glucose entering the systemic circulation postprandially. Thus, taken together GI and glucose response measurements provided information about the availability to digestive enzymes of retrograded *sbeII* starch and WT control.

Measurements of capillary blood glucose are limited by the number of finger pricks that can be collected per participant. This can be overcome by using a system for continuous glucose monitoring, even though so far, they have been used in a handful of dietary intervention studies involving healthy participants. One strength of the REST study described in chapter 6 was the relatively simple composition of the intervention meals and bread formulation, which was kept consistent across batches, and the *in vitro* characterisation of the material. Similarly, the semolina pudding used in the GIPIRS pilot study in chapter 5 was made using a very simple formulation aimed at controlling possible confounding factors. Most importantly, all *sbeII* and control foods were formulated to contain the same amount of starch so that any changes in glucose response could be attributed to changes in the starch characteristics. All foods were screened *in vitro* using the established amylolysis assay described in chapter 2; the starch susceptibility results gave an indication of the expected glycaemic impact difference between *sbeII* and WT control foods. Even though *sbeII* semolina and bread flour were characterised by just a small increase in amylose relative to WT products and relatively low RS compared to other studies,[39, 48] both *sbeII* semolina pudding and *sbeII* bread showed a lower starch susceptibility to hydrolysis *in vitro* than the respective controls. This did not translate into a significantly lower GI or glycaemic response when measured in capillary glucose *in vivo.* However, a borderline significant difference between *sbeII* and WT control breads was observed when the glucose response was

measured in the interstitial fluid by CGM, possibly because of the higher accuracy and precision of the measurements.

Previous studies have shown a good agreement between measurements taken in blood and interstitial fluid however, the interstitial fluid measurements can vary depending on the insertion site, particularly for sites that are less perfused.[370] In the REST study, there was moderate agreement between capillary blood glucose and interstitial fluid glucose measurements. The Kendal W coefficient calculated for these measurements was lower than expected based on a previous study by Dye *et al.* (2010).[120] This may be due to several factors including the choice and amount of foods, and the mechanisms of glucose delivery and removal during digestion of foods, which may cause different glucose excursions compared to the delivery of pure glucose (as in an oral glucose tolerance test).[371] The REST study had only 8 participants completing the intervention visits because of the COVID-19 outbreak and consequent lockdown period. The lower number of data points collected during the finger prick test and the limited sample size compared to the study by Dye *et al.* (2010)[120] may have contributed to the lower agreement between fingerprick and CGMs measurements observed in the REST study, as discussed in chapter 6.

Interstitial fluid responses are known to lag behind blood glucose responses measured in capillary and venous blood due to physiological fluctuations. The lag can appear wider at higher glucose concentrations or when glucose levels are changing rapidly, particularly after a meal.[372] In the REST study, the interstitial fluid glucose measurements lagged approximately 10 minutes behind capillary blood glucose measures, similar to what was previously reported by Dye *et al.* (2010)[120]. To overcome the lag between measurements, which could produce significant differences in blood glucose concentrations measured at different sites, the measurements were interpolated on a minute-by-minute basis to allow for a direct comparison. By comparing glucose measurements of the two CGM sensors worn by each participant, it was highlighted that a good concordance exists between different sensors of the same brand (Abbott). This has implications for the type of study design; particularly, the number of interventions or study days that future studies could require. Indeed, the sensor duration is currently a limitation as all intervention visits need to be completed within 14 days from the application of the sensors. However, based on the good agreement between sensors measurements observed in the REST study, it may be possible

to use consecutively multiple sensors per individual, to extend the study duration beyond the limit of 2 weeks.

Until recently, the fingertip has been considered the most practical sampling site to measure repeatedly blood glucose during intervention studies. Systems to measure glucose in interstitial fluid are gaining acceptance, particularly for studies aiming to monitor glucose changes outside the clinical setting. To date, the REST study was one of the first studies using a CGM device to monitor the effect of a food intervention on glycaemic response. Based on the results obtained, these devices are likely to replace the finger-prick test in future studies.

Overall, these *in vivo* studies showed that the small increases in amylose content in the *sbeII* wheat raw material can translate into *sbeII* foods with lower starch susceptibility to hydrolysis. However, to detect a significant decrease in the glycaemic response, larger number of participants and more accurate methods to measure glycaemic response are required to overcome some of the variability observed in these studies that may mask the effect of *sbeII* foods on glycaemic response.

7.4 Research impact and applications

The work presented in this thesis contributes to the existing knowledge of starch-based foods effect on postprandial glycaemia. The current nutritional labelling of foods does not provide the detailed characteristics of foods because it is limited to clustering macronutrients in four main categories (protein, fats, carbohydrates/sugars, fibre). The subtle differences in starch composition and characteristics that are not captured by food labels can greatly impact the metabolic response to foods.

Foods with identical macronutrients composition can have very different effects on the metabolic responses because of a spectrum of subtle differences within each macronutrient group. Moreover, manipulating the susceptibility to digestion of starchy foods could complement other nutritional strategies employed in the prevention and management of common chronic diseases, particularly type 2 diabetes. Tailoring the starch composition *in planta*, particularly increasing the amylose content of starch, offers a new route to generate wheat with desirable functional properties and to develop novel wheat-based foods that may elicit low glycaemic responses. A few other studies have shown the potential of such approach; recently Petropoulou *et al.* (2020)[139] showed that the altered starch structure and

increase RS in *rr* mutant pea seeds reduces postprandial glucose excursions and changes the microbiota composition associated with long-term metabolic health benefits. Based on the results presented in this thesis, the *sbeII* wheat could be utilised in the production of wheat-based foods that require a storage period. The small increment in amylose concentration allows production of bread of acceptable quality but with lower susceptibility to digestion than conventional white bread. This suggests that *sbeII* white bread could be used in deli counter foods that require cold storage and do not require re-heating such as sandwich bread.

While a higher proportion of high amylose in the starting raw material could be desirable to obtain further reductions of glycaemic response and GI, other studies have shown that a high intake of amylose can have undesirable side effects such as bloating and flatulence.[309] The careful modulation of the amylose proportion in wheat is paramount to avoid deleterious effects for the plant and grain development (pleiotropic effects on yield, grain size etc.) and for individuals susceptible to the side effects of consuming *sbeII* wheat products (e.g. bloating in IBS).

7.5 Conclusions and future work

The work presented in this thesis opens the door to several new research questions regarding the processing performance of *sbeII* wheat when other processes are applied, particularly regarding the type of product produced. While the straight-dough bread method is commonly used for the mass production of white bread, other artisanal processes that may enhance *sbeII* wheat properties should be investigated. Among others, sourdough bread is becoming a popular product. Sourdough bread making is a popular method particularly for flour with a high bran or fibre content; this process is thought to have a beneficial impact on the starch digestibility of wheat bread as the presence of lactic acid produced during fermentation favours RS formation.[322]

The *sbeII* bread used in the REST study showed that *sbeII* wheat could be used to improve glycaemic control, however, more research is needed on this topic.
The variety of responses to isoglucidic breads observed in the REST study was not surprising; recent studies by Zeevi *et al.* (2015)[327] and by Berry *et al.* (2020)[374] have shown that different individuals can respond differently to the same meals. These authors suggest that dietary intervention could benefit from personalised dietary recommendations to improve

glycaemic control. Zeevi and colleagues have shown that postprandial glucose response to the same meal varied greatly among individuals and similarly, Berry *et al.* found that a number individual-specific factors (from the time of the day when the meal is consumed to gut microbiome composition) can contribute to the postprandial response of a normo-glycaemic individual. This is an important finding considering that the UK dietary recommendations are based mainly on reduction of carbohydrate content, with little to no consideration for the carbohydrate characteristics or the inter-individual variation in glycaemic response.

Thus, future studies could benefit not only from a complete characterisation of the foods used but also from a characterisation of the metabolic type of the individuals consuming them. The physiological factors leading to glycemic variability appear to be heterogeneous, their relationship with specific patterns of glucose response needs to be studied further. Two studies have suggested the need to group normo-glycaemic individuals according to their glucose metabolic type. Halls *et al.*[371] suggested grouping individuals according to specific patterns of glycaemic responses, which the authors called "glucotypes". This categorisation revealed the heterogeneity of phenotypes present within groups of individuals that traditional diagnostic test, used in clinical screening, would group as normo-glycaemic. Berry *et al.*[362] observed the presence of individuals prone to experience glucose drops ('dips') in the post-prandial phase that appeared to be individual specific rather than meal-dependent. The glucose drop was identified as a recurrent characteristic of the early return of hunger, increased calorie intake during a subsequent meal and the overall calorie intake in a day. This relationship was not observed in the REST study possibly because of the limited number of participants completing the study; nevertheless, this seems to be an important observation that future studies aimed at measuring the effect of dietary intervention on satiety and energy intake may need to consider.

As discussed previously, the use of CGM in research is of great interest to monitor glucose changes to dietary interventions. While this technology is very promising, more research is needed on the accuracy of CGMs in normo-glycaemic individuals and on the study methodology when CGMs are used in clinical trials.

It should be considered that many types of CGM system exist and they rely on different methodologies to detect physiological glucose changes. Among the CGMs that measure real-time glucose by enzyme electrode method, differences may exist due to the application

site. A recent study on concordance between different real-time CGM devices highlighted a degree of disagreement between CGMs of different brands with different insertion sites.[373] This comes as no surprise since the devices are usually marked by a MARD[15] score of measurement accuracy, which currently ranges between 9% and 14.5% for CGMs of different manufacturing companies, depending on the model and the application site.[374] Among the CGMs based on an enzyme electrode method of detection, the main factors that determine measurement accuracy seem to be the insertion site and its perfusion. Comparison of measurements obtained from different anatomical sites can be expected to differ since different anatomical locations are characterised by specific glucose kinetics. The lag existing between measurements can be corrected by comparing interpolated measurements, however the overall response could differ when comparing mean glucose concentration across sites. Further work on identifying the correct outcome measure to compare the glucose kinetics of different sampling sites is needed.

Furthermore, the percentage of subcutaneous fat present at the different sampling sites could be contributing to the variation observed in glycaemic response measured by different devices; Howard *et al*.(2020)[373] showed that the mean within-subject discrepancy between the Abbott and Dexcom systems was directly proportional to the percentage of body fat. This is an interesting observation; future studies may use body fat measurements to complement other screening tests to identify metabolic subgroups within a cohort of normo-glycaemic individuals. The strong agreement between interstitial fluid data obtained from different Abbott sensors suggests that these could be used for chronic studies with duration longer than 14 days and outside the clinical setting. Such translation studies will be important to establish the applicability of *sbeII* bread as a nutritional tool for glycaemic control.

Overall, these post-prandial studies demonstrated that the limited susceptibility to digestion of *sbeII* starch after processing, mostly due to amylose retrogradation in cooked products, could attenuate glycaemic responses to starchy foods. The genetic variation in starch characteristics obtained by TILLING provides the opportunity to study the impact of different starch structures on the digestive process. Furthermore, these studies contribute

[15] Mean Absolute Relative Difference (MARD). A measure of average difference between the glucose sensor reading and a blood glucose reading; a low MARD score (<10%) indicates that CGM measurements are close to capillary blood glucose readings, providing a reliable measure of glucose concentrations.

to highlight the relevance of a crop to foods approach to generate foods for improved glycaemic control. Amylose-driven RS can be easily quantified at any stage of the processing chain, as shown in this thesis; this would enable the food industry to easily integrate forms of RS originated by an increase in amylose (because of the native semicrystalline structure or due to amylose retrogradation) into the food labelling system. Combining an improved labelling system for starch-based foods with the clustering of glycaemic response in to glucotypes would allow consumers to make informed decisions about the carbohydrate-based foods consumed, but it would also pave the way to personalised choices to help control glycaemic variability.

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Appendix A

Table A2 1 Bread wheat yield 2017-2018

Sample ID	Genotype	Harvest (kg)	Plot size (m2)	Yield (tons/ha)	Moisture (%)	Date Sown	Plot ID
1/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	1.96	6	3.266667	-	06/04/2018	FT18BH00026
2/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	2.32	6	3.866667	-	06/04/2018	FT18BH00029
3/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	2.36	6	3.933333	-	06/04/2018	FT18BH00033
4/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	2.12	6	3.533333	-	06/04/2018	FT18BH00036
5/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	2.01	6	3.35	-	06/04/2018	FT18BH00038
6/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	1.91	6	3.183333	-	06/04/2018	FT18BH00040
7/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	2.31	6	3.85	-	06/04/2018	FT18BH00045
8/Lassik RS Spring	*sbella/b*-AB, *sbella*-D	1.87	6	3.116667		06/04/2018	FT18BH00047
1/Lassik Spring	Wild-type	2.40	6	4	-	06/04/2018	FT18BH00025
2/Lassik Spring	Wild-type	2.62	6	4.366667	-	06/04/2018	FT18BH00030
3/Lassik Spring	Wild-type	2.19	6	3.65	-	06/04/2018	FT18BH00031
4/Lassik Spring	Wild-type	2.37	6	3.95	-	06/04/2018	FT18BH00035
5/Lassik Spring	Wild-type	1.82	6	3.033333	-	06/04/2018	FT18BH00039
6/Lassik Spring	Wild-type	2.71	6	4.516667	-	06/04/2018	FT18BH00042
7/Lassik Spring	Wild-type	2.30	6	3.833333	-	06/04/2018	FT18BH00043
8/Lassik Spring	Wild-type	2.48	6	4.133333	-	06/04/2018	FT18BH00048
1/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.44	6	5.733333	12.65	26/10/2017	FT18BH00002
2/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.48	6	5.8	12.69	26/10/2017	FT18BH00004
3/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.34	6	5.566667	11.62	26/10/2017	FT18BH00007

4/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.63	6	6.05	10.41	26/10/2017	FT18BH00011
5/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	2.90	6	4.833333	12.84	26/10/2017	FT18BH00014
6/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.26	6	5.433333	11.47	26/10/2017	FT18BH00018
7/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.46	6	5.766667	12.25	26/10/2017	FT18BH00020
8/Lassik RS Autumn	*sbella/b*-AB, *sbella*-D	3.48	6	5.8	11.66	26/10/2017	FT18BH00024
1/Lassik Autumn	Wild-type	4.24	6	7.066667	15.12	26/10/2017	FT18BH00001
2/Lassik Autumn	Wild-type	4.20	6	7	13.59	26/10/2017	FT18BH00006
3/Lassik Autumn	Wild-type	4.18	6	6.966667	13.55	26/10/2017	FT18BH00009
4/Lassik Autumn	Wild-type	4.31	6	7.183333	12.96	26/10/2017	FT18BH00010
5/Lassik Autumn	Wild-type	3.60	6	6	14.02	26/10/2017	FT18BH00015
6/Lassik Autumn	Wild-type	3.88	6	6.466667	14.61	26/10/2017	FT18BH00016
7/Lassik Autumn	Wild-type	4.38	6	7.3	13.55	26/10/2017	FT18BH00021
8/Lassik Autumn	Wild-type	4.30	6	7.166667	12.41	26/10/2017	FT18BH00023

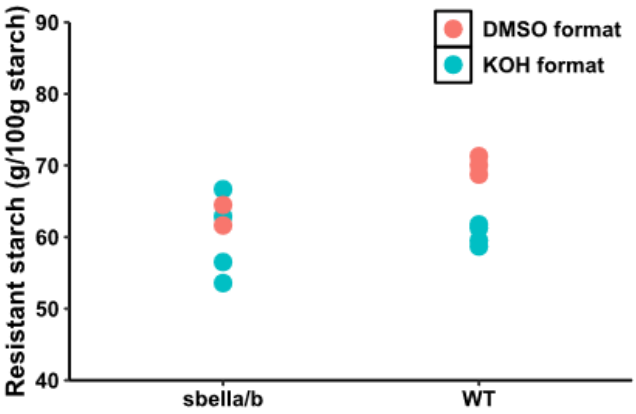

Figure A2 1. TS quantification using the Megazyme Total Starch kit as describe in Chapter 2, 2.2.2.1*. (AOAC 996.11. The graph shows the TS concentration of sbeII durum wheat semolina and wild-type control semolina (WT) using the DMSO format and the KOH format for samples not containing D-glucose and/or maltodextrins. in grey, the mean TS content.*

Table A3 1. Monosaccharides profiles. *Samples are independent replicates of bulked durum wheat semolina and bread wheat flour (n = 3). Durum wheat semolina was made into pudding: samples of Independent replicates of freeze-dried pudding are reported here (n = 7).*

Total monosaccharides

Sample	Genotype	Arabinose		Galactose		Xylose		Mannose	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Durum wheat semolina	*sbeII*	11.4535	0.728124	3.888567	0.541709	17.26183	0.909625	1.266833	0.62608
	WT	7.304467	0.325402	2.442633	0.242645	11.041	0.29733	0.81915	0.57925
Durum wheat Pudding	*sbeII*	9.95941	0.295793	3.048276	0.085165	14.21245	0.449171	0.979076	0.04943
	WT	7.201238	0.650758	2.154119	0.273856	10.35535	0.894504	0.937471	0.113535
Bread wheat flour	*sbeII*	9.21503	0.15240	3.51740	0.07881	14.39317	0.74607	2.99720	0.27839
	WT	8.12877	0.88129	3.05807	0.14478	11.2033	1.10793	1.86823	0.36588

Water extractable monosaccharides

Sample	Genotype	Arabinose		Galactose		Xylose		Mannose	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Durum wheat semolina	*sbeII*	1.8308	0.2746	1.7315	0.1463	1.2823	0.1978	0.1712	0.0707
	WT	1.3966	0.0146	1.2811	0.0333	0.9701	0.0513	0.0552	0.0078
Durum wheat Pudding	*sbeII*	1.844905	0.049324	1.953886	0.035205	1.601395	0.026227	0.435219	0.017454
	WT	1.27039	0.055	1.486367	0.027577	1.265081	0.051052	0.283267	0.009228
Bread wheat flour	*sbeII*	3.88027	0.13975	2.53227	0.02493	3.82183	0.10058	0.43673	0.02963
	WT	3.34160	0.08467	2.40777	0.05979	3.07740	0.18168	0.33947	0.02044

Table A3 2. Total (TOT) and Water Extractable (WE) Arabinoxylans (AX) of sbeII pudding compared to the wild-type control (WT). *Means ± SEMs, freeze-dried pudding n = 7 replicates prepared independently. Arabinoxylans are calculated as the sum of Arabinose and Xylose corrected for arabinogalactan (AGP) content as in[221, 375, 376] and multiplied by 0.89 to convert monosaccharide values, for arabinose and xylose, to that for the polysaccharide, arabinoxylan.*

Parameter	**Pudding WT control**	**Pudding *sbeII***
WE-AX (mg/g sample)	1.33 ± 0.03	1.84 ± 0.02
TOT-AX (mg/g sample)	14.28 ± 1.39	19.61 ± 0.73

Table A3 3. AXOS of sbeII pudding compared to the wild-type control (WT).

Means ± SEMs, freeze-dried pudding n = 7 replicates prepared independently from the same batch of bulked semolina.

Parameter	Pudding WT control	Pudding *sbeII*
TOT-AXOS[†]	11.84 ± 0.13	14.56 ± 0.09
AXOS Unsubstituted[†]	6.76 ± 0.09	7.93 ± 0.06
AXOS Substituted[†]	5.07 ± 0.05	6.63 ± 0.04
MLG (G3+G4) [†]	2.58 ± 0.04	3.11 ± 0.03

[†]*Sum of peaks relative to the internal standard*

Table A3 4. AXOS of sbeII bread flour compared to the wild-type control (WT).

Sum of peaks relative to the internal standard, n = 2 replicates of flour obtained from bulked grains in 2016.

Genotype	Replicate	AXOS Substituted	AXOS Unsubstituted	MLG (G3+G4)	TOT-AXOS
sbeII	1	9.041868	6.2116216	4.311359	15.25349
	2	8.483375	5.8896551	3.258627	14.37303
WT control	1	7.485587	5.0386823	2.320777	12.52427
	2	6.50514	4.170154	3.34794	10.67529

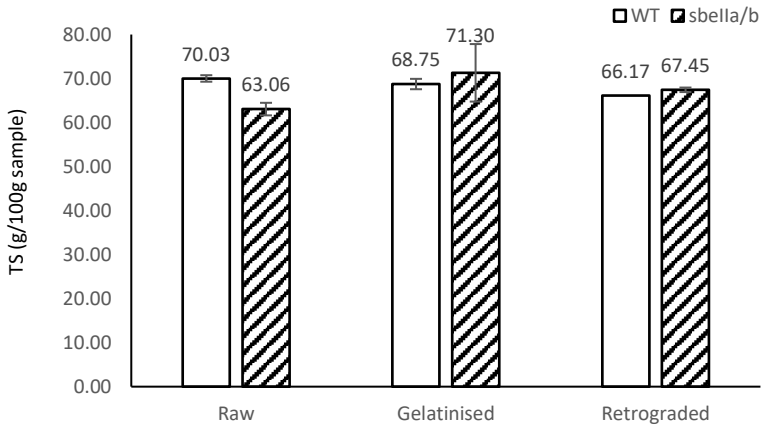

Figure A4 1. Total starch concentration during semolina hydrothermal processing

Table A5 1 GIPIRS capillary *blood glucose concentration 0-120 minutes*

***sbeII* pudding**								
Participant ID	**Time to consume the meal (mins)**	**0**	**15**	**30**	**45**	**60**	**90**	**120**
1	15.00	4.65	4.60	6.00	6.00	5.20	4.60	4.40
2	15.00	5.60	6.70	7.90	7.00	6.30	5.30	5.20
3	8.00	4.50	5.20	6.55	6.60	5.90	5.00	4.60
4	12.40	4.60	4.20	5.10	5.40	6.10	6.10	5.70
6	7.00	4.15	3.70	6.20	6.30	6.50	6.10	5.60
7	18.00	4.45	5.10	6.75	6.90	5.70	4.30	5.30
9	20.00	5.25	4.70	7.80	5.70	5.90	5.30	5.10
10	6.30	5.05	5.40	6.45	6.70	6.90	5.70	5.50
11	9.00	4.70	5.80	8.45	8.00	6.40	4.80	4.80
12	11.30	3.85	3.70	4.65	5.10	4.50	5.00	4.40
WT pudding								
Participant ID	**Time to consume the meal (mins)**	**0**	**15**	**30**	**45**	**60**	**90**	**120**
1	15.00	4.35	4.40	5.25	7.70	7.30	5.80	4.40
2	14.40	4.95	5.00	7.80	7.60	6.60	5.60	4.80
3	10.00	4.50	5.20	6.70	6.20	5.60	4.20	4.00

4	6.00	5.20	6.20	7.35	8.20	7.90	7.20	6.00
6	7.00	4.20	4.30	5.90	6.50	5.80	5.60	4.80
8	18.00	5.10	3.90	6.80	6.20	6.00	5.00	4.70
9	18.00	4.75	4.60	6.45	5.50	6.10	5.40	4.20
10	6.00	5.20	5.70	6.75	6.60	7.10	4.70	4.70
11	9.00	4.75	4.50	7.35	7.70	7.70	5.60	5.30
12	11.30	4.65	5.70	6.05	6.30	6.60	6.20	5.60

Table A6 1. REST bread end-use quality parameters *reported as the mean ± SE of four independent replicates of bread.*

	Parameter	WT control bread	*sbeII* bread
Specific volume (cm^3/g)		2.952 ± 0.05	2.651 ± 0.04 *
Bulk density (g/ml)		0.339 ± 0.008	0.378 ± 0.008 *
Colour crust	L*	78.20 ± 0.68	83.73 ± 0.88 ***
	a*	11.58 ± 0.63	6.66 ± 0.80 ***
	b*	40.72 ± 0.82	35.39 ± 1.27 ***
	Hue angle	105.75 ± 0.72	100.26 ± 1.05***
	Chroma	42.41 ± 0.90	36.12 ± 1.37***
	Browning Index	587.40 ± 0.03	587.63 ± 0.039***
Colour crumb	L*	92.33 ± 0.35	93.06 ± 0.42
	a*	0.52 ± 0.05	0.58 ± 0.09
	b*	19.56 ± 0.27	20.19 ± 0.47
	Hue angle	91.52 ± 0.17	91.65 ± 0.24
	Chroma	19.58 ± 0.27	20.21 ± 0.47
	Browning Index	588 ± 0.003	587.99 ± 0.007
Texture	Hardness	993.484 ± 28.00	1238.68 ± 12.24 ***
	Springiness (g*sec)	0.98 ± 0.01	0.99 ± 0.009
	Cohesiveness	0.74 ± 0.002	0.69 ± 0.002
	Chewiness	702.86 ± 11.43	871.89 ± 6.35 ***
	Resilience	0.41 ± 0.004	0.42 ±0.005

Parameter values that are significantly different between the two bread types are indicated with an asterisk; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (unpaired Students t-test).

Figure A6 1 breads produced with the straight-dough method described in chapter 6. *Breads in figure a proofed for 15 minutes resulted in variable colour, often a cracked on the surface with an uneven surface and shape. Breads in figure b were proofed for 30 minutes, as the sponge breads, resulting in a very even and regular shape and colour.*

Table A6 2 Capillary glucose concentrations REST study

Capillary glucose concentrations after consuming *sbeII* bread											
Participant ID	Time (min)										
	0	15	30	45	60	75	90	120	150	180	210
1	4.9	6.7	6.7	6.8	7	6.4	6.7	5.8	5.9	6.3	5.3
2	4.9	4.9	6.7	7.1	6	5.8	5.2	5.3	5.1	5.8	5
3	4.8	4.8	4.9	5.9	5.7	5.2	5.8	5.3	5.5	5.3	5.4
4	5.2	5.4	6.9	6.9	6.1	6	6.1	5.9	5.5	4.8	4.8
5	5.2	5.9	8.3	7.7	7.2	6.6	6.7	6	5.2	5	4.8
6	5.1	6.3	7.6	6.2	6.6	5.5	5.2	6.2	5.2	5	4.8
7	4.9	5.3	6.6	6.8	5.3	5	4.8	5.7	5.7	5.5	4.8
8	5.9	6.4	7.1	6.5	6.7	6.3	6.3	7.2	6.4	5.8	5.3
Capillary glucose concentrations after consuming WT bread											
Participant ID	Time (min)										
	0	15	30	45	60	75	90	120	150	180	210
1	5.4	5.3	7.4	6.5	8.2	6.5	6.4	6.5	5.3	5.2	5.2
2	4.7	5	6.2	6.3	5.2	5.2	5.3	5.9	5.3	6.8	5.4
3	4.8	4.9	5.4	5.9	6.2	5.5	5.4	6.4	5.4	5.2	5

4	5.6	5.5	7	7.4	7.8	6.5	6.5	7.2	5.7	5.2	5.2
5	5.1	5.2	8	8	7.7	7.3	6.6	5.8	5	4.4	4.2
6	4.8	6	6.6	6.2	6	5.7	5.7	5	4.8	4.7	4.3
7	4.6	4.9	7.2	7	6.2	5.8	5.6	5.9	5.2	5	4.5
8	5.3	6.3	7.9	7.5	6.3	6.2	6.6	6.3	6.2	6.2	5.2

Time = 0 is the average of three measurements collected at -15 min, -10 min, -5 min (before starting to eat the test meal)

Table A6 3 Interstitial glucose concentrations REST stud (sbeII bread)

Interstitial glucose concentrations after consuming *sbeII* bread																		
Participant ID	Sensor	Time (min)																
	A	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**	**195**	**210**	**225**	**240**
1	A	5.2	5.4	6.3	6.9	6.9	6.6	6.2	6.1	6.1	5.9	5.6	5.6	5.6	5.6	5.4	5.2	5.1
2	A	4.3	4.4	4.9	5.8	5.8	5.3	5	5	5.1	5.2	5.2	4.9	4.8	4.9	4.8	4.6	4.4
3	A	4.1	4.3	4.7	5.2	5.2	4.7	4.4	4.6	4.7	4.8	4.8	5	4.9	4.7	4.7	4.8	4.7
4	A	4.4	4.7	5.6	6.1	5.8	5.3	5.3	5.6	5.6	5.2	4.9	4.7	4.6	4.4	4.3	4.2	4.1
5	A	4.7	4.6	5.7	7.6	7.9	7.1	6.7	6.5	5.9	4.8	4.2	4.3	4.2	3.8	3.6	3.4	3.4
6	A	4.6	4.4	5	5.9	6.1	5.7	5.3	4.9	4.9	5.1	4.9	4.8	4.7	4.6	4.6	4.5	4.4
7	A	4.7	4.7	5.4	6	5.6	4.8	4.6	5	5.5	5.8	5.7	5.3	5.2	5	4.7	4.4	4.2

8	A	5.0	5.9	6.8	6.6	5.9	5.7	5.8	6.2	6.4	6.1	5.7	5.6	5.3	5.2	5.1	4.9	4.9
1	B	4.9	5.2	6	6.4	6.4	6.2	5.9	5.8	5.7	5.5	5.3	5.3	5.3	5.3	5.1	5.1	4.9
2	B	4.9	4.8	5.3	6.7	7	6.1	5.5	5.6	5.8	5.9	5.8	5.4	5.2	5.6	5.5	5.1	4.9
3	B	5.2	5.1	5.4	5.9	6.2	5.8	5.4	5.6	5.7	5.7	5.8	5.9	5.9	5.8	5.8	5.7	5.8
4	B	4.1	4.2	4.6	5.3	5.5	5.2	5.2	5.4	5.3	5.1	4.8	4.6	4.4	4.3	4.2	4	3.9
5	B	5.1	5.4	6.9	8.6	8.4	7.4	7.1	7	6.3	5.1	4.7	4.7	4.4	4.1	4	4.1	4.1
6	B	4.6	4.6	4.7	5.2	5.8	5.9	5.6	5.3	5.1	5	5.1	4.8	4.7	4.6	4.6	4.6	4.6
7	B	4.7	4.7	5	5.7	5.9	5.4	4.9	5	5.2	5.6	5.9	5.9	5.7	5.4	5.1	4.7	4.6
8	B	5.2	5.1	5.8	6.7	6.7	6	5.7	5.7	6.2	6.4	6.2	5.8	5.7	5.5	5.3	5.2	5.1

Time = 0 is the average of three measurements collected at -15 min, -10 min, -5 min (before starting to eat the test meal)

Table A6 4 Interstitial glucose concentrations REST stud (WT bread)

Interstitial glucose concentrations after consuming WT bread																		
Participant ID	Sensor	Time (min)																
	A	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**165**	**180**	**195**	**210**	**225**	**240**
1	A	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

2	A	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
3	A	4.4	4.7	5.1	5.4	5.3	5	5.1	5.2	5.1	4.9	4.8	4.8	4.7	4.6	4.7	5.1	5.3
4	A	4.8	5.2	6.1	6.9	6.8	6.3	6.1	6.3	6.6	6.2	5.6	5.1	4.9	4.7	4.6	4.4	4.4
5	A	5.4	5.7	7.6	8.8	7.7	6.8	6.8	6.4	6.1	5.5	5.1	5	5	4.9	4.8	4.8	4.8
6	A	5.0	5.4	6.4	6.9	6.1	5.6	5.8	5.8	5.6	5.1	4.8	4.7	4.4	4.6	4.8	4.9	4.8
7	A	4.6	4.9	6.3	7.4	6.8	5.7	5.4	5.9	6.1	6.1	5.8	5.4	5.2	4.8	4.4	4.2	4.1
8	A	5.0	5.2	6.4	7.2	6.4	5.6	5.7	5.9	5.8	5.9	6	6.1	6.1	5.6	4.8	4.4	4.3
1	B	3.2	3.3	4.6	5.5	5.4	4.9	4.4	4.4	4.4	4.1	3.6	3.3	3.2	3.2	3.1	2.9	2.7
2	B	4.9	4.7	5.1	5.9	6.6	6.1	5.4	5.7	6.1	6	5.8	5.6	5.5	5.9	5.9	5.4	5
3	B	5.1	5.1	5.4	5.9	6.2	5.8	5.6	5.7	5.8	5.7	5.6	5.4	5.3	5.2	5.1	5.4	5.7
4	B	3.2	3.2	3.6	4.6	5	4.5	4.1	4.2	4.5	4.5	3.8	3.4	3.2	3.1	2.9	2.8	2.8
5	B	5.5	6.1	7.9	8.7	7.4	6.8	6.8	6.4	6.2	5.5	5.1	5.2	5.2	5.1	4.9	4.9	5.1
6	B	4.7	4.9	5.7	6.4	6.3	5.8	5.6	5.5	5.4	5.2	4.8	4.6	4.4	4.3	4.3	4.4	4.6
7	B	4.8	4.8	5.6	6.8	7.1	6.3	5.6	5.7	6.1	6.1	6.1	5.7	5.3	5.1	4.8	4.6	4.3
8	B	5.0	5.3	6.3	6.8	6.2	5.7	5.7	5.9	5.9	5.9	6.1	6.2	6.2	5.7	4.9	4.5	4.3

Time = 0 is the average of three measurements collected at -15 min, -10 min, -5 min (before starting to eat the test meal)

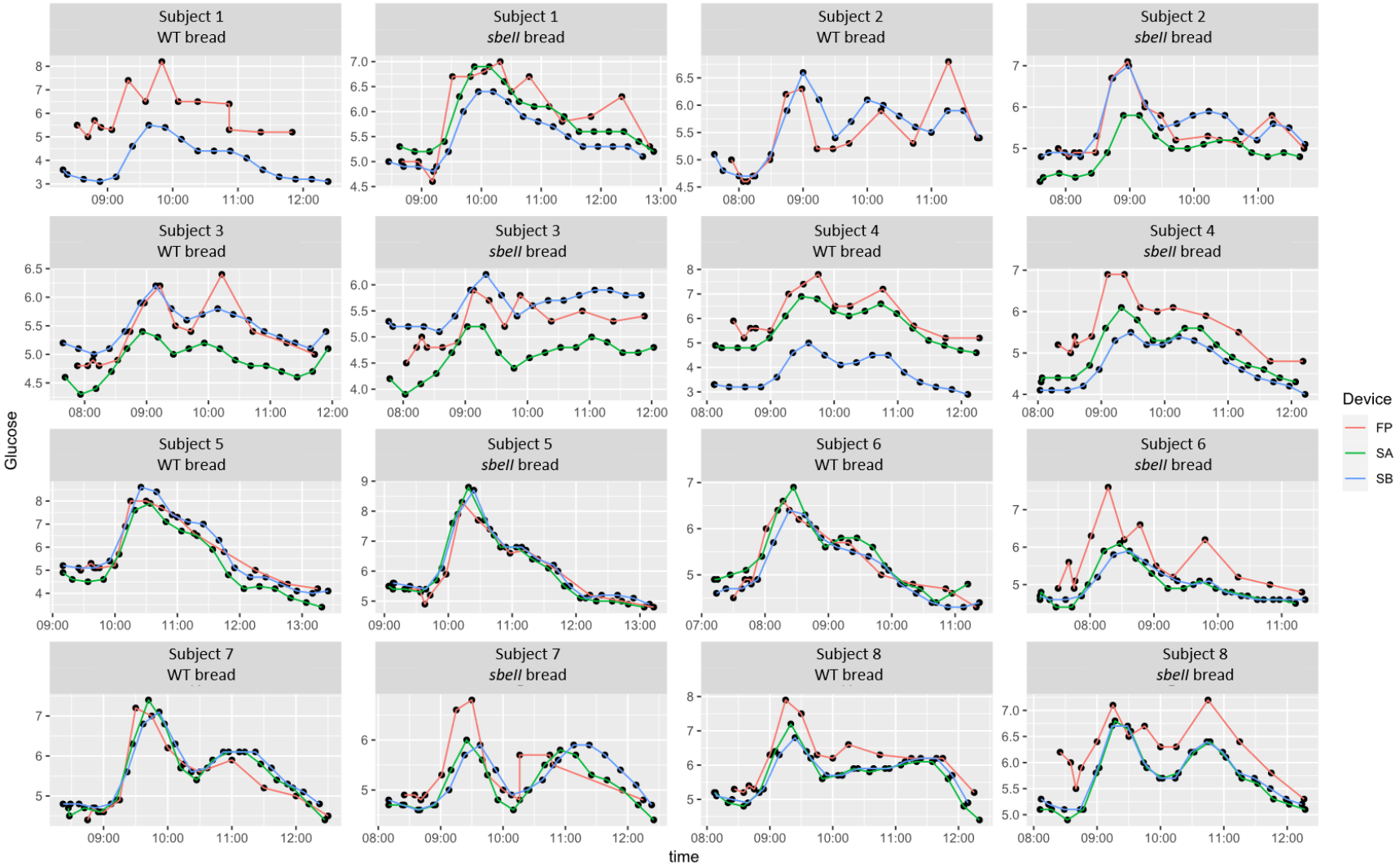

Figure A6 2. Post-prandial interstitial glucose measured with Sensor A (SA) and Sensor B (SB) and capillary glucose measured by Finger Prick test (FP).

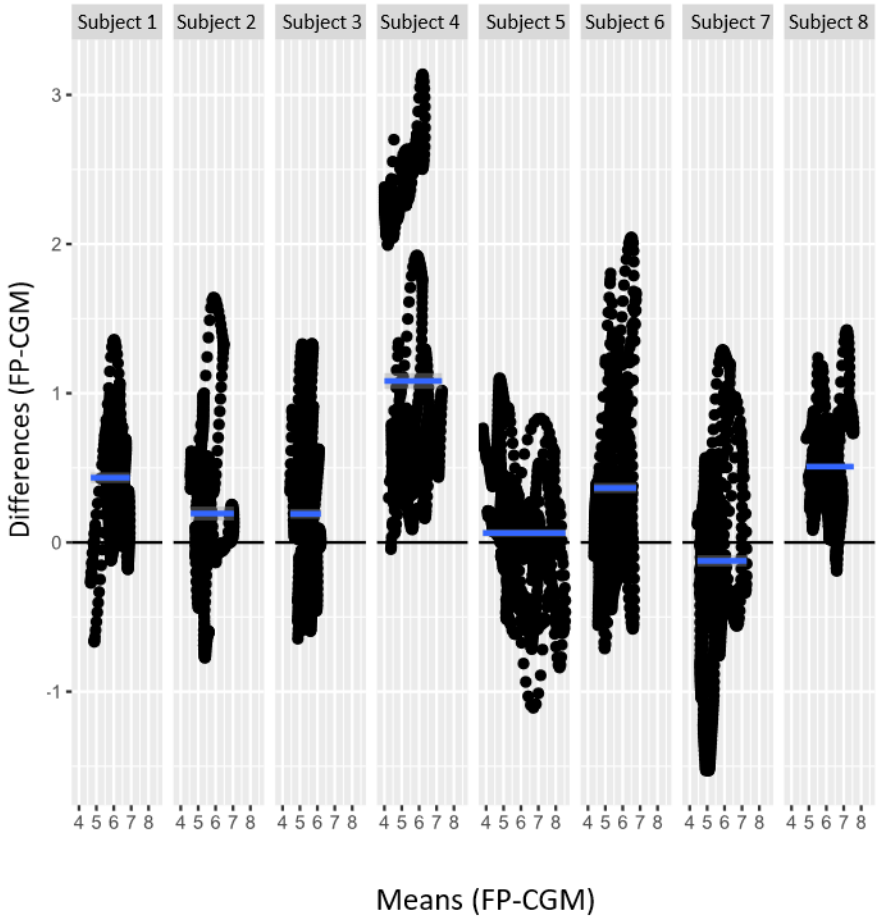

Figure A6 3. Bland-Altman plot by participant *showing glucose measurements of subject 4 deviating from the overall tendency of agreement between capillary and interstitial fluid glucose values.*

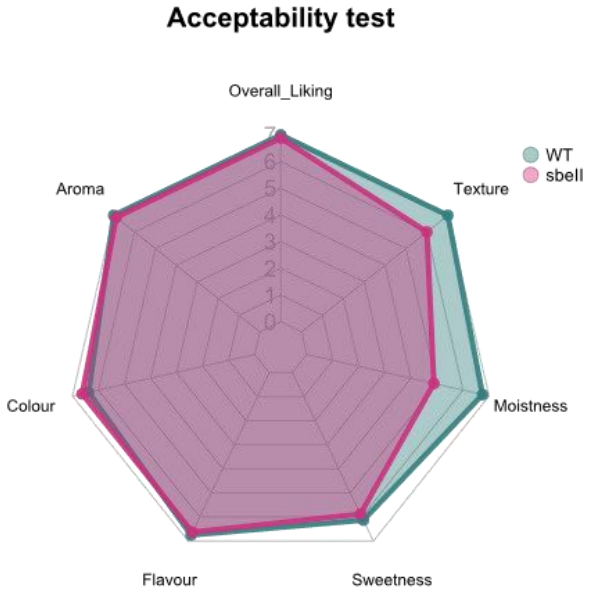

Figure A6 4. Bread acceptability scores*. sbeII bread (pink) and WT control bread (green), n=8.*

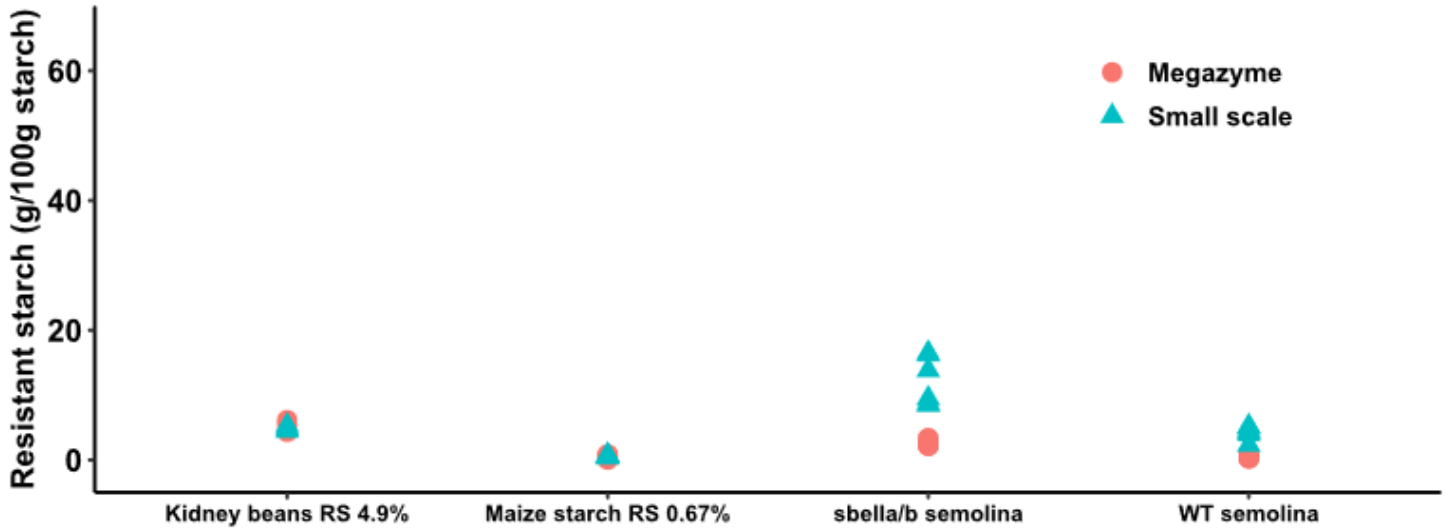

Figure A6 5. RS concentration of wild-type control (WT) and sbeII semolina and other standards provided by Megazyme. *Samples include: dried and milled kidney beans, n=3, moisture = 1%; Maize starch 0.67% RS n = 6, moisture = 14%; Maize starch 44% RS, n = 9, moisture = 14%; Semolina sbeII and WT, n = 6, moisture = average of 12% between replicates.*

Appendix B: REST study

1. Eligibility assessment

Volunteers who express interest in the study will be invited to attend an eligibility screening visit at QI CRF with a member of the QI CRF team. Eligibility screening will be carried out following QI CRF standard operating procedures. Prior to their appointment, participants will receive a letter or email or text message to confirm the date time of their screening appointment (Annex 6, 6a and 6b) before the appointment, and a reminder one or two days before the appointment. When study participants attend the QI CRF and clinical assessments are to be made, two members of the QI CRF team will always be present. Participants will also be reminded in the 'appointment card' by post or email or text message (Annexes 6, 6a and 6b) to bring with them details of any prescribed medication, herbal remedies or dietary supplements that they take (i.e. name of medication, dose taken). On arrival at the QI CRF the participant will be taken into a confidential room where a member of the study team (QIB PI or study investigator) or the QI CRF research nurse will explain the study again, show the participant the finger-prick and CGM devices and answer any questions. The QI CRF research nurse and/or a study team member will then go through a 'consent form REST' for study participation (Annex 7) with the participant and encourage any questions they may have at that stage. Participants will then be asked to sign one copy of the consent form for study participation and two copies of the signed form will be made. One copy will be returned to the participant, two copies will be retained by the study team. The participants will also be asked to sign three copies of the 'medical declaration form' (Annex 8) agreeing to inform the study team of any medication they may have to take, illnesses suffered, or if they become pregnant during the study; two will be retained by the study team and one copy will be returned to the participant.

Following consent, participants will be able to practice pricking their finger to collect drops of capillary blood. Participants that are not able to carry out the finger-prick may be asked to withdraw from the study as this is required to successfully study completion.

The QI research nurse or a member of the study team will collect the participants' contact details and background information which will be used to contact the participant during the study (Annex 28). Participants' background information will be used to describe the study cohort as a group. The QI research nurse supported by a member of the study team will

complete an 'eligibility screening questionnaire' with the participant and record their preferred study dinner/lunch preferences (Annexes 9 and 9a). They will measure and record participants' blood pressure (BP), pulse, height (cm) and weight (kg) and calculate Body Mass Index (BMI, kg/m2) according to the Norwich and Norfolk Hospital (NNUH) standard operating procedure. A blood sample (20 mL) will be collected by venepuncture (according to the NNUH standard procedure for venepuncture) into specific vacutainer tubes for HbA1c (this test will give an indication of the participants' average glucose levels over a period of approximately 2-3 months) and fasting glucose as a marker of glucose metabolism. Lipid profiles will be measured as lipid metabolism is closely linked to glucose metabolism (30–32). Full blood count, renal and liver function, will also be measured to evaluate participant's overall health (bloods taken for eligibility screenings will be sent to NNUH laboratories for analysis in tubes labelled with participant name and date of birth).

During the eligibility screening, volunteers will be given instructions on how to prepare for intervention visits, how to wear and care for the CGM device and how to prick their finger to measure glucose concentration in capillary blood. At the end of the visit, participants will be accompanied by a member of the study team to the QI Cafe' to receive a complimentary breakfast.

If blood results indicate that exclusion from the study is appropriate (refer to inclusion and exclusion criteria), the decision to exclude the participant from the study will be taken by the QI medical advisor. The participant will be advised by phone call or letter (Annex 17) to speak to their GP regarding their results. The GP will be informed of the reason why their patient did not qualify for the study (annex 12).

Copy of all the eligibility screening results (blood test results, blood pressure, pulse, weight, BMI) will be made available to the GP via the hospital electronic system, and/or by letter (Annex 12).

2. Eligibility criteria

Eligible participants will be contacted by the method they indicated to be their preference on their consent form. Participants that prefer to be contacted by email or post will be sent an 'invitation letter to join the study' (Annex 25) and will be asked to contact the study team to book their study visits.

Inclusion criteria

- Men and women
- Aged 18 to 65 years
- BMI between 18-25 kg/m2 (Participants who do not have a BMI between 18 (underweight) and 25 (overweight) kg/m2 will be excluded from the study on the grounds that their glucose metabolism may be impaired; severe malnutrition and excessive weight gain are associated with reduced insulin secretion and glucose intolerance (33,34).
- Non-smokers
- Those that live within a 40-mile radius of Norwich

Exclusion criteria

Participants will not be able to join the study if:

- Fasting glucose >6.1 mmol/L and/or HbA1c>42 mmol/mol, as assessed by a fasting blood test
- Eligibility screening results indicate they are not suitable to take part in this study (from the blood test or eligibility screening questionnaire results). These include blood test results deemed by the medical advisor to be indicative of a health problem that may compromise the well-being of the participant or the study outcome.
- Smokers (if they have smoked within the 6 weeks prior to the study or during the study).
- Suffer from allergy, intolerance, or sensitivity to gluten, yeast or any of the food ingredients used in this study (see Annex 9a)
- Have a known allergy to adhesives that would prevent proper attachment of the CGM sensors
- Are pregnant and/or have been pregnant in the last year or are lactating and/or breastfeeding
- Are currently suffering from, or have ever suffered from eating disorders, any diagnosed gastrointestinal disease, gastrointestinal disorders including regular diarrhoea and constipation (excluding hiatus hernia unless symptomatic) or other inflammatory

diseases like rheumatoid arthritis (RA), polymyalgia rheumatica or other connective tissues diseases

- Have undergone gastrointestinal surgery; this will be assessed on an individual basis
- Have been diagnosed with diabetes, anaemia as this may affect the study outcome
- Have been diagnosed with any long-term medical condition that may affect the study outcome (e.g. cardiovascular diseases, cancer); this will be assessed on an individual basis
- Have been diagnosed with any long-term medical condition requiring medication that may affect the study outcome
- Regularly take over-the-counter medications for digestive/gastrointestinal conditions
- Use medications likely to interfere with energy metabolism, appetite regulation and hormonal balance, including long-term steroids, antibiotics. They may be able to participate if 4 weeks or more have passed from the end of a course such medication (this will be assessed on an individual basis depending on dose and duration of the prescription)
- Regularly take laxatives (once a month or more) as this may affect blood glucose levels
- Take certain dietary supplements or herbal remedies and are unwilling to stop taking them (if required) for two weeks prior to and during the study period; this will be assessed on an individual basis
- Are on, or plan to start, a diet programme that may affect the study outcome (e.g. 5:2 fasting diet) unless willing to abstain for 1 month prior to and during the study period; this will be assessed on an individual basis
- Went through a weight change of ≥ 3kg in the preceding 2 months as this could indicate a change in metabolism that could affect the study outcome
- Have a recent history of substance abuse
- Regularly consume more than 14 units of alcohol a week
- Are unwilling to suspend smoking and vaping for the duration of the study.

- Are participating in another research project that involves dietary intervention or blood sampling.
- Are unwilling to provide GPs contact details
- Are related to or living with any member of the study team.
- Are unable to provide written informed consent
- Have not donated blood or taken part in another dietary intervention in the last 16 weeks and are unwilling to wait until 16 weeks have elapsed
- Those with abnormal blood pressure measurements (160/100 will be regarded as an exclusion value)

Eligible participants will be contacted by phone/letter/email (Annex 25) to schedule the three study visits. The GPs of those successfully recruited onto the study will be informed of their patient's participation in the study by 'letter of study involvement' (Annex 10) along with a study description (Annex 11). It is expected that all participants who successfully pass the screening, and who wish to continue, will commence on the study within 3 months of their eligibility screening appointment otherwise a re-screen will be necessary.

3. Participant Information Sheet

Quadram Institute
Science Health Food Innovation

The *REST* Study

Glycaemic response to high REsistant STarch bread

NHS
Norfolk and Norwich University Hospitals
NHS Foundation Trust

REST Participant Information Sheet
Version 3
Date 25 September 2019
Investigator: Marina Corrado
IRAS 262271

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CONTACT DETAILS

This booklet contains information about the REST study (Measuring the Effects of High Resistant Starch bread on glycaemic response).

Please read it carefully. If you'd like to know more, please get in touch with the study team.

Marina Corrado is QIB Principal investigator of the REST study

01603 255101

REST@quadram.ac.uk

https://quadram.ac.uk/reststudy/

Quadram Institute Bioscience
Norwich Research Park
Norwich, Norfolk
NR4 7UQ
UK

The mission of the Quadram Institute is to understand how food and the gut are linked and to promote health and the prevention of disease. We aim to fine-tune the impact of foods on health, from early life to the extension of a healthy lifespan in old age and reduce the economic and societal costs of chronic diseases. The Quadram Institute is an interdisciplinary institute founded on a cluster of academic and clinical expertise at the Norwich Research Park, working alongside the food and pharmaceutical industries

What is REST

REST is about white bread made from wheat with high **RE**sistant **ST**arch, a type of fibre. Wheat bread made from white flour is one of the UK's favourite foods but normally, it has very low levels of fibre, which is an essential part of a healthy diet. Fibre exerts a protective role in reducing the risk of developing many common diseases that are diet related. However, in the UK, 91% of the adult population does not meet the recommended fibre intake of 30g per day[1]. A correct fibre intake helps to control blood sugar levels and body weight, reducing the risk of developing type II diabetes and obesity.

We have developed a type of white wheat flour that appears similar to conventional white flour but has higher resistant starch content. In this study, we want to compare body sugar levels (called glucose) after eating a high resistant starch bread with eating a conventional bread, low in resistant starch. This will help us understand whether high resistant starch bread can help to boost fibre intake of healthy people. In the long term, we might be able to determine whether high resistant starch bread can help reduce the risk of common chronic diseases such as type II diabetes and obesity.

We are looking for:

- **Healthy** people
- Between **18 and 65** years of age
- Living within 40 miles from the **Norwich Research Park**

[1]The average fibre intake of adults in the UK is 19g per day. The recommended average intake for adults is 30g per day. From National Diet and Nutrition Survey (NDNS) from 2014 to 2016 by Public Health England

Meet the REST team

The study is led by Miss Marina Corrado, a PhD student at Quadram Institute Bioscience (QIB).

Dr. Jennifer Ahn-Jarvis and Mr Brendan Fahy are the study co-investigators helping the study to run.

Dr. Cathrina Edwards and Dr. Brittany Hazard are the study advisors.

The QI Clinical Research Facility (QI CRF) is managed by Dr. Melanie Pascale.

The study team is supported by a Medical Advisor and a Research Nurse.

Our Clinical Research Facility is equipped with Wifi and TV.

The Quadram Café is open from 8am to 6pm and parking is free of charge for REST study participants during study visits.

Why am I invited?

We are inviting you because you showed an interest in the study. In this booklet, we explain what participating in this study involves. Please read it carefully.

If after reading this, you think you would like to participate, or you want to know more about the REST study, let us know so we can contact you by phone to talk you through the details of the study, and answer any questions you may have.

At the end of the talk, we will leave you some time to think about whether you want to book an appointment for eligibility screening at the QI Clinical Research Facility (QI CRF).

We will then ask you to sign a consent form, once you have understood what you are being asked to do for the study.

If you would like more information or have any concerns, please get in touch with us.

About the REST Study

REST happens during the week! Visits are scheduled according to your availability during week days, usually in the morning. We aim to complete the study within 2 weeks.

Screening visit
Sign consent, measure blood pressure, height, weight, do a blood test and health questionnaire

Calibration visit
Wear 2 glucose sensors until the end of the study
Training on how to complete a food and activity diary

Prepare for intervention day - at home
Eat a standard dinner provided by the study team then fast for 10h

Intervention visit 1
Breakfast: bread with Flora diary free spread and water
Finger prick blood tests
Fill in Questionnaire about your hunger and about the bread
Lunch is served!

Food diary - at home
Log ALL you eat and drink for 3 days in the app or paper (eat as you would normally do)

Prepare for intervention day - at home
Eat a standard dinner provided by the study team then fast for 10h

Intervention visit 2
Breakfast: bread with Flora diary free spread and water
Finger prick blood tests
Fill in Questionnaire about your hunger and about the bread
Lunch is served!

Follow up
Remove glucose sensors, complete follow up questionnaire. Return them to the study team

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How does it work?

We will invite you for a screening visit for a health assessment **(visit 1)**. We will ask you to sign a consent form to join the study and we will then measure your blood pressure, weight and height. We will also ask you some questions about your general health and collect a blood sample.
We will ask you for your contact details and the contact details of your GP.
Once you are enrolled, there are 3 visits to complete at the QI Clinical Research Facility, in Norwich. During two of the three visits, you will be served breakfast bread, one with low resistant starch and one with high resistant starch.
You will not be told which bread you are eating on each day, but you will have both bread types, if you complete the study.
After eating the bread portion, you will be asked to measure your blood glucose level with a "finger-prick test" and to complete some questionnaires.

At calibration (visit 2) you will be asked to complete a general health assessment after which you will be given 2 glucose sensors to wear for the whole duration of the study. These automatically record your body sugar levels. We would like you to scan the devices every 6 to 8h to collect body glucose readings. Because we would like you to eat and drink as you would do normally, you will not be able to see your readings. The sensors should not interfere with your daily routine. While the sensors warm up, we will also show you how to record your food and drink intake while at home. This visit will last approx. 3h.

Before the intervention, we will give you instructions on how to prepare at home for the intervention day. You will be

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asked to avoid caffeine, alcohol and very calorific meals[2], as well as intense exercise (such as hiking, jogging at 6mph, basketball or soccer game). You will be provided with a standard dinner to consume the night before each intervention, you will need to fast for 10h before your next visit.

On intervention day (visit 3), you will be at the QI CRF from approx. 8.30am until 1.30pm. You will be provided with breakfast and lunch. Between breakfast and lunch, you will be asked to collect a series of blood drops from the tip of your fingers to measure blood sugar level and complete a series of questionnaires. A member of the study team will be there to support you with this.

Between visit 3 and 4, we would like you to complete a non-consecutive 3-day food diary at home. This means recording everything you eat and drink for 3 days (2-week days and 1 weekend day) using an app or paper-based diary.

Before the intervention, you will be asked to prepare as you did for your previous visit (No caffeine, alcohol, very calorific meals[2]). As before you will be provided with a standard dinner.

The second and last intervention visit (visit 4) will proceed exactly as visit 3.

The day after visit 4, you will be asked to remove the glucose sensors and return them to the study team together with a follow up questionnaire as soon as possible.

Once the study is completed, we will summarise the results and let you know the outcome of REST.

[2] a very calorific meal is a meal 1000kcal (4184 kj) or more

Expenses refund

We will refund you for travel expenses and for taking the time to take part in the study.
See EXPENSES AND PAYMENTS for more details.

Are you eligible?

Please read the statements below. If you answer YES to all of them, you might be eligible to take part in this study.

- ☐ I am between 18 and 65 years of age
- ☐ I do not smoke
- ☐ I am not pregnant
- ☐ I do not have type I or II diabetes
- ☐ I do not have allergies or intolerances to gluten, yeast
- ☐ I am not allergic to adhesives which could prevent the attachment of the glucose monitor
- ☐ I am not suffering from an eating disorder
- ☐ I do not have Inflammatory bowel disease (IBD), or other inflammatory diseases like rheumatoid arthritis, polymyalgia rheumatica or other connective tissues diseases
- ☐ I have not donated blood or taken part in another dietary intervention in the last 16 weeks or I am willing to wait until 16 weeks have elapsed
- ☐ I have not had cancer in the last 3 years
- ☐ I am not suffering from a gastrointestinal disease or disorders including regular diarrhoea and constipation
- ☐ I have not had a heart attack (myocardial infarction) or stroke in the last 6 months
- ☐ I have not taken antibiotics in the last 4 weeks or I am willing to wait until 4 weeks after the treatment is completed
- ☐ I am able/willing to consume one of the lunch choices and one of the dinner choices provided (see REST day-lunch)
- ☐ I am not or do not plan to start a diet programme that cannot be postponed
- ☐ I do not take dietary supplements, or I am willing to stop taking them during the study period if required
- ☐ I am willing to wear the glucose sensors for the duration of the study
- ☐ I will be able to fast for 10h the night before the interventions

Please note: some medications may preclude you from taking part in the study. This will be assessed on an individual basis

Eligibility Screening

When you arrive at the Quadram Institute we will welcome you and walk you through the study and answer any questions. To join the study, you will be required to provide written consent.

We will ask you whether you have fasted for at least 10 hours. If you have not, we might have to reschedule the screening visit to another day.

We will ask you a series of questions about your health, similar to those we listed on page 5 under 'Why am I invited?' but in more detail. We will ask you details about any medications you are taking, please bring the details of this with you.

If you meet the study criteria, we will measure your blood pressure, height and weight and calculate your BMI

If these measurements are within the study criteria, we will proceed to draw blood samples from a superficial vein in your forearm. We will collect a maximum of 20mL of blood (about 2 tablespoons) to check your glucose metabolism, fat levels in the blood and general health status.

Once we have your blood test results, we will let you and your GP know if you are eligible or not to participate in the REST study. Your GP will have access to your test results, we may advise you to discuss these results with your GP.

Because **you have to be fasted** for the blood test (nothing to eat or drink apart from water since the night before), at the end of the visit, we will accompany you to the QI café for a complimentary breakfast.

This visit will last approximately 3 hours.

Calibration

During this study we would like to measure your body glucose levels in two different ways: using a glucose sensor applied to the back of your arm (see picture below) and by collecting finger prick blood samples.

The sensor that you will wear measures your sugar level every 15 min for the duration of the study. This device is currently used by diabetic patients to monitor their sugar level.

After you arrive at the CRF on the calibration day, you will be given two *FreeStyle Libre* (www.freestylelibre.co.uk) sensors to apply to the back of your upper arm. The sensors are waterproof and should not interfere with your daily activities. However, you should avoid strenuous activities for the duration of the study. We would like you to wear the sensors day and night and you will scan them every 8h with a reader device to store the measurements collected by the sensors. Once applied, the device takes one hour to warm up.

Meanwhile we will introduce you to the *Libro* app or form that is used to record your food intake. The *Libro* app is based in the UK and is GDPR compliant (see **WHAT WILL HAPPEN TO MY DATA AND SAMPLES)**

We would like you to record what you eat and drink accurately for 3 non-consecutive days during the study. This

can be done between visits and preferably on two-week days and one weekend day. This information will be used by the research team to characterise your normal diet.
We will also match what you eat and drink with your glucose levels measured by the sensor you will be wearing. We do not want you to change your dietary habits. You should feel free to consume any food or drink as you would normally do.
We will demo the app and practice recording your intake while your sensor warms up.

On the intervention day, you will be asked to measure your blood sugar level using a blood drop from your finger. During the calibration visit, we will show you how to do this and answer any questions you may have at this stage.

This visit will last approx. 3 hours.

Preparing to a study day

The day before your intervention days (visits 3 and 4), we would like you to avoid caffeine, alcohol and strenuous exercise.
You will be given a standard meal to consume the night before your visit.
You should consume this meal at least 10h before your morning visit (not later than 9pm).
On the morning of your visit we will ask you to arrive fasted for at least 10h. You can drink water during this period but no other drink such as coffee or tea.
You will be sent reminders during the study on how to prepare for a visit.
If you have queries during the study, you can contact the study team at +44 (0)1603 255101 or email us at REST@quadram.ac.uk.

ReSt day - Intervention

There are two intervention visits. The only difference between the two visits is the type of bread you consume.

On arrival at the QI CRF, we will go through the plan for the day. We will provide a study bundle explaining the tasks of the day. A member of the study team will be with you during the visit.

We will ask you to arrive at the QI CRF having fasted for at least 10h and we will give you breakfast: a serving of bread with Flora diary free spread with a glass of water.

- You will be asked to scan the sensor on your upper arm.
- You will also be asked to complete a series of questionnaires on satiety. Satiety is the feeling of fullness and we want to determine how long before you feel hungry after breakfast. We will ask you to write on a scale between 0 and 10 how hungry, full, thirsty etc. you feel during the visit.
- We would also like you to complete a questionnaire about how the bread taste to you.
- After this You will be asked to collect a series of blood drops from your fingers tip to measure blood sugar levels.
- Four hours after breakfast, you will be served lunch. We would like you to eat lunch within 1.5/2h.

The intervention visits will last approx. 5 hours.

REST day- Lunch

During the intervention day, you will be given two options for lunch. You will be asked to consume the same lunch option on both intervention visits.
Additional helpings will be served until you let us know that you feel 'comfortably satisfied'.

Option A is white rice with tomato and basil sauce[2] and parmesan cheese.

Allergens recipe A: Milk, Parmesan cheese

Option B is white rice with beef bolognese sauce[3].

Allergens recipe B: Celery

[2]Tomato and Basil ingredients are Tomato (88%), Tomato Puree, Rapeseed Oil, Demerara Sugar, Garlic Puree, Basil (0.5%), Salt, Balsamic Vinegar (Red Wine Vinegar, Grape Must Concentrate), Black Pepper.

[3]Bolognese sauce ingredients are Minced British Beef (26%), Tomato Concentrate, Water, Tomato, Carrot, Onion, Tomato Puree, Celery, Red Wine (3%), Rapeseed Oil, Cornflour, Salt, Garlic Puree, Oregano, Sugar, Black Pepper.

Follow up

The day after the final visit (visit 4), you will be asked to remove the sensor from the back of the arm and return both the sensor and reader to us in a pre-paid envelope supplied.

We would also like you to complete a follow up questionnaire, which can be returned along with the sensor.

FAQs

DO I HAVE TO TAKE PART IN THIS STUDY?

No, taking part in this study is your choice. To help you decide, we will give you detailed information about the study over the phone or in an email, and in person during your first visit.

If you decide to take part, we will ask you to sign a consent form agreeing to take part.

CAN I WITHDRAW FROM THE STUDY?

Yes, you are free to withdraw from the study at any time without giving a reason. This will not preclude you from taking part in future QI studies. Data collected up to the point of withdrawal will be kept and, if possible, used in our data analyses to answer the study aims. Study data is analysed as a group and not by individual, so it will not be possible to identify you personally.
To withdraw, please contact the study team. We will send you a confirmation of your withdrawal from the study by email or mail.

WILL I BE GIVEN ANY MEDICATIONS DURING THE STUDY?

No, this is not a drug trial. We will not ask you to take any medicine.

WHO WILL CARRY OUT THE MEDICAL PROCEDURES?

The study will be carried out in the QI CRF. The QI CRF is an NHS-governed facility and all clinical procedures for this study will be carried out by the QI CRF team following NNUH

standard operating procedures. Clinical assessments and procedures will be performed by the QI CRF research nurse supported by a REST study team member. Non-clinical assessments or interventions (for example during intervention days) will be conducted by either the QI CRF research nurse or QI CRF Healthcare professional who is trained in NNUH emergency procedures, or a REST study team member.

WILL I GET MY TEST RESULTS?

As a volunteer you are valuable to us, but we are unable to tell you any of your individual results. However, the general findings of the study will be given to you in the form of talk or letter once the study is complete. Your GP will have access to your blood test results from the eligibility visit and will discuss any clinically relevant result with you directly.

WHY SHOULD I TAKE PART IN THIS STUDY?

You will be able to taste a high fibre white bread made from wheat grown in Norwich and baked at the Quadram Institute. This bread is not commercially available at the moment.

At the end of the study you will receive a report about the study outcome.

Your results will contribute to advancing nutritional research and the search for foods that can have a positive impact on our health. This study will contribute to understanding whether white flour with resistant starch helps prevent blood sugar levels from spiking and delay the return of hunger. By controlling blood sugar and the return of hunger, we hope to help reduce the risk of chronic diseases such as type II diabetes and obesity.

ARE THERE ANY DISADVANTAGES OR RISKS?

You should not experience any side effects by taking part in this study. You may feel a slight discomfort when giving a blood sample or when pricking your finger to take a blood

drop. This area may be slightly tender, sore or bruised after the procedure but these should fade within a few weeks. The continuous glucose monitoring device (the sensor) will be applied to the back of your upper arm using an applicator. Applying the sensor should not be more painful than a finger prick. As with a finger prick, you may feel a slight discomfort when applying the sensor and the application area may be slightly tender or sore after the procedure.

The sensor is kept in place by an adhesive that sticks to your skin. You may experience some irritation when removing the sensor which should fade with time.

Our test bread is a white bread with higher resistant starch content (a type of fibre). The resistant starch content of the bread is higher than in normal white bread but not higher than in a whole-wheat bread. If you are not used to consuming fibre, you may experience wind or bloating due to the change in diet.

WHAT IF THERE IS A PROBLEM?

If you have any concerns about the study, you should ask to speak to the QIB Principal investigator, Marina Corrado on 01603 255101 who will do her best to answer your questions. If you are still unhappy, and wish to complain formally, you can do this through the chairperson of the QI Human Research Governance Committee (HRGC) – Dr Antonietta Melchini on 01603 255030.

QIB accepts responsibility for carrying out trials and as such will give consideration to claims from participants for any harm suffered by them as a result of participating in the trial, with the exception of those claims arising out of negligence by the participant. QIB has liability insurance in respect of research work involving human volunteers. Please note that

the Institute will not fund any legal costs arising from any action unless awarded by a court.

If you wish to complain or have any concerns about the way you have been treated whilst taking part in this study at the QI CRF, there will be a local hospital complaints procedure that you can follow. If you wish to complain you should contact the Patient Advice and Liaison Service (PALS) at the NNUH on 01603 289036 (email: pals@nnuh.nhs.uk). Their offices are located next to Kimberley Ward, East Block Level 2 or please ask at the main reception desks at the Inpatient and Outpatient NNUH hospital entrances. The office has an answerphone which is available 24 hours a day and messages will be responded to as quickly as possible. As this study involves the QI CRF, which is an NHS facility, indemnity is provided through NHS schemes.

KEEP IN TOUCH!

For your safety and the success of the study, it is important that you let us know if your health changes.
Please tell us if you

- Have any episodes of illness, even if it is just a headache
- Are injured in any way
- Feel unwell during or after a visit to the unit
- Become pregnant

Some medicines affect the information we are collecting. Please tell us if you take any medication including over-the-counter medicines and those you purchase at the chemist or supermarket (e.g. acetaminophen, also known as paracetamol).
You should bring details of any medication (i.e. name of the medicine and the dose taken) you are taking when you come for your screening visit (visit 1).

EXPENSES AND PAYMENTS

Participation in this study is on a voluntary basis. However, we do recognise that taking part can cause some inconvenience and there are associated travel costs. Thus, you will receive £162.50 as an inconvenience payment upon completion of the study; if you withdraw or are excluded from the study, *payment will be pro-rata*. This means that you will be paid up until the point of withdrawal/exclusion from study. Travelling expenses to and from the QI CRF will be reimbursed on presentation of a receipt for buses or trains, or at the current QIB mileage rate for private cars. QI parking fees will be lifted. If you require transport to and from the QI CRF, please let us know and we will arrange and pay for a taxi.

All payments are liable to tax and you are responsible for declaring your own payments for tax purposes. Members of staff at QIB are free to participate in this study provided they meet the study criteria. However, we would like to point out that their inconvenience payment will be taxed at source in accordance with Biotechnology and Biological Science Research Council (BBSRC) and QIB rules and HM Revenue and Customs (HMRC). If you are in receipt of benefits this payment may affect your benefits.

HOW WILL YOU CONTACT ME?

We will contact you by phone, email or mail, depending on your preference. We will ask you how we should reach you during the first visit at QI.

WHO ORGANISES AND FUNDS THIS PROJECT?

This study is led by Miss Marina Corrado, based at QIB in the Food Innovation and Health Programme. The study is funded by the BBSRC and Norwich Research Park through the Biosciences Doctoral Training Partnership (grant number

BB/M011216/1) and the BBSRC QIB Institute Strategic Programme Food Innovation and Health BB/R012512/1 and its constituent projects BBS/E/F/000PR10343 (Theme 1, Food Innovation) and BBS/E/F/000PR10345 (Theme 2, Digestion in the Upper GI Tract).

WHAT WILL HAPPEN TO MY DATA AND SAMPLES?

We will store your clinical data and diet information anonymously in an electronic database at QIB. All information collected about you during the course of the study will be kept strictly confidential.
Hard copies of the clinical data will be archived separately from your personal information. Access to your personal records is restricted to the study team, the QI CRF research nurse and your GP.

Your biological samples collected during the screening visit will be processed at QI Clinical Research facility and sent to the Norwich and Norfolk University Hospital for analysis. These blood tests results will be logged on the hospital electronic system where your GP can access them if needed.

Your personal information is confidential! We follow Good Clinical Practice (GCP) and strict ethical and research governance rules. All information about you will be handled in
confidence. Data will be managed by the study team in compliance with EU General Data Protection Regulation (GPDR) and the UK Data Protection Act (DPA; 2018).

Any information that could show who you are will be held safely with strict limits on who can access it. We will store all your data using a unique a code number (ID) to protect your privacy and we work with trustworthy services (Libro app for dietary intake and/or the SMS reminder service) to ensure

your personal information are safe. QIB acts as data controller of the information collected during the study. This means that at the end of the study the information stored by the service providers will be returned to the data controller (QIB) and deleted from the Libro and SMS reminder service platforms.

The research team will record the study data and combine it with the information from everyone else in the study. The group data will be published in scientific journals and/or presented to the scientific community to share the outcome of the study. We will make sure that any other information that could show who you are is removed so you will not have a claim on the material collected or the data resulting from this study.

QIB is the sponsor of this study. We will be using information from you in order to complete this study and will act as the data controller for the study. This means that we are responsible for looking after your information and using it correctly. QIB will keep identifiable information about you for 15 years after the study has finished. Any archived identifiable data will not be used to contact you after the end of the study.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
The only people at QIB who will have access to information that identifies you personally, will be people who need to contact you under emergency unblinding procedures or audit

the data collection process. Other researchers won't be able to contact you to ask you about future research.

All research is subject to inspection and audit. Although your records may be accessed for this purpose, any personal information remains confidential. Please note, QI has CCTV cameras in use for security purposes.

You can find out more about how we use your information by contacting the QIB Data Protection Officer [Mr Mohamed Imran; mohamed.imran@nbi.ac.uk] or the QIB Human Studies coordinator (Dr Antonietta Melchini; antonietta.melchini@quadram.ac.uk).

WHO HAS REVIEWED AND APPROVED THE STUDY?

At QIB this research project has been reviewed by the QIB Human Research Governance Committee (HRGC), as well as an external Local Research Ethics Committee (REC). These are groups of independent people who review research to protect your safety, rights, well-being and dignity. This study has been reviewed by all committees and given a favourable opinion. Following ethical approval, the study protocol will also be registered at Clinicaltrials.gov, a publicly available database. It is good practice for all research projects to be registered in a publicly-accessible database and this supports our duties to promote research transparency.

REST Study reply slip

Please check the box if you are interested in taking part in this study or if you would like to know about it

I am interested in taking part in this study

☐

Please provide your contact details.

We will contact you to give you more information

Name .....

Address .....

.....

Telephone number .....

Email .....

REST Participant Information Sheet
Version 2
Date 27 June 2019
Principal Investigator: Marina Corrado
IRAS 262271

Get in touch

If you have any questions or concerns, please speak to a member of the REST study team during your visit or contact the Principal Investigator, Marina Corrado.

01603 255101
REST@quadram.ac.uk
https://quadram.ac.uk/reststudy/

Quadram Institute Bioscience
Norwich Research Park
Norwich, Norfolk
NR4 7UQ
UK

BBSRC | UK Research and Innovation
norwich research park Doctoral Training Partnership

4. REST study Standard Operating procedures

a. Study talk SOP

Greet Participant and make sure they have time to go through the study talk ~30min

The purpose of the study talk is to explain the PIS (which they should have seen), check the eligibility criteria and answer any questions they may have

1. Do they know QI? Have they been to QI before – new building? Explain where it is, where the study will take place: CRF, a hospital managed facility
2. Introduce yourself: you are a study investigator, the chief investigator is.. other investigators are.. and there will be a nurse if a medical procedure is involved
3. What is REST (page 3 – keep it simple, do not use the word "mutant")
4. REST is made of 4 visits (page 6)

a. Screening visit: describe activities, fasting 10h, results forwarded to GP - MAX 2h

b. If eligible: calibration: wear the sensors (talk about the sensors, wear for whole duration of the study, picture on page 7..), wait one hour to give reading and complete food diary. PROMPT: have they ever completed a food diary? No, we use an app but paper version also available and they get trained during the calibration visit – MAX 2h

c. Preparation to intervention the day before (no caffeinated drinks or alcohol 24h before, no strenuous exercise), can resume all these after the visit. Standard dinner (choice between meat based, or fish based, or vegetable based – the same before both interventions.

d. Intervention 1 : within 14 days from calibration. Breakfast with one type of bread, spread and water. Finger prick tests and satiety/sensory questionnaires.

PROMPT: have they ever done a finger prick test before? If not, can try at screening.

4h after breakfast, lunch ad libitum – eat until comfortably satisfied – rice-based meal with either tomato sauce+parmesan cheese or beef Bolognese sauce

e. 4 days between intervention 1 and 2

f. Complete the food diary: 3 days, 2 week and 1 weekend, not necessarily consecutive

g. Preparation to intervention and intervention 2 as previous

h. Day after last visit: remove sensors, complete follow up questionnaire and send back to study team

i. End of study description

5. Check eligibility criteria – specify that we are not recording anything, but it is important so that we do not make them come in for a screening visit for nothing
6. Check BMI: we have a specific range of BMI because we are measuring a specific metabolic response. DO you know your BMI? If not, do you know your weight and height? Offer to check for them on the NHS BMI calculator

If between 18 and 25 (included 25.5) then continue to eligibility checklist, otherwise thank participant for the time and stop here.

ELIGIBILITY CHECKLIST

- ☐ Are you between 18 and 65?
- ☐ Do you smoke? – casual smokers may be eligible if they can suspend smoking for 1 month before screening and for the duration for the study
- ☐ Are you pregnant?
- ☐ Do you suffer from any medical conditions such as diabetes, IBS, IBD or any other gastrointestinal disorder?
- ☐ Have you ever been diagnosed any form of cancer?
- ☐ Any history of cardiovascular disease?
- ☐ Are you on any medication? Prescribed or self-prescribed? MAKE A NOTE AND CHECK WITH MEDICAL ADVISOR
- ☐ Are you taking any dietary supplements? Check if willing to stop taking them during the study period if required MAKE A NOTE AND CHECK WITH MEDICAL ADVISOR
- ☐ Do you have any food allergies or intolerances?
- ☐ Have you ever experienced any allergic reaction to adhesives or medical glue?
- ☐ Are you a blood donor? – If yeas when was your last donation? Need to await 4 months since last donation for screening, needed for planning the visit
- ☐ Are you on a diet or plan to start a diet?
- ☐ Happy to wear the glucose sensors?
- ☐ Happy to consume the lunch and dinner choices mentioned?
- ☐ Can fast for 10h before visits, when required? DRINK PLENTY OF WATER
- ☐ Are you happy for us to contact your GP?

If eligible so far – go to page 17

7. Withdrawal: can withdraw at any time and without indicating the reason

8. Disadvantages and risks (page 19)
9. Keep in touch (page 21) – encourage communication with the study team
10. Expenses and payments (page 21)
11. Highlight that the PIS contains all our contact details
12. Any other questions?

If inclined to take part, can ask for availability or suggests dates for screening but the booking can be confirmed only 24h after the study talk.

Thank participant

b. REST Screening SOP

- ☐ Take consent – <u>COPY to participant</u>
- ☐ Data collection (personal information form)
- ☐ Fill in medical declaration form – <u>COPY to participant</u>
- ☐ Fill in inclusion /exclusion information (Pages 2,3,4 of the Case Report Form, this is a repeat of check at study talk)
- ☐ Observations (Page 5, CRF)
- ☐ Screening questionnaire take from observations
- ☐ Ready meal options form
- ☐ Bloods (Page 11, CRF) Page 11
    - 1 purple tube
    - 1 yellow tube
    - 1 grey tube
- ☐ Travel expenses form – fill in bank details

c. REST Calibration SOP

QUADRAM INSTITUTE BIOSCIENCE

STANDARD OPERATING PROCEDURE

Quadram Institute
Science Health Food Innovation

Title: REST Calibration visit

Applies to staff in: CRF	*Reference number:* (To be completed by QA Office)
Author: Marina Corrado	*Approver:*
Issue date: 10/01/2020	*Review date*: (To be completed by QA Office)
QA and H&S Review: (To be completed by QA Office)	*Version Number: 1*

1. PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

REST Calibration visit SOP

2. EQUIPMENT AND REAGENTS NEEDED

Gloves, sanitizing wipes

3. STEPS IN PROCEDURE (including safety information)

- Take participant folder, participant pack (2 sensors, 2 readers, scale) and food diary practice material (plate with post it)
- Take an envelope with return address and freepost code, a large zip bag and two small sip bags.
- Take out form participant folder the case report form, the CGM user manual, food-diary paper version and the preparation to intervention guidelines
- Show participant the sensors and reader. Explain the application process. To explain this you can use the flier in the CGM reader.
- Sensors are applied to the non-dominant arm, confirm with participant on which arm they should be applied.
- Ask participant to rest their forearm on the desk
- check that sensor and applicator serial number match. If they don’t replace them.
- Wear gloves
- Load sensor into applicator
- Clean application area with Abbott wipes
- Identify the area for application: sensor A is applied to the lower part of the deltoid, sensor B is applied below the deltoid, between bicep and tricep.
- Hold the arm and apply the applicator, when ready press firmly.
- To release the applicator gently pull and rotate until the applicator detaches.

- Check that the sensor is correctly applied (check adhesive), verify with participant that all is well.
- Apply stripe of Tegaderm to prevent detachment. Explain participant sensors features (robust and waterproof) but encourage care when getting dressed or using towels.
- Scan sensor with corresponding reader
- Set reader alarms
- Check that sound is configured s ON, LOW and vibration in ON
- repeat for the other sensor
- Proceed to Nutritics account set and training while waiting for sensors readings

SAFETY INFORMATION

Principal Hazards: biohazards

Method Steps involving Higher Risk (and precautions to minimise risk): Wearing PPE and wash hands before and after sensor application.

Essential Precautions:

Personal Protective Clothing: Gloves

Any restrictions on use: N/A

Exceptions or Warnings: In case of emergency, stay with the participant and press emergency button behind the green chair

4. RISK STATEMENT

A statement of the H&S risks associated with the method or instrument must be made, e.g. low, medium or high. The risk of individual elements of the procedure may also be stated where these are out from the general risk for the procedure, e.g. use of a specific chemical presenting a medium risk, whereas the overall risk is low.

All individuals using this procedure will be shown the risk assessment and given appropriate information, instruction and training in the risks and precautions necessary, including the use of any personal protective equipment required

SOP HEALTH RISK ASSESSMENT			
[1] Activity:	Calibration visit		
[2] Location of activity:	CRF Clinic room		
[3] Who is involved:	REST study team		
[4] Frequency of activity:	N/A		
[5] Duration of Activity:	2 hours		
[6] Chemical Hazard Name:	**Hazard Statements**	**Route of Exposure***	**Quantity Used**
[7] Details of biological agents of risk to human health:			**GMRA Number**
Contact with participant			

[8] Other Hazards:		Please ☒ as necessary			
Hot or Cold Burns	☐	Ionising Radiation**	☐	Ultra Violet or Infra Red	☐
Dust	☐	Noise	☐	Pollen Sensitizer	☐
Repetitive Action	☐	Extreme Cold Environment (< 0^{o}C)	☐	Lifting / Manual Handling	☐
Asphyxiation	☐	Cuts	☐	Electrical	☐

Slips / trips / falls	☐	Display Screen Equipment	☐	Nanomaterials (man-made particles, tubes, rods, or fibers ≤100nm in size)	☐
Other (give details)	☐				
[9] Control Measures:		Please ☒ as necessary			
Fume Cupboard	☐	Microbiological Safety Cabinet	☐	Total Containment Cabinet	☐
Ventilated Bench	☐	Spill Tray	☐	Trained personnel only	☒
Signs	☐	Reduce frequency/alternate activity	☐	Reduce duration of activity	☐
Sub divide a load	☐	2 persons lift of equipment	☐	Not for more than 1 hour	☐
Regular, short breaks	☐	Alternate activities	☐	Other (give details)	☐
[10] Personal Protection:		Please ☒ as necessary			
Lab coat	☐	Safety Glasses	☐	Face Shield	☐
Goggles	☐	Gloves *(specify)*	☒	Protective Gloves *(specify)*	☐
Ear defenders	☐	Other (give details)	☐		
[11] Is personal monitoring and/or health surveillance required?			Yes	☐	No ☒
Details:					
[12] Restrictions:		Please ☒ as necessary			
No lone working	☐	Not to be left unattended	☐	Named persons only	☐
In restricted area	☐	Risk Assessment Required for New or Expectant Workers	☐	Under constant supervision	☐

Not by under 18's	☐	Other (give details)	☐		
[13] Level of Residual Risk:		Please ☒ as necessary			
Low	☒	Medium	☐	High	☐
Name of Assessor:				Date :	

* Route of exposure; S = skin, I = ingestion, B = inhalation

** A special risk assessment for ionising radiation is required. For more information or advice see Radiation Safety Information on the intranet (http://intranet/infoserv/support/Safety/Radiation/index.htm), or refer to your local Radiation Protection Supervisor (RPS).

A special risk assessment may be required for specific hazards where there is significant risk e.g. manual handling of heavy/large items.

Activities involving new or expectant mothers and young persons also require additional risk assessment.

For specific risk assessment forms please refer to Risk Assessment on the intranet (http://intranet/infoserv/support/Safety/Risk/index.htm).

d. REST Intervention Visit

QUADRAM INSTITUTE BIOSCIENCE

STANDARD OPERATING PROCEDURE

Quadram Institute
Science Health Food Innovation

Title: REST Calibration visitCOVID19 situation

Applies to staff in: CRF	*Reference number:* (To be completed by QA Office)
Author: Marina Corrado	*Approver:*
Issue date: 10/03/2020	*Review date*: (To be completed by QA Office)
QA and H&S Review: (To be completed by QA Office)	*Version Number: 1*

1. PURPOSE OF PROCEDURE/METHOD AND ITS SCOPE

Intervention visit safety measures to reduce risk of exposure to Coronavirus for study participants and study team

2. EQUIPMENT AND REAGENTS NEEDED

Gloves, sanitizing wipes

3. STEPS IN PROCEDURE (including safety information)

In the multifunction room:

- Chair, pillow and table to be sanitised on Friday morning.
- Sanitise also all pens and timers, electric heat pads.
- Sanitise glucose meters before calibration.
- Take paperwork from the participant folder: case report form, study bundle, lunch sheet and checklist
- Calibrate meters using the two calibrating solutions, use gloves and record on booklet
- If you open a new vial of strips, you need to re-calibrate the meter.
- record meter Serial Number as the same meter needs to be used for all visits of a participant.
- Always wear gloves when using the meters, the strips or the unistick needles.
- Write time schedule on white board – reflect the checklist. If two participants are present, they need to be staggered by half hour.
- Participant is to wash hands after entering the multifunction room.
- If using a laptop/book/phones or other, these should be placed on a separate table and kept away when pricking. Offer to wipe these as well if possible so they can be used during the visit. If they cannot be wiped, then hand washing needs to be more frequent between pricks.
- Wipe the CGM readers using wipes when taken out
- Go though case report for intervention visit. Record how the participant travelled for the visit
- Explain the visit and the study bundle to the participant, answers any questions
- Do not talk while pricking to avoid contamination.

- Let participant record the glucose value on the study bundle and record time.
- Dispose of used strip in the sharp bin
- Change gloves after every prick.
- Participant to wash hands before and after breakfast, before and after lunch. Do not use alcohol wipes or any other wipes on the strips or on the fingers, if blood on fingers, ask the participant to wash their hands.
- After the last prick (before lunch) the table, pen and timer need to be sanitized
- After the visit clean room as follows – wear gloves:
- Chair, pillow and table to be sanitized on intervention morning.
- Use wipes to clean also all pens and timers, electric heat pads.
- Use wipes to clean glucose meters.
- Store meters in Clean Utility room
- discard gauzes in the clinical waste bin
- Discard needles in the sharp bin

In the kitchen

- Wash hands when entering the kitchen and clean the prep area with Dettol.
- Wear gloves and plastic apron thought-out
- Avoid talking while preparing foods to prevent contamination.
- If you have to sneeze, please step away, sneeze in the elbow. Change gloves and apron and resume prep activities.
- Stay in the area that was sanitized, do not share utensils while preparing
- Ask other study investigators or nurses to keep a safe distance when entering the kitchen.
- Start dishwasher if off.
- Take trolley with Basta boxes form the Test kitchen
- Weigh out plates with dedicated spoon and record the weight on the lunch sheet
- Place plates in the oven at 60C to keep the temp – use oven mitts to handle.
- Set trays, tissue and water bottles
- follow Lunch prep SOP
- After serving, clean up as usual.

SAFETY INFORMATION

Principal Hazards: biohazards

Method Steps involving Higher Risk (and precautions to minimise risk): finger-pricks and lunch preparation. Wearing PPE and wash hands frequently and thoroughly. Wipe any object the participant is carrying that will be in contact with hands during the visit, keep the object on a separate table.

Essential Precautions: Avoid talking while pricking fingers or preparing study meals.

Personal Protective Clothing: Gloves. Plastic apron the kitchen

Any restrictions on use: N/A

Exceptions or Warnings: In case of emergency, stay with the participant and press emergency button behind the green chair

4. RISK STATEMENT

A statement of the H&S risks associated with the method or instrument must be made, e.g. low, medium or high. The risk of individual elements of the procedure may also be stated where these are out from the general risk for the procedure, e.g. use of a specific chemical presenting a medium risk, whereas the overall risk is low.

All individuals using this procedure will be shown the risk assessment and given appropriate information, instruction and training in the risks and precautions necessary, including the use of any personal protective equipment required

SOP HEALTH RISK ASSESSMENT			
[1] Activity:	Intervention visit		
[2] Location of activity:	CRF multifunction room/ participant kitchen		
[3] Who is involved:	REST study team		
[4] Frequency of activity:	N/A		
[5] Duration of Activity:	6 hours		
[6] Chemical Hazard Name:	**Hazard Statements**	**Route of Exposure***	**Quantity Used**
[7] Details of biological agents of risk to human health:			**GMRA Number**
Coronavirus situation			

[8] Other Hazards:		Please ■ as necessary			
Hot or Cold Burns	☐	Ionising Radiation**	☐	Ultra Violet or Infra Red	☐
Dust	☐	Noise	☐	Pollen Sensitizer	☐
Repetitive Action	☐	Extreme Cold Environment (< 0°C)	☐	Lifting / Manual Handling	☐
Asphyxiation	☐	Cuts	☐	Electrical	☐

Slips / trips / falls	☐	Display Screen Equipment	☐	Nanomaterials (man-made particles, tubes, rods, or fibers ≤100nm in size)	☐
Other (give details)	☐				
[9] Control Measures:		Please ■ as necessary			
Fume Cupboard	☐	Microbiological Safety Cabinet	☐	Total Containment Cabinet	☐
Ventilated Bench	☐	Spill Tray	☐	Trained personnel only	■
Signs	☐	Reduce frequency/alternate activity	☐	Reduce duration of activity	☐
Sub divide a load	☐	2 persons lift of equipment	☐	Not for more than 1 hour	☐
Regular, short breaks	☐	Alternate activities	☐	Other (give details)	☐
[10] Personal Protection:		Please ■ as necessary			
Lab coat	☐	Safety Glasses	☐	Face Shield	☐
Goggles	☐	Gloves *(specify)*	☒	Protective Gloves *(specify)*	☐
Ear defenders	☐	Other (give details)	☐		
[11] Is personal monitoring and/or health surveillance required?			Yes	☐	No ☒
Details:					
[12] Restrictions:		Please ■ as necessary			
No lone working	☐	Not to be left unattended	☐	Named persons only	☐
In restricted area	☐	Risk Assessment Required for New or Expectant Workers	☐	Under constant supervision	☐
Not by under 18's	☐	Other (give details)	☐		

[13] Level of Residual Risk:		Please ■ as necessary			
Low	☒	Medium	☐	High	☐
Name of Assessor:			Date:		

* Route of exposure; S = skin, I = ingestion, B = inhalation

** A special risk assessment for ionising radiation is required. For more information or advice see Radiation Safety Information on the intranet (http://intranet/infoserv/support/Safety/Radiation/index.htm), or refer to your local Radiation Protection Supervisor (RPS).

A special risk assessment may be required for specific hazards where there is significant risk e.g. manual handling of heavy/large items.

Activities involving new or expectant mothers and young persons also require additional risk assessment.

For specific risk assessment forms please refer to Risk Assessment on the intranet (http://intranet/infoserv/support/Safety/Risk/index.htm).

e. Lunch Serving in CRF Multifunction room SOP

1. The food will be microwaved as per manufacturer instructions in the serving plate.
2. Once ready, the plate will be placed on a clean serving tray and covered with a clean plate cover.
3. The tray will be paced in the clean isolating food container. Before closing, we will take the food temperature with a laser probe – this needs to be at least 63C.
4. The container will be closed and placed on the trolley to be transported to the multifunction room.
5. Once in front of the participant in the multifunction room, the container will be opened.
6. The tray will be removed from the isolating container and placed on the side table.

While participants consume the portion (1), a new portion (2) will be prepared in the kitchen – steps 1-3.

When participant is about to finish portion 1, portion 2 will be brought over as is step 4 using a clean tray, cover and container.

Steps 5 and 6 will follow.

Meanwhile the previous tray with empty plate (1) will be placed in the original container and transported back to the kitchen on a trolley.

Containers and tray will be wiped with Dettol and leave for 2 min. The tray cover and plate and cutlery will be washed in the dishwasher for 3 min cycle.

f. Lunch preparation SOP

REST Study

REST Study

Intervention lunch SOP

Tomato sauce and parmesan cheese (A)

For big eaters – up to 12 servings:	For normal eaters – up to 8 servings:
2 3 rice pouches	3 2 rice pouches
4 3 tubs of tomato sauce	5 2 tubs of tomato sauce
6 1 parmesan cheese pouch	7 1 parmesan cheese pouch

8 Defrost 16h before visit in fridge (participant kitchen)

9 Open **tomato sauce**

9.1.1 Microwave for 2 minutes at 700W - HIGH

10 Squeeze the pouch to separate the rice.

11 Open rice pouch

11.1.1 Microwave for 2 minutes in a 700W - HIGH

12 Open cheese pouch, add **7g parmesan cheese** to the bowl

13 Weigh 85 g of tomato sauce

14 Weigh 80 g of rice and mix

15 Check temperature = **63C**

16 Place bowl/plate on a clean tray

Intervention lunch SOP

Beef bolognese sauce (B)

For big eaters – up to 12 servings:	For normal eaters – up to 9 servings:
2 3 rice pouches	3 2 rice pouches
4 4 tubs of beef sauce	5 3 tubs of beef sauce

6 Defrost 16h before visit in fridge (participant kitchen)

7 Open **beef Bolognese sauce**

7.1.1 Microwave for 2 minutes at 700W - HIGH

8 Squeeze the pouch to separate the rice.

9 Open rice pouch

9.1.1 Microwave for 2 minutes in a 700W - HIGH

10 Weigh 90 g of beef bolognese sauce

11 Weigh 80 g of rice and mix

12 Check temperature = **63C**

13 Place bowl/plate on a clean tray

14 Place tray inside clean food box and transport