Impact of processing on the carbohydrate quality and digestibility of pearl millet (*pennisetum glaucum*)

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Publications

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Igbetar, B.D., Bosch, C., and Orfila, C., ‘Pearl millet: a sustainable cereal with low glycemic potential’ (2018), the Nutrition Society summer Conference-Getting energy balance right, Leeds, England.

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Abstract

Pearl millet (*Pennisetum glaucum*) is a climate resilient cereal consumed widely in traditional diets in rural regions of Africa. It is a source of complex carbohydrates, and has a high content of protein and minerals compared to maize, rice and sorghum. Previous research has suggested beneficial effects of consumption of complex carbohydrates. However, there is limited research on the impact of processing on carbohydrate quality and digestibility of traditional millet products.

In this study, the impact of domestic processing on the carbohydrate composition and digestion pearl millet was investigated. Nigerian pearl millet was milled into flour particles ranging from 250 to 1000 µM in size and flours were used to prepare unfermented cooked porridge (*ibyer*) and fermented porridge (*ibyer-i-angen*) up to 72 h fermentation. The protocols for carbohydrate analysis and starch digestion used in this study were optimised using millet flour and validated with cooked samples.

The optimisation of carbohydrate analysis and starch digestion indicated that agitation is a key factor for the extraction of soluble sugars and effective digestion of millet starch. Some commercial enzymes used in carbohydrate digestion contained high levels of glucose and other sugars that can could interfere with the digestion and measurement of starch. The soluble sugar was low in millet flour (2.61%) and increased 2 folds after cooking and 6 folds after fermentation for 72 hours. This suggests the carbohydrate hydrolase activities in flour and fermentation medium. The starch content of millet was not altered by

cooking (68.80%) but decreased to 35.88% following 72 h of fermentation. Digestion of starch using optimised static digestion protocol showed that the gastric phase significantly improved the hydrolysis of millet starch ($p<0.05$), increasing starch digestion from 15.27 to 47.07% in millet flour. The starch digestibility of *ibyer* was around 56.01% and increased to 69.39 and 98.62% after 12 and 72 h of fermentation of *ibyer-i-angen*. Millet samples contained both rapidly digestible starch (RDS) and resistant starch (RS). The RDS and RS contents were 41.37 and 43.98% for (*ibyer*), 52.78 and 30.98% for 12 h and 64.96 and 18.35% for 24 h fermented *ibyer-i-angen*. The relatively high RS of *ibyer* was is in part explained by the high susceptibility of millet starch to retrogradation suggested by differential scanning calorimetry (DSC) of *ibyer*. The estimated GI of millet was low (<55) for *ibyer*, and intermediate (<69) 12 h and 24 h and high (>70) for 48 and 72 h fermented *ibyer-i-angen*. In conclusion, domestic processing had a significant impact on carbohydrate quality and digestion of millet with low glycemic potential. The modulation of starch resistance by processing could be exploited to produce *ibyer* and *ibyer-i-angen* to address specific nutritional requirements in both over and undernourished populations.

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Abbreviations

AMG ……. Amyloglucosidase

CHO …….. Carbohydrate

DMSO …… Dimethyl sulfoxide

DNS …… 3,5-Dinitrosalicylic acid

DSC …… Differential scanning calorimetry

eGI …….. Estimated glycemic index

FEG-ESEM …..... Field emission gun environmental scanning electron microscope

HPAEC-PAD …….. High performance anion exchange chromatography coupled with pulse amperometric detector

IMS …….. Isolated millet starch

PTFE ……. Polytetrafluorethylene

RDS ……. Rapidly digestible starch

RS ……. Resistant starch

SDS …….. Slowly digestible starch

SEM …… Scanning electron microscope

SGF ……. Simulated gastric fluid

SIF …….. Simulated intestinal fluid

Chapter 1: Introduction

1.1. Background

1.1.1. Nigeria as a multicultural nation

Nigeria is presumed to be the most populous black nation in the world. The population of Nigeria from the 2006 census was 140 431 790 with recent speculations putting the population between 185 990 000 to 190 000 000 (National Population Commission Nigeria, 2006; National Bureau of statistics, 2010; WHO, 2016; United Nations Department of Economics and Financial Affairs/Population Division, 2017). Nigeria is a multicultural country made up of 36 States located in the Western part of Africa. The States in Nigeria are grouped into six geo-political zones, each of which is made up between 5 to 7 States. The geo-political zones are the North West, comprised of Kaduna, Katsina, Kano, Kebbi, Sokoto, Jigawa and Zamfara States; the North East, comprised of Adamawa, Bauchi, Gombe, Yobe, Taraba and Borno States; the Middle belt, comprised of Benue, Kogi, Nasarawa, Federal Capital Territory (FCT) Abuja, Kwara, Niger and Plateau States; the South West, comprised of Lagos, Ogun, Oyo, Osun, Ondo and Ekiti States; the South East, comprised of Abia, Anambra, Enugu, Imo and Ebonyi States; and the South South, comprised of Edo, Bayelsa, Delta, Akwa Ibom, Rivers and Cross-River States (National Population Commission Nigeria, 2006; Nigerian government, 2016). Religious wise, the North is predominantly Muslims and the South predominantly Christians, and the Middle is partly Muslims and partly Christians.

1.1.2. Cultivation seasons and staples in Nigeria

In terms of climate, there are two major seasons in Nigeria, rainy and dry season. The rainy season begins in April and ends in October measuring between 500 to 2000 mm with a temperature range of 30 to 45°C and peak period between June and September. On the other hand, the dry season also referred to as “harmatan period” starts in November and ends in March with temperature between 12 to 37°C and peak period between December and March (Shiru *et al*., 2018). Nigeria produces most of the staples consumed by its population and this includes tuber and root crops like yam, cassava and sweet potato; and cereals like pearl millet, maize, sorghum, and rice. It is also a home for the cultivation of legumes and pulses like black eye beans, soybeans, pigeon peas and groundnut also referred to as peanut or monkey nut; and fruits and vegetables like banana, plantain, tomato, pepper, onions, spinach, oranges, and mangoes (Nigeria Government, 2016). These crops are cultivated all year and Table 1 shows the period of cultivation of some major staples in Nigeria, most of which are planted in the rainy season (FAO, 2018).

Table 1. Cultivation period of the major food crops grown in Nigeria within a year. P = Planting, G = Growing, H = Harvesting periods (FAO, 2018).

Crops	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Cassava	H				P	P	G	G	G	G	G	H
Rice	H				P	P	P	P	P	H	H	H
Maize1(North)					P	P	G	H	H			
Maize 2 (North)	H							P	P	G	G	H
Maize 3 (South)			P	P	G	H	H	H				
Pearl millet						P	G	G	H	H		
Sorghum					P	P	P	G	H	H	H	
Yam	H	P	P	G	G	G	H	H	H	H	H	H

1.1.3. Pearl millet production in Nigeria

In Nigeria, pearl millet is one of the main food crops and the only type of millet cultivated (Nigeria Government, 2016; FAO, 2018). Other known types millet grown globally include foxtail, finger, proso, khodo, little, and barnyard (Ren *et al.,* 2016; Saleh *et al.,* 2013; Dias-Martins *et al.,* 2018). In some studies, pearl millet was reported to be the fourth cereal-produced crop in the world with India, Niger, Nigeria and Mali being some of the largest producers (Osman, 2011; Saleh et al., 2013) and is a routinely consumed food crop in developing countries. However, the most recent millet record (Fig. 1) showed a decline in pearl millet production from 2010 to 2013. The decline in traditional cultivation of millet may be due to consumer food choice and industrial preference

(known grains that people like in different parts of the word determine products developed) as consumers may not be aware of the nutritional importance of millet. In developed countries such as the United States of America and Canada, millet is used primarily as forage (Dias-Martins *et al.,* 2018). It is important to note that there has been no recent update on millet production, last being 2015.

Figure 1. World's largest producers of millet as reported by FAO, (2015)

Generally, pearl millet is a climate-resilient cereal and cultivated at a relative wide temperature range. In Nigeria, the temperature range for growing millet is between 30 to 45°C and it takes about 2 months from cultivation to harvest (FAO, 2018; Shiru *et al.,* 2018). Millet is cultivated in many States in Nigeria (Fig. 2) and mainly in the Northern and Middle belt region of Nigeria.

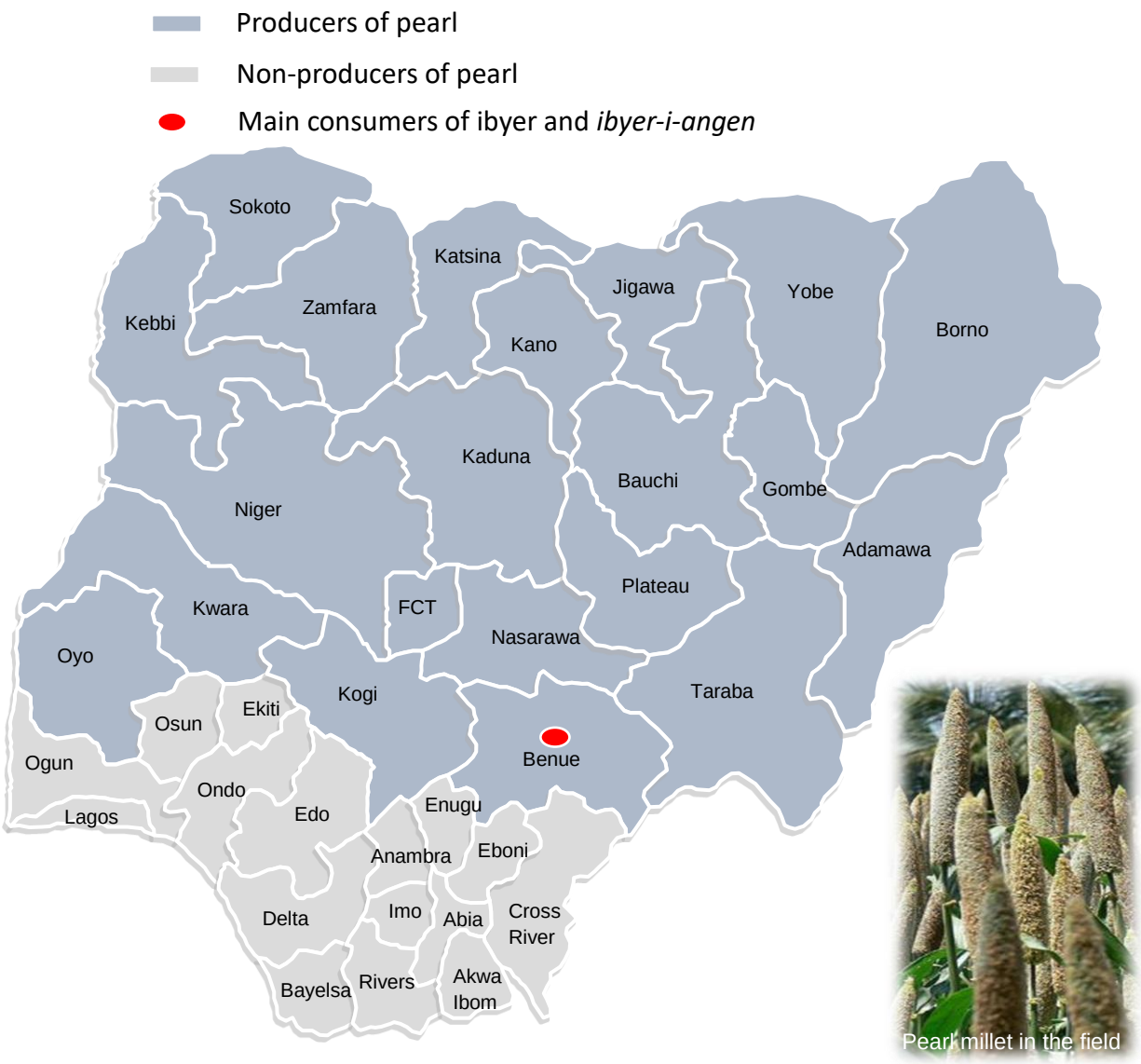

Figure 2. Nigerian map showing States where pearl millet is grown and State where *ibyer* and *ibyer-i-angen* is consumed.

1.1.4. Processing and consumption of millet

Millet is eaten as a meal after the combination of two or more domestic scale processing methods including milling, fermentation, roasting and cooking with water to make a porridge or gruel. Processing has been extensively used to enhance the utilization of millet (Chhiva and Sarita, 2012; Coulibaly *et al*., 2012; Saleh *et al*., 2013; Jaybhaye *et al.*, 2014). Other traditional and advanced technological methods of processing common to millet include; dehulling, milling and sieving, malting, extrusion cooking, popping, soaking, fortification and supplementation, blending into composite flour and drying enhance the texture, nutritional quality, digestibility and the acceptability of millet (Akinleye *et al.,* 2014; Jaybhaye *et al.,* 2014; AM and Bankole, 2015). Dehulling is one of the first steps carried out in processing millet (Chandrasekara and Shahidi*,* 2011; Jaybhaye *et al.,* 2014) by pounding, milling, or the use of centrifugal shellers. A study by Chandrasekara and Shahidi, (2011) investigated the effect of dehulling on the total polyphenolic content of millet and demonstrated that pearl millet is considerably rich in nutrients, particularly phytochemicals. Significantly high amounts of polyphenol are present in the hull of pearl millet compared to other types of millets (Chandrasekara and Shahidi*,* 2011). This means that the removing hulls is likely to result in a lower concentration of total polyphenol in dehulled millet flour. Although this is the case, it is important to remove the hulls in millet prior to food preparation because the hull is inedible and indigestible.

Fermentation is one of the oldest methods of processing in history. It is characterised by the breakdown of carbohydrates by the activities of microorganisms and

endogenous enzymes, and its application in millet products has been studied (Dhankher and Chauhan, 1987; Sharma and Kapoor, 1996; Elyas *et al.,* 2002; Abdelgadir *et al.*, 2005; Adebiyi *et al*., 2016; Akinola *et al*., 2017). These studies highlighted the importance of fermentation in reducing anti-nutritive properties in foods and enhancing the bioavailability and accessibility of nutrients, as well as altering the microstructure of starch. In Africa, traditional fermentation has remained a major pre-treatment step in the preparation of most cereal-based foods. Cereal flour or grains are soaked in water and left in the open under non controlled temperature conditions to allow for microbial growth to take place before or after cooking, depending on intended product (Mugula *et al.,* 2003; Muyanja *et al.*, 2003; Tou *et al.*, 2006; Nwachukwu *et al.,* 2010; Owusu-Kwarteng *et al.,* 2012).

1.1.5. Traditional Nigerian pearl millet based diets

In Nigeria, pearl millet is consumed daily as a gruel or porridge called *ibyer* and its fermented counterpart *ibyer-i-angen* (Fig. 3 and 4). More recently, a variety of products have been developed for larger scale commercial production including beverages, ready-to-eat porridges, fermented and unfermented breads, breakfast cereals, cookies and snacks (Wu *et al.,* 2014; Zhu 2014a; AM and Bankole, 2015; Farinde 2015; Izah *et al.,* 2016). Products made from millet are consumed as breakfast, lunch or dinner. Traditional cereal based food products are prepared mainly from maize, sorghum, rice, fonio and pearl millet and are grouped as thin porridge (*kunu*), thick porridges (*ogi, ibyer, ibyer-i-angen*), thick gel (*fufu, agidi*), puff batter (*masa*) or dough (*fura*). There are other very common meals in Nigeria (Fig. 4) prepared from roots and tubers,

legumes and pulses, and vegetables. Most Nigerian meals are eaten in combination with other meals and take longer than 1 hour to prepare.

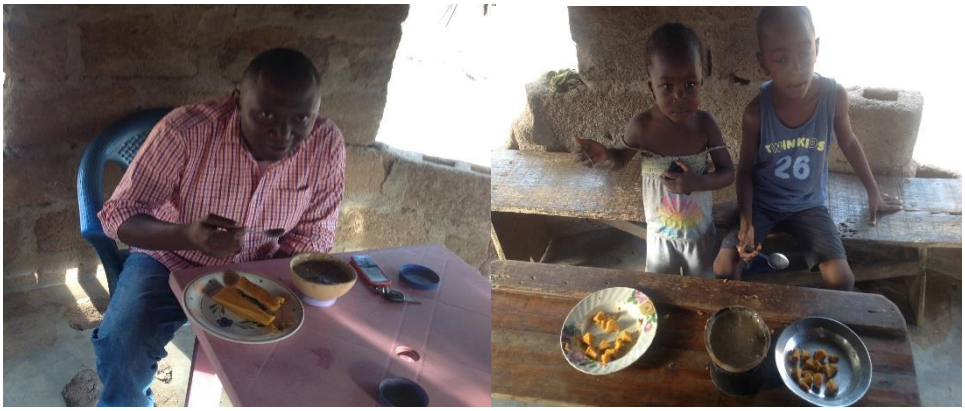

Figure 3. A village setting showing people eating *ibyer* with *moimoi* (bean pudding) in Makurdi, Benue State.

1.1.5.1. *Ogi*

This is one of the most popular gruels consumed in Nigeria, Ghana, and Benin (Greppi *et al.*, 2017; Akinleye *et al.,* 2014). Its preparation, physicochemical properties, microbial analysis, nutritional profile has been reported (AM and Bankole, 2015; Farinde, 2015; Izah *et al*., 2016). Millet, sorghum or maize is steeped in water and left to ferment over a period of 24 to 72 h in an open container, milled, sieved, decanted and boiled water is added to the slurry to cook it. *Ogi* is usually served hot and eaten with an accompanying meals such as *akara* (bean cake) and *moimoi* (bean pudding).

1.1.5.2. *Kunu*

Maize, millet or sorghum is used in the preparation of *kunu*. This gruel is indigenous to people in the Northern and middle-belt parts of Nigeria. Grains are steeped and left to

ferment for 24h, milled, sieved, boiled water is added to the mix without decanting, and stirred until cooked and left to cool before consumption (Wakil *et al*., 2013; Oluwajoba *et al.,* 2013).

1.1.5.3. *Ibyer* and *ibyer-i-angen*

This traditional cereal cooked porridge is prepared mainly from pearl millet and consumed as breakfast porridge by children and adults of the Tiv ethnic group from Benue State, Nigeria. The unfermented porridge is called *ibyer* and the fermented is referred to as *ibyer-i-angen*. The process of preparation is by adding of water to millet flour and cooking (*ibyer*), while for *ibyer-i-angen*, the reconstituted porridge is left in the open to ferment at 27 to 30 ºC overnight, and cooked by boiling with a predetermined volume of water. The prepared porridge is eaten hot with akara or moimoi. There is limited information on the preparation and nutritional profile of this gruel. A study by Kure and Wyasu, (2013) investigated the physicochemical properties of a similar gruel made from sorghum.

1.1.5.4. *Fufu*

Fufu is a term used to describe thick gel-like meal eaten with soup by the majority of Nigerians on a daily basis as part of lunch and dinner. It is made from cereals such as millet, maize, wheat, fonio, sorghum, and rice flour, and root and tuber plants such as yam and cassava. Preparation of *fufu* from cereal starts with milling grains, sieving to remove chaff, cooking in boiled water until a thick highly viscous gel is formed. *Fufu* from yam is prepared by peeling and drying then it is milled to flour for the preparation of *amala* (black coloured *fufu*) or peeled and boiled in water, and the cooked yam is then pounded while hot in a traditional wooden mortar and pestle. For cassava, it is

either peeled and dried to mill as flour, or peeled and fermented to for the preparation of *akpu*, or peeled and grated and then fermented for the preparation of *garri*. The processed cassava flour is cooked in boiling water into a thick gel, while the *akpu* is moulded in balls and cooked and pounded. On the other hand, the processed *garri* is made into *eba*. All the types of *fufu* are eaten with an accompanied soup (Fig. 4) as lunch or dinner.

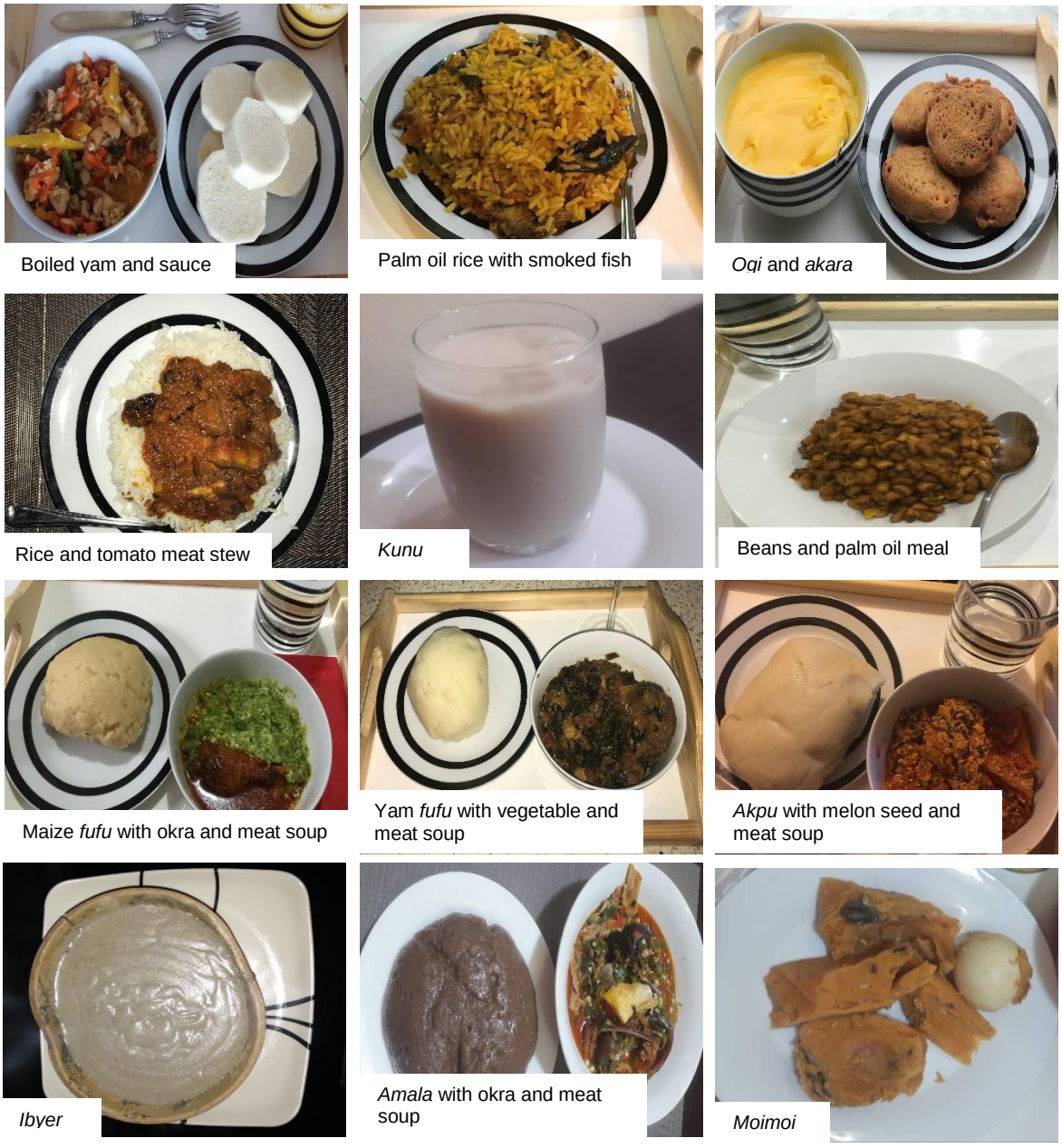

Figure 4. Common traditional Nigerian meals.

Chapter 2: Literature review

2.1. Pearl millet

Pearl millet also referred to as '*amine*' amongst the Tiv ethnic group of Benue State, Nigeria. It is one of many types of small seeded plants of the poaceae family. Pearl millet is known under different species names such as *pennisetum glaucum*, *pennisetum typhoides*, *pennisetum americanum* and *pennisetum spicatum* (Sripriya and Chandra, 1997; Amadou *et al.,* 2014; Annor *et al.,* 2017; Lemgharbi *et al.,* 2017; Dias-Martins *et al.,* 2018). Other common species of the poaceae family are finger millet (*eleusine coracana*), proso (*panicum miliaceum*), and foxtail millet (*setaria italic*). The grains of pearl millet are small (between 1.5-5.5 mm) and have different shapes (supplementary data: Fig. S1), and typically greenish-brown in colour. Millet is one of the oldest food crops grown, and its cultivation dates back to 5550 BC in Asia (Crawford, 2006).

2.2. Nutritional properties of millet

Millets are gluten free, which makes them an important seed crop as part of a diet by everyone. The nutritional composition of pearl millet has been studied previously (Hulse *et al.,* 1980; Chhavi and Sarita, 2012; FAO, 2012; Amadou, 2014; Saleh *et al.,* 2013; Devisetti *et al.,* 2014; Akinola *et al*., 2017). The carbohydrate, protein, crude fibre, fat and ash content of pseudocereals was reported (Table 2) and have similar nutritional properties as pearl millet. A review by Saleh *et al.,* (2013) reported that compared to wheat, sorghum, rice and maize,

pearl millet is richer in protein, crude fibre, and minerals. It also has significant amount of isoleucine, leucine, histidine, glutamic acid, methionine and threonine and other amino acid when compared to other commonly consumed cereals (Osman, 2011; Chhavi and Sarita, 2012; Saleh *et al*., 2013; Amadou *et al*., 2013). Adding to the uniqueness of pearl millet, its grains have reasonable amount of polyphenols (832.2 µg/g) and trace nutrients like calcium, phosphorus and magnesium as shown on Table 3 (Saleh *et al.,* 2013; Hithamani and Srinivasan, 2014; Muthamilarasan *et al*., 2016). The most abundant nutrient component in millet and cereals is carbohydrate.

Table 2. Proximate composition of selected cereals. Data are presented as g/100 g dry matter for protein, fat, crude fibre and carbohydrate (CHO), and mg/100 g for ash (from Saleh *et al.,* 2013).

Food	Protein	Fat	Ash	Fibre	CHO
Rice (brown)	7.90	2.70	1.30	1.00	76.00
Wheat	11.60	2.00	1.60	2.00	71.00
Maize	9.20	4.60	1.20	2.80	73.00
Sorghum	10.40	3.10	1.60	2.00	70.00
Pearl millet	11.80	4.80	2.20	2.30	75.00
Finger millet	7.70	1.50	2.60	3.60	72.00
Foxtail millet	11.20	4.00	3.30	6.70	63.20
Common millet	12.50	3.50	3.10	5.20	63.80
Little millet	9.70	5.20	5.40	7.60	60.90
Barnyard millet	11.00	3.90	4.50	13.60	55.00
Kodo millet	9.80	3.60	3.30	5.20	66.60

Table 3. Trace mineral composition of different species of millet. Data are presented as mg/100 g dry matter (from Saleh *et al.,* 2013)

Grain	P	Mg	Ca	Fe	Zn	Cu	Mn	Mo	Cr
Pearl millet	379	137	46	8.0	3.1	1.06	1.15	0.07	0.023
Finger millet	320	137	398	3.9	2.3	0.47	5.49	0.10	0.028
Foxtail millet	422	81	38	5.3	2.9	1.60	0.85	–	0.070
Proso millet	281	117	23	4.0	2.4	5.80	1.20	–	0.040
Little millet	251	133	12	13.9	3.5	1.60	1.03	–	0.240
Barnyard millet	340	82	21	9.2	2.6	1.30	1.33	–	0.140
Kodo millet	215	166	31	3.6	1.5	5.80	2.90	–	0.080

2.3. Carbohydrates in millet

Carbohydrates are the most abundant nutrients in cereals that contribute to human nutrition. Carbohydrate serves as a storage source for plant seeds, including cereals, and is primarily located in the endosperm (Fig. 5). Carbohydrates make up 56.10 to 76.10% of the total seed weight in pearl millet (Oshodi, 1999; Dias-Martins *et al.,* 2018; Amadou *et al.,* 2014; Saleh *et al.,* 2013). The variation in carbohydrate composition is influenced mainly by the method of determination and variety of millet analysed. Nevertheless, cereals, especially millets, maize and sorghum have similar carbohydrate composition. Carbohydrate comprises of mono, di and polysaccharides. In brief, mono and disaccharides are small molecular weight and relatively soluble in water. In food composition and nutrition, carbohydrates soluble in water are referred to as ‘sugars or simple sugars.’ Polysaccharides are high molecular weight molecules, categorised in nutrition as starch and fibre (or non-

starch polysaccharides). When starch is found within the plant cell structure (i.e surrounded by plant cell wall), the carbohydrate is referred to as ‘complex’ carbohydrates (Southgate, 1995; Malleshi et al., 1986). The bioavailability of carbohydrate in traditional pearl millet diet will provide a good understanding on how to develop functional foods.

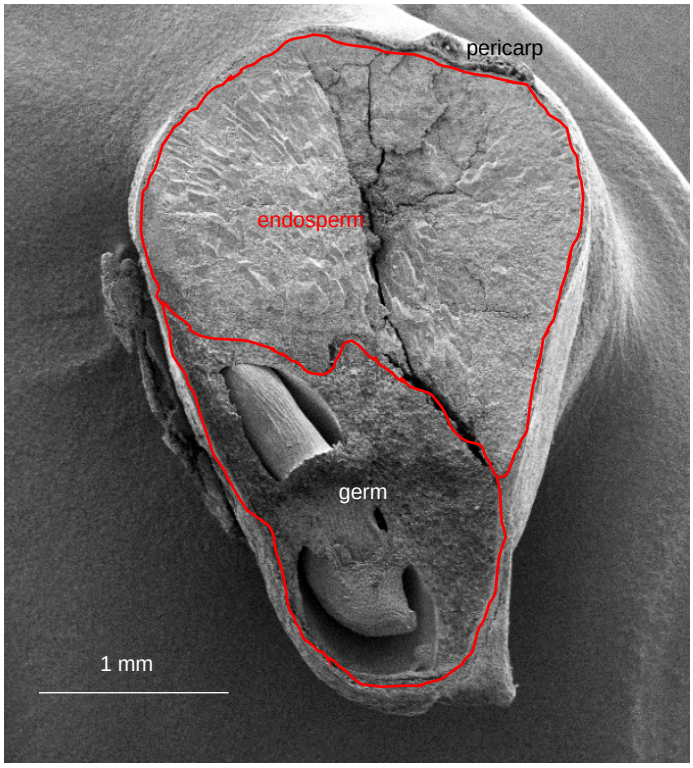

Figure 5. SEM microscopy of split pearl millet grain showing pericarp, endosperm and germ.

2.3.1. Sugars

The composition of common sugars in the diet, their digestion and metabolism are relatively established (Southgate, 1995; Wong and Jenkins, 2007). Glucose, fructose, maltose and sucrose are the main sugars in millet products (Malleshi et al., 1986, Rao and Muralikrishna, 2001; Rao and Nagasampige, 2011; Amadou

et al., 2014; Oshodi, 1999). However, galactose, and fructo-oligosaccharides raffinose and stachyose have also been reported (Malleshi et al., 1986; Oshodi, 1999). The sugars from millet have been mostly extracted by suspension in methanol, ethanol solution, water, copper-reagent and simulated intestinal fluid, and quantified using different detection methods (Malleshi et al., 1986, Rao and Muralikrishna, 2001; Rao and Nagasampige, 2011; Amadou *et al.,* 2014; Oshodi, 1999). The sugar yield from different studies differed significantly. For example, the extraction with copper-reagent gave a higher sugar yield (5.28%) compared to ethanol solution (1.44%) method (Malleshi et al., 1986; Oshodi, 1999) and different identified sugars. Thus, a more harmonised method for sugar extraction and quantification if developed will be useful in order to compare results of millet products.

2.3.2. Millet starch

Starch is the main carbohydrate component of cereal grains acting as an energy source for the developing plant during germination. The starch content of millet grains ranges from 51.35 to 74.12% of the grain's dry weight. The variation is attributed to genotype, season of cultivation, processing treatment, region and experimental methods used to measure starch (Saleh *et al.,* 2013; Yang *et al.,* 2013; Zhu 2014a; Lemgharbi *et al.,* 2017; Djurle *et al.,* 2016; Srichuwong *et al.,* 2017). Genotypic variation was evident in the highly variable starch content measured in different pearl millet cultivars in Algeria (Lemgharbi *et al.,* 2017). It

is therefore important to consider the cultivar of pearl millet when comparing starch data between studies.

Starch consists of two different molecular components, amylose and amylopectin (Fig. 6). Amylose is a linear polymer of α-(1→4) linked glucose while amylopectin has an amylose-type backbone with frequent branches of α-(1→4) linked glycans, linked to the backbone through (1→6)-α-linkages (Li *et al.,* 2017; Srichuwong *et al.,* 2017; Wang *et al.,* 2013; Zhu, 2014). Most millet starches are composed of around 20-30% amylose and 70-80% amylopectin (Zhu 2014 and Annor *et al.,* 2017). During starch synthesis in the grain, amylose and amylopectin self-assemble into compact ordered structures within membrane bound organelles called amyloplasts, which contain starch granules (Zhu, 2014). Pearl millet starch is composed of between 6-32.5% amylose content of which is higher than little millet (18%), barnyard (20%) and similar to foxtail (11.2-32%), but lower than the estimation for finger (14.4-38.6%) and proso (17.2-33.9%).

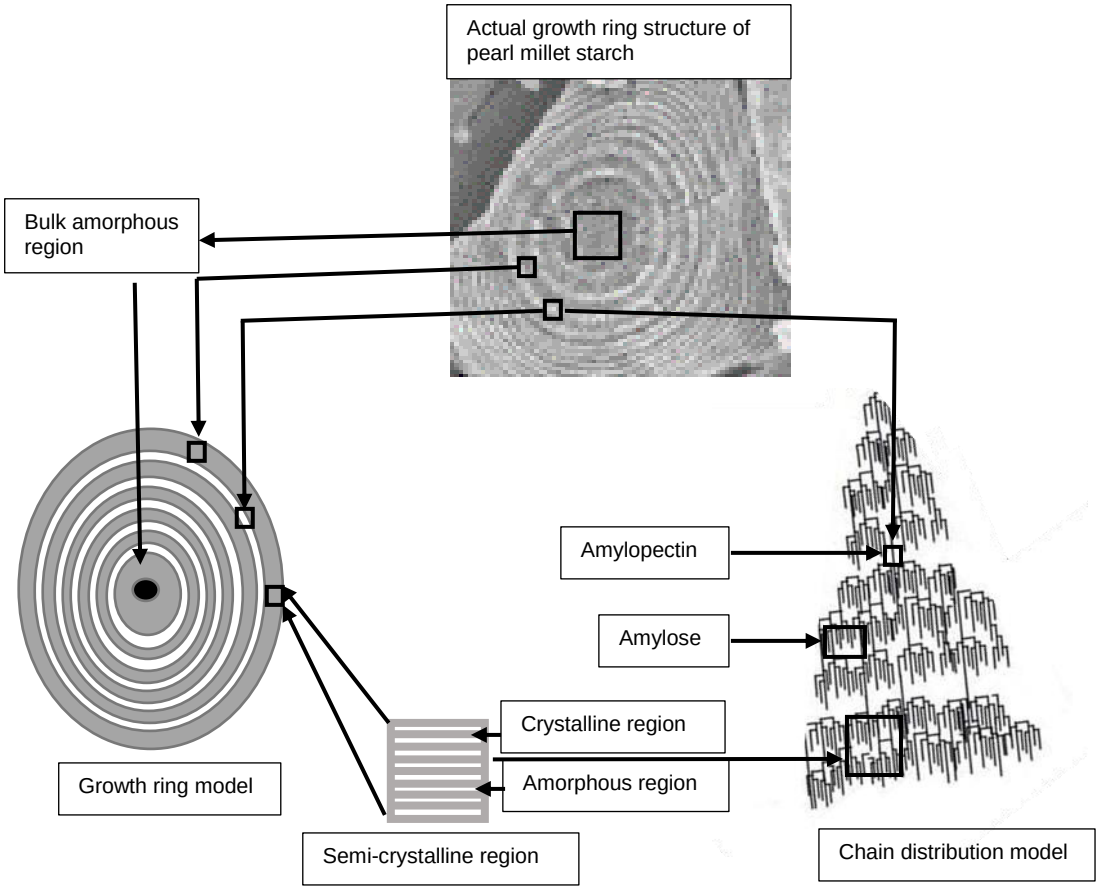

Figure 6. Starch granule growth rings in the amorphous region of pearl millet starch granule.

2.3.2.1. Properties of millet starch

Millet starch exhibits the A-type polymorphic arrangement, found typically in cereals, where little water is found within the amylopectin crystalline forms, compared to the more open structure of the type-B polymorph, found typically in potatoes and other tubers (Perez and Bertoft, 2010; Annor *et al.,*2015; Zhu,

2014). The granules of millet starch are typically smaller in comparison to wheat, sorghum, maize, but bigger than quinoa, and mostly polyhedral in shape with apparent pores at the surface (McDonough and Rooney, 1989; Srichuwong *et al.,* 2017; Saccomanno *et al.,* 2017). The granule size and pores are thought to facilitate gelatinisation and digestion of starch by providing access to amylases. Also, the importance of other grain components including protein and lipids in regulating starch digestion is an emerging area of research (Wong *et al.,* 2009; Ren *et al.,* 2016; Zou *et al.,* 2016).

2.3.2.2. Starch crystallinity and gelatinsation

Starch crystallinity is the imperfect order in which the starch polymers are arranged within a granule. The crystalline structure of starch is altered by either gelatinisation or melting (Cooke and Gidley., 1992). Gelatinisation of starch results from heating starch in the presence of water whilst melting resulting in the heating of starch in the absence of water. During gelatinisation, the amorphous region of starch first takes up water, hydrating the granule and causing it to swell, hence, allowing the crystalline region to also take up water, which destabilises the granule structure (Slade and Levine., 1988). Cereal starches including millet, maize, rice, wheat and sorghum gelatinise at temperatures between 60 to 85 °C (Hsu *et al.,* 2015; Srichuwong et al., 2017; Ye *et al.,* 2018). The variation in gelatinisation temperature reported in these studies are dependent of variety of starch, moisture-starch ratio, and rate of heating temperature per minute. For instance, variety difference was evident in the higher gelatinisation temperature

of millet compared to wheat and quinoa (Srichuwong et al., 2017). Therefore, it will be interesting to conduct gelatinisation analysis of millet in comparison to how its relative food product as eaten is prepared.

2.3.2.3. Starch isolation and content estimation

The methods for the estimation of starch content and starch isolation in cereals has been developed and widely applied (Englyst *et al.,* 1992; Goñi *et al*., 1997; Al-Rabadi *et al.,* 2011; Decker *et al*., 2014; Suma and Urooj, 2015; Akinola *et al.,* 2017). Starch isolation has been a key step to study the properties of starch. Common methods used for starch extraction include wet milling alone, alkaline-protease wet milling, alkaline, acidic, and neutral buffer method (Bustos *et al.,* 2004; Zhu, 2014; Van Hung et al., 2016; Ren *et al.,* 2016; Srichuwong *et al.,* 2017). These methods may have profound effect on the starch yield. Zhu, (2014) reviewed the isolated starch yield of different cereals and showed varying content amongst reports.

Starch content is the amount of glucose in carbohydrates determined after the chemical or enzymatic degradation of complex carbohydrate and the detection of glucose produced. The amount of free glucose (i.e the amount of glucose soluble in water) or sugars is deducted from the overall glucose yield (Englyst *et al.,* 1992). Therefore, if not carefully corrected, the free sugars or glucose present in food sample may give a false contribution to starch yield in the analysed product. The most common methods of estimating starch in food samples is the dissolution

of starch by acid hydrolysis followed by an enzymatic hydrolysis of maltose to glucose (Englyst *et al.,* 1992; Goñi *et al.,* 1997). Amyloglucosidase is the most commonly used enzyme for starch content analysis. Typically, food samples analysed are subjected to different pre-treatment processing including milling, gelatinisation and fermentation, and concentration of enzyme used in experimental procedure differ from sample to sample (Goñi *et al.,* 1997; Englyst *et al.,* 1992; Amadou *et al.,* 2014). Thus it may be important to understand the properties of the samples been analysed, especially their homogeneity in solution in order to determine the best method to estimate starch. For instance, compared to cereal food samples, the starch content in legumes is more easily analysed when subjected to pre-treatment like prolonged gelatinisation prior to analysis because of the hardness of the grains (Goñi *et al.,* 1997).

2.4. Classification of starch digestion (digestion, absorption and glycemic response)

Generally, classification of starchy food samples is based on their digestion (accessibility), and how the digestion products (sugars) are metabolised (bioavailability) and used for different physiological functions (Englyst *et al.,* 1997; Heaton *et al.,* 1989; Ren *et al.,* 2016). High bioavailability and accessibility of products of carbohydrate digestion have been associated to obesity and type-2 diabetes and when low, it can have potential health benefits (Heaton *et al.,* 1988; Edward *et al.,* 2015; Mlotha *et al.,* 2015; Van Hung et al., 2016; Ren *et al.,* 2016). Therefore, it is necessary to provide information on commonly consumed food products. To give a better understanding on carbohydrate digestibility, Englyst *et*

al., (1992) characterised starches based on speed of digestion: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RSD, SDS and RS are starch products available after the activities of digestive enzyme within 20min, 20 to 120 min and starch not hydrolysed after 120 min under a simulated *in vitro* condition. The RS content and starch digestion are very important factors in determining the glycemic index.

2.4.1. *In vitro* starch digestion of millet

The starch digestibility of different species and varieties of millets has been studied (Hu *et al.,* 2004; Alka and Shruti, 2012; Annor *et al.,* 2015; Ren *et al.,* 2016; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017; Yang *et al.,* 2018) with results varying from 14.24 to 78.50% up to 120 min incubation for the intestinal phase of digestion. It is important to note that the physical state of samples analysed and starch digestion methods used in these studies varied greatly. For example, Lemgharbi *et al.,* (2017) reported low digestibility ranging from 14.24 to 22.83% for different cultivars of millet flour using only the intestinal phase of digestion, while up to 45% was reported when gastric digestion preceded the intestinal phase (Srichuwong *et al.,* 2017). It should be noted that when the PhD project started in 2015, little evidence was available for pearl millet.

2.4.2. *In vitro* digestion methods

In vitro starch digestion protocols are listed in Table 4. The parameters and conditions in protocols including pH of buffer, type of enzymes, concentration of enzymes and incubation time vary greatly between studies. In fact, only few studies have evaluated the effect of the gastric phase on starch digestion, especially in pearl millet. It is therefore necessary to compare starch digestion data using harmonised methods. The methods developed by Englyst *et al.,* (1992) and Goñi *et al.,* (1997) are most common starch digestion methods used in research. Recently, the INFOGEST designed static digestion protocol to harmonise previously developed methods to give better interpretation of data (Minekus *et al.,* 2014). However, examples of the current research using the INFOGEST protocol focused on lipid and protein digestion (El *et al.,* 2015; Mat *et al.,* 2016). In a few cases, it has been used to investigate the release of other nutrients, such as carotenoids from spinach (Eriksen *et al.,* 2017) and the intactness of cell walls during simulated digestion of fruit smoothie, puree and juices (Chu *et al.,* 2017). There has been only one study on starch digestion of cereal products using INFOGEST, where the starch digestibility of processed products; white bread, pasta, spaghetti and cookies made from wheat and gluten free bread made from rice were measured (Bustos *et al.,* 2017). Interestingly, the INFOGEST protocol has not yet been applied to millet or its products.

Table 4. *In vitro* starch digestion methods used in different cereals

Method of sugar detection	Type of millet and treatment	buffer	Phases	Type of enzyme and unit (U) used	Incubation time, pH and temperature	Starch digestibility (%)	Author
Glucose oxidase and peroxidase kit	Pearl millet (unfermented flour)	HCl solution	Gastric	Pepsin, unit not given	5 min, pH not given, 37°C	58 to 70	Srichuwong, *et al* 2017
		Maleate buffer	Intestinal	Pancreatic α-amylase, 22 U and amyloglucosidase unit 6.6 U	240 min, pH 6, 37°C		
Glucose oxidase and peroxidase kit	Pearl millet (unfermented flour)	Phosphate buffer	Intestinal	Pancreatic α-amylase, unit not given	120 min, pH 6.9, 37°C	14.24 to 22.83	Lemgharbi *et al.,* 2017
		Sodium acetate buffer	-	Amyloglucosidase unit 0.25 U	45 min, pH 4.8, 60°C		
Glucose-peroxidase kit	Foxtail millet (cooked)	Acetate buffer	Gastric	Pepsin, 3570 U	30min, pH 5.2, 37°C	75.20 to 91.20	Ren *et al.,* 2016
		Continued with gastric digesta	Intestinal	Pancreatin, 150 U amylase activity Amyloglucosidase, 33.64 U and Invertase, 112.15 U	30min, pH 5.5, 37°C 120min, pH 5.5, 37°C		
DNS reagent	Pearl millet (unfermented and fermented flour)	Phosphate	Intestinal	Pancreatic α-amylase, unit not given	120min, pH 6.9, 37°C	15.00 to 29.00	Alka and Shruti, 2012

Oxidase-peroxidase kit	Proso millet (cooked)	Phosphate	Intestinal	Pancreatic α-amylase, 20.33 U and amyloglucosidase, unit 2.7 U	120min, pH 5.2, 37°C	81.01 to 91.88.	Yang *et al.,* 2018

2.4.3. Glycemic Index of millet

The glycemic index is a tool that uses data generated from starch digestibility of foods *in vivo* to provide a circulated comparison of the potential glycemic response in individuals, compared to a standard carbohydrate load (Englyst *et al.,* 1992; Goñi *et al.,* 1997; Heaton *et al.,* 1988). In most cases, the reference food is either glucose or white bread. Glycemic index can be predicted from *in vitro* digestibility data. Hence, estimating the glycemic index is useful in the development of foods to meet specific nutrition and health needs of individuals, especially in managing the diet of the growing diabetic population. The estimated glycemic index (eGI) of pearl millets has been reported (<70) (Annor *et al.,* 2015; Lemgharbi *et al.,* 2017). It has not been as studied as other types of millet which have presented data that correlates with *in vitro* studies (Heaton *et al.,* 1989; Annor *et al.,* 2015; Ren *et al.,* 2016; Lemgharbi *et al.,* 2017). Following the classification of glycemic index of foods i.e low (<55), intermediate (between 56 to 69) and high (>70), pearl millet falls between low and intermediate (Goñi *et al.,* 1997; Trinidad *et al.,* 2010; Wolter *et al.,* 2013; Lemgharbi *et al.,* 2017; Annor *et al.,* 2015). Limited information is available for the GI of pearl millet compared to cereals such as rice and maize. Considering how important pearl millet is to millions of people globally, information on how other food preparation can influence GI will be useful.

2.5. Factors affecting starch hydrolysis of millet

Many factors have been previously attributed to the starch digestibility (especially the RS fraction) and glycemic index of millets; they include processing, particle size of flour, and genotype of cultivar, amylose content, and fermentation (Ren *et al.,* 2016; Lemgharbi *et al.,* 2017; Annor *et al.,* 2015).

2.5.1. Particle size

The particle size of flour has been suggested to contribute to starch digestion. Though there is scarce information on the effect of particle size of pearl millet on starch digestion, there have been studies on barley, wheat and oat flour (Heaton *et al.,* 1988; Al-Rabadi *et al.,* 2011; Al-Rabadi *et al.,* 2009). These studies demonstrated that manipulating the particle size of flour before food preparation was an important factor modulating digestion rate of starches. To explain this, a study on sorghum and barley by Al-Rabadi *et al.,* (2009) points to more intact cell walls in larger flour particles (>500 µM) limiting enzyme access to starch during hydrolysis.

2.5.2. Cooking

Cooking by boiling a food sample in water remains one of the oldest methods of food processing used to improve the nutritional profile of starch-based foods. The ratio of food and water, and extent of cooking is thought to have a pronounced effect on the physical and chemical properties of foxtail millet, rice and beans

(Goñi *et al.,* 1997; Amadou *et al.,* 2014; Ren *et al.,* 2016; Hu *et al.,* 2004; Ye *et al.,* 2018). For instance, cooking of foxtail millet resulted in more than 2-fold increase in starch digestion. Ren *et al.,* (2016) reported higher digestibility of foxtail millet cooked with higher ratio of water and lower with less water. The ratio of water to sample during cooking is thought to influence the rate of diffusion into granule and complete disruption of starch (Al-Rabadi *et al.,* 2011; Ren *et al.,* 2016). The starch digestibility of pearl millet has been reported (Srichuwong *et al.,* 2017; Lemgharbi *et al.,* 2017; Annor *et al.,* 2015; Alka and Shruti, 2012), majority of these studies were on raw flour. Therefore, it is useful to provide information on the how cooking methods can contribute to the starch digestibility of pearl millet.

2.5.3. Fermentation

Fermentation is important because it is thought to improve the palatability, nutritional profile and the preservation of foods (El Hag et al., 2002; Alka and Shruti, 2012; Saleh *et al.,* 2013; Amadou *et al.,* 2014). Traditional fermentation remains a major processing step in many African cereal based diets. *Kunu* and *ogi* are the most common fermented porridges that form part of breakfast meals across Nigeria (Oluwajoba *et al.,* 2014; Akinleye *et al.,* 2014; AM and Bankole, 2015) with *ibyer-i-angen* only popular in Benue state. *Kunu* and *ogi* are prepared from maize, sorghum or pearl millet, but *ibyer-i-angen* is mainly prepared from pearl millet porridge. Studies on fermented millet products are mainly focused on their proximal composition (Kheterpaul Chauhan, 1989; Kheterpaul and Chauhan, 1991; Oluwajoba *et al.,* 2014; Akinleye *et al.,* 2014; AM and Bankole, 2015) with limited studies reporting starch digestion (Sharma and Kapoor 1996;

Alka and Shruti, 2012). Alka *et al.,* (2012) investigated the effect of fermentation of pearl millet, maize and sorghum up to 36 h and reported significant improvement in their starch digestibility.

2.5.3.1. Microbiological profile of fermented millet

The microbial profile of fermented of food product are important indicators of the quality of that food. Traditionally, fermentation of millet are mainly by alcoholic formation from 2 h with lactic acid from 8 h and yeast fermentation (Lei and Jakobsen, 2004; Tou et al., 2006). Groups of Lactic acid bacteria isolated from *koko*, a fermented Ghanaian beverage was predominantly *Weisella confuse*, *Lactobacillus fermentum*, *Lactobacillus salivarius* and *Pediococcus species,* which were shown to be active during in vitro digestion analysis (Lei and Jakobsen, 2004) and may have probiotic potentials to gut health. Consequently, it is likely that the *ibyer-i-angen* may have similar benefit to gut health.

2.6. Potential health benefits of millet

In vivo studies provide evidence on the physiological metabolism of food and their influence on health. Evidence from *in vivo* studies such as randomized controlled trials (acute and chronic) study has shown that the quality of carbohydrate in plant based diet i.e GI and RS content are critical in managing degenerative health conditions such as diabetes and obesity (Southgate, 1995; Heaton *et al.,* 1989; Fayet-Moore *et al.,* 2017; Ren *et al.,* 2016; Thathola *et al.,* 2010). In recent times, there have been emerging claims that suggest millet based diets have potential in combatting diabetes. Evidence to support this was provided from a study on

low GI foxtail millet products by Thathola *et al.,* (2010). This study showed a positive impact on type-2 diabetic patients by the lowering effect of foxtail millet on blood glucose, triglyceride and cholesterol. Another example still on foxtail millet also reported strong correlation between low GI and reduced blood glucose (Ren *et al.,* 2016). Considering that available data suggests that pearl millet has low to intermediate estimated GI (Annor *et al.,* 2015; Lemgharbi *et al.,* 2017), it may play a critical role in controlling postprandial glucose if ingested by individuals with compromised blood glucose regulation.

Recently, Annor *et al.,* (2017) and Dias-Martins *et al.,* (2018) reviewed and highlighted the potential benefit of pearl millet as an alternative healthy dietary choice. It was clear from the reviews that more evidence based researches are needed for pearl millet and its products, especially in providing information on GI and important starch fraction contents (SDS and RS). Furthermore, pearl millet is gluten free (Dias-Martins *et al.,* 2018) and can therefore be an alternative to some of the most utilized grains such as wheat, oat, rye and barley, especially for people with celiac disease. Hence, pearl millet is arguably a good choice as part of a diet for everyone globally.

2.7. Justification of millet research

Pearl millet is a very popular cereal in Africa and is used in the preparation of a wide range of products consumed by adults and children. It is a staple source of nutrients to millions of people in developing countries (Lemgharbi *et al.,* 2017)

and is suggested as an emerging food in developed countries whose populations are interested in sustainable plant-based diets. A large number of people consume pearl millet daily as a gruel or porridge called *ibyer* and its fermented counterpart *ibyer-i-angen* in the Benue State of Nigeria. However, there is limited data on nutritional profile of pearl millet and its products.

There is need to harmonise methods for estimating the carbohydrate composition in food, especially in millet as it is difficult to compare results between studies. Understanding the starch digestibility of millet is a good example why it is important to harmonise methods. So far, different methods, enzyme concentration, different digestive enzyme and have been used to estimate carbohydrate composition in pearl millet (Alka and Shruti, 2012; Annor *et al.,* 2015; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017) all yielding widely varied results.

Furthermore, compared to other traditional millets like foxtail and proso, only a few studies have been reported on the digestibility of pearl millet or its products both in *in vitro* and *in vivo* studies (Ren *et al.,* 2016; Lemgharbi *et al.,* 2017; Yang *et al.,* 2018). Majority of pearl millet studies are on flour. The effect of particle size reduction, food preparation, and fermentation of foxtail millet, sorghum, oat and wheat has been studied (Heaton *et al.,* 1989; Al-Rabadi *et al.,* 2011; Souilah *et al.,* 2014; Ren *et al.,* 2016). Particle size of flour is thought to have a positive impact on nutrient availability. This is evident as research on grain fractions of sorghum and barley flour showed similar trend of higher starch digestion in

smaller particle sizes of flour compared to larger ones (Al-Rabadi *et al*., 2011). Hence, manipulating the particle size of pearl millet to what is obtainable (250 to 1000 µM) will provide data on the relationship of particle size in relation to digestibility. Considering that pearl is an emerging food promoted to meet the dietary needs of the growing population of the world, studies on its products will be useful. Interestingly, traditional methods of processing pearl millet such as milling, fermentation and cooking are cheap and easy to apply.

Furthermore, though *in vivo* methods are expensive to conduct, methods used in *in vitro* analysis have presented data that had strong correlation with *in vivo* studies (Ren *et al.,* 2016). It is important to note that *in vitro* methods are cost effective and easy to use to obtain data. In using a harmonised *in vitro* method, the gastric and intestinal phase of digestion will be a factor when comparing the digestibility of millet products. This will add to the already researched data on starch digestibility of food (Goñi *et al.,* 1997, Englyst and Englyst, 2005; Germaine *et al.,* 2008) and provide one of the first results for starch digestion using INFOGEST protocol (Minekus *et al*., 2014).

There has been a growing rate of non-communicable diseases globally, particularly with prevalence of diabetes, which is among the common health conditions in Nigeria, and predicted to be the 7th major cause of death globally in the near future (WHO, 2015). However, dietary intervention is suggested as a way to manage diabetes (WHO, 2015; Uloko *et al.,* 2018), especially the ingestion of foods which are high in resistant starch and low in glycemic index. Pearl millet

has these attributes and an added advantage of being a gluten free grain (Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017; Annor *et al.,* 2015; Annor *et al.,* 2017, Dias-Martins *et al.,* 2018) and it is therefore an excellent option that could meet the nutritional needs of diabetes and also celiac disease, vegetarian and malnourished populations. In fact, more recently, there has been a sharp increase in the global production of millet (Wu *et al.,* 2014; FAO, 2015), especially in countries that were not originally millet producers. This may be due to the realization of the nutritional potential of millet.

2.8. Outline and structure of research

2.8.1. General aim of thesis

This research attempts to explore the impact of processing on the carbohydrate quality and digestibility of pearl millet products, and its benefit to human nutrition.

2.8.2. Objectives

1. To optimise protocols for the carbohydrate digestibility and analysis of pearl millet products.
2. To evaluate the impact of particle size reduction of flour and cooking on the carbohydrate quality and digestibility of pearl millet.
3. To evaluate the impact of fermentation time on the carbohydrate quality and digestibility of pearl millet products.
4. To use in vitro data to estimate the starch fractions and GI of pearl millet products.

2.8.3. Hypothesis

Domestic processing i.e size reduction, cooking and fermentation will have a positive contribution on the digestion of starch *in vitro*. Considering that the GI of millet has been reported in other studies, the pearl millet samples used in this study might show a GI of <70.

Chapter 3: Materials and methods

3.1. Plant material

Dehulled pearl millet (*pennisetum glaucum*) used for this project was procured in September 2015, from Makurdi Modern market, Benue State, Middle Belt of Nigeria.

3.1.1. Preparation of porridge

Pearl millet used for this study was washed with tap water to remove grit and dirt. The clean millet was dried at 38°C, for 24 h in a SANYO incubator (MIR-262). The dried millet was milled with a kenwood food mixer in combination with a spice grinder and sieved into different particle sizes of flour (250, 500 and 1000 µM) using Endecolt test sieve shaker. Modified traditional method of preparation of *ibyer* (unfermented porridge) and *ibyer-i-angen* (fermented porridge) was adopted (Fig. 7). For the preparation of *ibyer,* millet flour of different particle sizes were reconstituted in water at a ratio of 1:5.5 (w/v) and mixed by stirring and subsequently cooked on a Fisherbrand ARE hot-plate stirrer set at 300°C for 30 min. For the *ibyer-i-angen*, millet was reconstituted in water and placed in a SANYO incubator (MIR-262) set at 30°C and allowed to ferment (natural fermentation) for up to 72 h before cooking by boiling as previously described for *ibyer*. The cooked porridge was allowed to cool to room temperature, and then frozen at -80°C. The frozen samples were freeze-dried using a Labconco Freezone 2.5 L benchtop free dryer and stored in a dark cupboard for subsequent analysis.

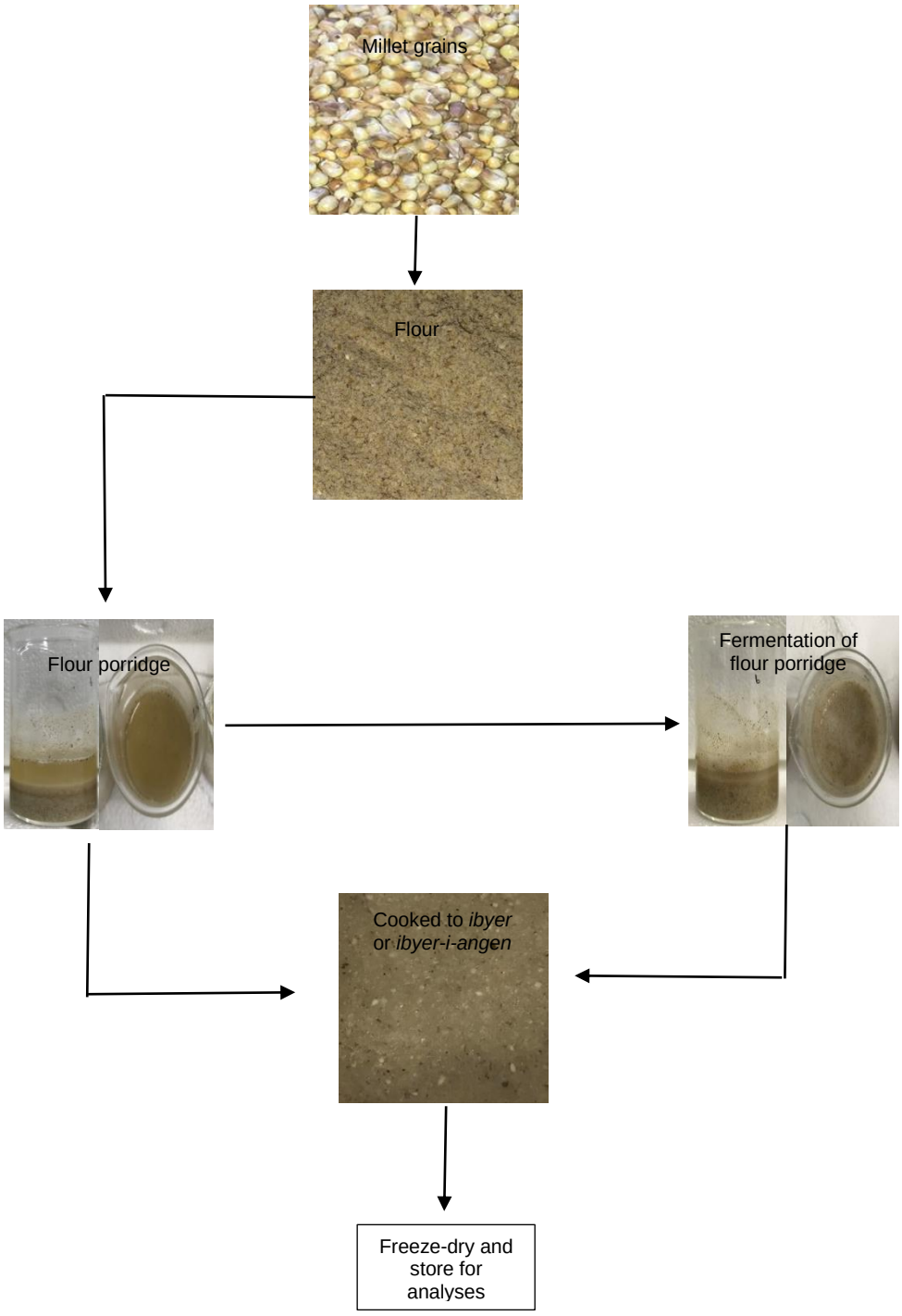

Figure 7. Summary of the preparation of *ibyer* and *ibyer-i-angen.*

3.2. Carbohydrate digestion

3.2.1. Preparation of buffers

3.2.1.1. SGF and SIF

Buffer solutions of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by Minekus *et al.,* (2014) and presented in Table 5. Each simulated fluid was made up to a volume of 500 mL with Millipore water, and 2 M HCl and 2 M NaOH was used to adjust the pH to 3 and 7 using Hanna instruments HI-2210-02 pH bench meter. The prepared simulated fluids were stored at 4°C and used for 4 months.

Table 5. Preparation of simulated gastric and intestinal fluids (adopted from Minekus et al 2014)

Constituents	Stock conc. (mol L^{-1})	SGF (pH 3)		SIF (pH 7)	
		Vol. of stock (mL)	Conc. of SGF (mmol L^{-1})	Vol. of stock (mL)	Conc. of SGF (mmol L^{-1})
KCl	0.5	6.9	6.9	6.8	6.8
KH_2PO_4	0.5	0.9	0.9	0.8	0.8
$NaHCO_3$	1	12.5	25	42.5	85
NaCl	2	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	0.15	0.4	0.1	1.1	0.33
$(NH_4)_2CO_3$	0.5	0.5	0.5	-	-

3.2.1.2. TRIS-EDTA-sodium metabisulfite buffer (pH 7.5)

To make the final concentration of buffer, Tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid (TRIS-EDTA)-sodium metabisulfate buffer consisted of 50 mM TRIS, 10 mM EDTA and 3 mM sodium metabisulfite. The pH was adjusted to 7.5 using 2 M HCl and 2 M NaOH. The buffer is stored at 4°C overnight before use.

3.2.1.3. Sodium acetate buffer (pH 5)

To make the final concentration of sodium acetate buffer, 0.2 M sodium acetate and 0.2 M acetic acid solutions were prepared separately. From the solutions made, a ratio of 59:41 of sodium acetate solution and acetic acid solution were mixed. The pH was adjusted to 5 with 2 M acetic acid. Afterwards, the buffer was stored at room temperature.

3.2.1.4. Sodium phosphate buffer (pH 7.0)

To prepare the final concentration of phosphate buffer used for enzyme activity assay, a solution consisting of 20 mM sodium phosphate monobasic and 6.7 mM sodium chloride was made, and pH adjusted to 7 with 2 M HCl and 2 M NaOH. Phosphate buffer was then stored at room temperature.

3.2.2. Enzyme preparation and α-amylase activity measurement

3.2.2.1. Preparation of pancreatic pepsin, α-amylase and pancreatin and AMG

Table 6 shows the enzymes used in this study and solutions which were prepared on the day of analysis. A 35 mg of pepsin (Sigma Aldrich) was suspended in 1 mL of SGF to make a stock of 25000 U/mL. From the pepsin stock made, 200 U per mL was used in the final digestion of gastric phase. A 1 mg α-amylase (Sigma Aldrich) was suspended in 15 mL of sodium phosphate buffer for enzyme activity measurement. For pancreatin (Sigma Aldrich) 1700 mg was suspended in 5.313 mL of SIF to prepare a stock of 1600 U/mL α-amylase activity. From the pancreatin stock made, 200 U per mL α-amylase activity was used in the final digestion of intestinal phase. Amyloglucosidase (AMG) from Sigma Aldrich and Megazyme were already prepared. From the Megazyme stock, 0.23 mL was added to 4.77 mL of sodium acetate buffer (buffer had sample in it) to obtain 150 U per mL in digesta. While for AMG from Sigma Aldrich, 2.5 mL was added to 2.5 mL of sodium acetate buffer to obtain 150 U/ml in digesta.

The final units of enzyme used in carbohydrate digestion were optimised using millet and discussed in Chapter 4.

Table 6. Summary of enzymes used in this study.

Enzyme	Code	Manufacturer	Activity if the bottle	Organism/origin	Storage (°C)
Pepsin	P7000	Sigma Aldrich	714 U/mg	Porcine gastric mucosa	4
α-amylase	A3176	Sigma Aldrich	15 U/mg	Porcine pancreas	4
Pancreatin (8X USP)	P7545	Sigma Aldrich	5 U/mg α-amylase activity	Porcine pancreas	-20
AMG	A7095	Sigma Aldrich	300 U/mL	*Asperigillus niger*	4
AMG	E-AMGDF	Megazyme	3260 U/mL	*Asperigillus niger*	4

3.2.2.2. Determination of sugars in enzymes

For the determination of sugars in the enzyme solutions, enzymes were prepared as described previously (final units used in digestion protocol) but simulated fluid was substituted with water. The pepsin (Sigma Aldrich), α-amylase (Sigma Aldrich), pancreatin (Sigma Aldrich), Sigma AMG and Megazyme AMG were diluted at a ratio of 1:200, 1:20, 1:10, 1:1600 and 1:100 within limit of detection. Sugar standard solutions used were glucose, fructose, maltose, sucrose, maltotriose, arabinose, galactose, raffinose, stachyose and fucose (internal standard) and glycerol sugar alcohol. Sugar standards and enzyme solutions were analysed by HPAEC-PAD (see section 3.2.5.1).

3.2.2.3. Determination of α-amylase enzyme activity in pancreatin

To measure the enzyme activity of α-amylase in pancreatin, pancreatic α-amylase was used as positive control. Prior to preparation soluble potato starch, enzyme solution of 1 mg mL^{-1} of pancreatic α-amylase and 0.05 mg mL^{-1} of pancreatin were prepared separately with phosphate buffer. Afterwards, 1% w/v soluble potato starch (substrate) was suspended in phosphate buffer, and heated on a hot-plate fitted with a vortex at 70°C for 15 min. The suspension was mixed constantly using a magnetic stirrer until starch was completely dissolved. The dissolved potato starch was allowed to cool to room temperature. Then, two sets of tubes were labelled; for amylase activity in pancreatic amylase, and pancreatin. Enzyme solution was added to potato starch solution as described in Table 7, before incubating the mix at 20°C for 3 min in a shaking water bath. After 3 min of incubation, 1 mL DNS colour reagent was added to inactivate enzyme (see DNS method section 3.2.5.2). Maltose standard and potato solutions were prepared for DNS analysis as described in section 3.2.5.2.3 and boiled for 15 min in a water bath (Grant SBB 14). The concentration of maltose standard solution prepared was between 0.05-2 mg mL^{-1}. Enzyme activity was calculated using equation 1, 2, and 3 and the dilutions during enzyme preparation were factored.

$$\text{Units/mL} = \frac{\boldsymbol{Amount\ of\ maltose\ produced\ (mg)}}{\boldsymbol{volume\ of\ enzyme\ used\ (mL)}} \quad \textbf{Equation 1}$$

$$\text{Units/mg of enzyme} = \frac{\frac{\boldsymbol{units}}{\boldsymbol{mL}}\ \boldsymbol{of\ enzyme\ used}}{\frac{\boldsymbol{mg}}{\boldsymbol{mL}}\ \boldsymbol{solid\ of\ enzyme\ used}} \quad \textbf{Equation 2}$$

$$\text{Units/mg protein in enzyme} = \frac{\frac{\boldsymbol{units}}{\boldsymbol{mL}}\ \boldsymbol{of\ enzyme\ used}}{\frac{\boldsymbol{mg}}{\boldsymbol{mL}}\ \boldsymbol{protein\ in\ enzyme\ used}} \quad \textbf{Equation 3}$$

Table 7. Preparation of substrate-enzyme solution for enzyme activity analysis.

Sample	Tube A	Tube B	Tube C	Substrate blank
Potato starch solution (mL)	1.00	1.00	1.00	1.00
Pancreatic α-amylase/pancreatin solution (mL)	0.50	0.70	1.00	(-)
Water (mL)	0.50	0.30	(-)	1.00

3.2.3. *In vitro* digestion of millet

Two *in vitro* protocols were used to measure the starch digestibility of millet; a simple non-gastric method (Goñi) and INFOGEST method by Goñi *et al.,* (1997) and Minekus *et al.,* (2014) with modifications as shown in Table 8. The oral phase of the digestion was excluded for both methods. Once the final method for the INFOGEST was determined, it was used for all digestion measurements and discussed in future Chapters. The original INFOGEST protocol involved oral, gastric and intestinal digestion of food samples. However, it was modified for this current study because the protocol was not standardised previously for starch digestion.

Table 8. Summary of non-gastric Goñi and INFOGEST starch digestion methods.

	Goñi	**INFOGEST**
Gastric phase	No SGF	SGF
	No pepsin	Pepsin
	NO $CaCl_2$	$CaCl_2$
Intestinal phase	SIF	SIF
	No bile	Bile
	No $CaCl_2$	$CaCl_2$
	Pancreatin	Pancreatin
	AMG	AMG
Detection method	HPAEC-PAD	HPAEC-PAD

3.2.3.1. Non-gastric Goñi *in vitro* starch digestion method (Goñi)

The non-gastric method (Goñi) starts from the intestinal phase of digestion. A 250 mg of test sample in dry weight was suspended in SIF and incubated with pancreatin (200 U of α-amylase activity in final digesta) at 37°C for 3 h in a shaking water bath with intermittent mixing by inversion. Enzyme activity was inactivated and pH was adjusted to 5 with 2 M HCl and centrifuged for 15 min at 5000 rpm at 20°C. The supernatant was collected and incubated with 150 U AMG (Megazyme) for 45 min at 60°C. The enzyme activity was inactivated and 4

volumes of absolute ethanol was added to sample and centrifuged for 15 min at 20°C and at 5000 rpm for 15 min at 20°C.

3.2.3.2. INFOGEST *in vitro* starch digestion method

For INFOGEST protocol, 250 mg of test sample was weighed and first suspended in SGF containing 75 µM $CaCl_2$ and the pH was adjusted to 2.5 before incubating with 2000 U pepsin and for 2h at 37°C in a shaking water bath. Enzyme was inactivated by boiling at 100°C and cooled to room temperature. Then, 300 µM $CaCl_2$ and 160 mM bile salt solution were added to the digesta and the pH was adjusted to 7 with 2 M NaOH before adding SIF. After the pH was adjusted, digesta was incubated with pancreatin (200 U/mL of α-amylase) for 3 h and at 37°C in the shaking water bath. During the intestinal digestion in shaking water bath, digesta was mixed intermittently by inversion method. At the end of the intestinal digestion, enzyme activity was inactivated by boiling at 100°C. The pH was adjusted to 5 and sodium acetate buffer was added and as well centrifuged at 5000 rpm at 20°C for 15 min. The supernatant collected was incubated with 150 U AMG for 45 min at 60°C. The enzyme activity of AMG was inactivated and 4 volume of absolute ethanol was added, centrifuged at 5000 rpm for 15 min at 20°C.

The supernatant collected from the Goñi and INFOGEST digestion methods were diluted to obtain concentrations between 0.5-12 µg mL^{-1} and spiked with internal standard (fucose, final concentration of 5 µg mL^{-1}). Glucose released from millet samples and enzyme blanks (supernatant with only enzyme) was detected by

HPAEC-PAD and results from enzymes were subtracted the results of millet digestion. The final results were presented as total starch digested in dry weight after multiplying with 0.9 to convert glucose to starch.

3.2.3.3. Estimation of the glycemic index of millet

The hydrolysis index (HI) and glycemic index (GI) of pearl millet samples was estimated by first fitting the starch digestion data to the first-order equation (Eq. 4) as established by Goñi *et al.,* (1997). Then the HI and GI were calculated from the area under curve (AUC_{exp}) relative to 0 to 120 min starch digestion of millet samples (Eq. 5). The results obtained from equation 1 and 2 were expressed as the ratio of the area under curve of white bread (9453) using INFOGEST digestion to give the HI of millet samples. The estimated GI (eGI) was calculated by integrating the HI of millet to equation 6 (Goñi *et al.,* 1997) and then the result was multiplied by 0.7 to obtain the estimated amount of glucose compared to reference sample (white bread).

$C_t = C_\infty (1-\exp [-kt])$……**Equation 4**

$AUC_{exp} = C_\infty t_f - C_\infty/k(1-\exp [-kt_f]$……**Equation 5**

eGI = 39.71 + 0.55(HI)……...**Equation 6**

Where C_t = starch digestion at time t.

C_∞ = the equilibrium digestion of starch at 120 min.

k = kinetic constant at time t

t_f = final time

3.2.4. Extraction of soluble sugar in millet

An optimised method was used to extract, quantify and identify the free sugars in millet flour and the cooked porridges. The determination of free sugars was to test the hypothesis that type of solvent, degree of mixing of suspension and processing will significantly affect the sugar content of millet. 250 mg of unfermented and fermented millet samples (flour of different particle sizes and cooked porridge) was suspended in 10 ml of water was agitated for 1 min and up to 25 min with SIF on a Scientific industries SI-0236 vortex-Genie 2 mixer (120 V). This was followed by centrifugation for 15 min at 5000 rpm and at 20°C by ThermoFisher Scientific centrifuge (Heraeus megafuge, 16R). The supernatant was diluted to obtain maltose or glucose concentrations within the ranges of 0.05-2 mg mL^{-1} before the detection of sugars in samples by DNS method. The results generated were presented as percentage reducing sugar.

After the optimal sugar extraction method was established as suspension of sample in SIF followed by agitation for 10 min, 4 volumes of absolute ethanol were added to the extracted sugar supernatant to precipitate protein and soluble polysaccharides. This was further diluted to concentrations between 0.5-12 µg mL^{-1} and spiked with internal standard (fucose, final concentration of 5 µg mL^{-1}). Sugars were detected and identified by HPAEC-PAD and results generated were presented as percentage free soluble sugars in dry weight of flour.

3.2.5. Analytical methods for carbohydrate quantification and qualification

3.2.5.1. HPAEC-PAD analysis

3.2.5.1.1. Equipment

A high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) Dionex ICS-5000 ion chromatography system (Thermo Scientific) was used.

3.2.5.1.2. Principle of HPAEC-PAD

The HPAEC-PAD quantifies carbohydrates by measuring charges due to oxidation of sugars in alkaline solution on the surface of a gold electrode. The HPAEC is equipped with GP40 gradient pumps, a PAD apparatus consisting of electrochemical detectors with gold and silver reference electrodes, an autosampler (AS) and a chromatography enclosed column oven. A CarboPac Pa20 analytical column (3x150 mm with a capacity of 65 µeq) was used to provide high resolution and good separation of simple sugars and oligosaccharides.

The mobile phase used was 300 mM NaOH with a flow rate of 0.4 mL/min. The autosampler and column temperature was 20 and 30°C respectively to maintain baseline stability for reproducibility. The injection volume for samples by AS was set at 10 µL and pressurized by helium gas (3500 psi). Before the injection of each sample by the autosampler, the column was flushed for 10 min with 90 mM NaOH (Table 9). Afterwards, the concentration of NaOH was increased steadily from 90 mM to 300 mM in 10 min, before eluting with decreasing concentration of NaOH from 300 mM to 90 mM for 5 min. Therefore separation of sugars was

achieved within 25 min and detected by PAD. Data generated from the process was collected in real time by Dionex chromeleon program (version 6.8) on a computer. The final result of carbohydrate detection as deduced from the area (nC) of the chromatogram was calculated as percentage soluble sugars in dry weight. For total starch content and *in vitro* digestion analysis, glucose result was multiplied with 0.9 to convert glucose to starch (Goñi *et al.,* 1997).

Table 9. Program of gradient elution for soluble sugars separation by HPAEC-PAD

Run time (min)	Mobile phase A (%)	Mobile phase B (%)	Concentration of NaOH (mM)
0-10	70	30	90
10-15	70-0	30-100	90-300
15-18	0	100	300
18-20	0-70	100-30	300-90
20-25	70	30	90

3.2.5.1.3. Preparation of HPAEC-PAD eluent

Two eluents were prepared for HPAEC-PAD analysis; eluent A and B. Eluent A was 100% Millipore water and eluent B was prepared using 50% sodium hydroxide (HPLC grade; Fluka 71686) to obtain 300mM. Eluents were made on the day of analysis and used for 1 week.

3.2.5.1.4. Sample preparation for HPAEC-PAD

For the analysis of detection of soluble sugars by HPAEC-PAD, millet test samples and sugar standards including glucose (Glu), fructose (Fru), sucrose (Suc), arabinose (Ara), raffinose (Raf), galactose (Gal), stachyose (Sts), glycerol (Gro), maltose (Mlt) and maltotriose (Mtr) (all from Sigma Aldrich) and were prepared at concentrations between 0.5-12 µg mL^{-1}. Test samples were diluted to the concentration of sugar standards. Both test samples and standards were spiked with internal standard (fucose (Fu), final concentration of 5 µg mL^{-1}) and passed through 0.2 µM Polytetrafluorethylene (PTFE) membrane filters into snap vials prior to injection.

3.2.5.2. 3, 5 Dinitrosalicylic acid assay (DNS)

3.2.5.2.1. Principle of DNS

The DNS is used to detect the presence of the carbonyl group (C=O) of reducing sugars (glucose, maltose and fructose) when in an alkaline solution under high temperature (Miller, 1959). The carbonyl group of reducing sugar (glucose) reacts with 3,5-dinitrosalicylic acids and reduces it to 3-amino-5-nitrosalicylic acid, thereby producing a yellow to deep orange colour absorbed at 540 nm wavelength. The intensity of colour of the reaction depends on the concentration of sugars present.

3.2.5.2.2. Preparation of DNS reagent

To prepare DNS colour reagent, sodium hydroxide (Sigma) and 3,5-dinitrosalicylic acid (Sigma) stock solutions were made separately. Firstly, 2 M sodium hydroxide solution was prepared and heated at 60°C on a Fisherbrand ARE hot-plate stirrer, and sodium potassium tartrate (Sigma) was added to the solution to obtain a concentration of 5.3 M. The solution consisting of sodium hydroxide (NaOH) and sodium potassium tartrate was mixed with a magnetic stirrer on the hot-plate until the solute was completely dissolved. Thereafter, a 96 mM DNS was suspended in water, the solution was heated at 60°C as previously described (for NaOH) and mixed constantly until the solute was dissolved.

Finally, to complete the preparation, a ratio of 30:20:50 of water (50°C), solution consisting of 2 M sodium hydroxide and 5.3 M sodium potassium tartrate and 96 mM DNS solution respectively, was mixed together. The prepared DNS colour reagent was protected from light and stored at room temperature and used for 6 months.

3.2.5.2.3. Sample preparation for detection of reducing sugars by DNS

To measure reducing sugar, stock solution of glucose or maltose standard was prepared. Sugar standards were diluted serially to obtain concentration from 0.1 to 2 mg mL^{-1}. Samples were diluted within the concentration limit of standard prepared. Two mL of sample or standard was transferred into a test tube and 1 mL of DNS reagent was added. The tubes were placed in a boiling water bath

(Grant SBB 14) set at 100°C for 15 min to allow the reducing sugars to react with DNS. At the end of 15 min, the tubes were quickly cooled on ice and 9 mL of Millipore water was added. Absorbance was read at 540 nm by spectrophotometry (Jenway 6715 UV/vis). A blank was prepared by substituting the sample or standard with distilled water to correct for any interferences. A standard curve of absorbance (nm) against concentration of standard was used for calculating (Equation 7 and 8) the amount of either maltose or glucose.

ΔA_{540nm} Standard = A_{540nm} Standards - A_{540nm} Blank…….**Equation 7**

ΔA_{540nm} Sample = A_{540nm} sample - A_{540nm} Blank……..…..**Equation 8**

3.2.6. Starch

3.2.6.1. Extraction of starch

Starch extraction also shown in this study as isolated millet starch (IMS) was carried out as described by Bustos *et al.,* 2004. Briefly, 1g of millet flour was suspended in TRIS-EDTA-sodium metabisulfate buffer. Preparation of buffer is described in section 3.2.1.2 of this Chapter. The suspension solution was homogenized using a polytron (IKA T-25 ULTRA-TURRAX Digital High-Speed homogenizer systems) set at 10000 rpm for 5 min and filtered through 4 layers of muslin cloth. The filtrate was collected and centrifuged at 5000 rpm for 15 min at 20°C. The supernatant was discarded and the pellet was washed with 50 mL of pH 7.5 buffer and centrifuged, a process repeated 3 times. Afterwards, the pellet was washed 3 times with 20 mL 100% acetone, then homogenized, and centrifuged. The final starch pellet was dried in the fume cupboard at room

temperature condition. The starch was stored in a dark cupboard for subsequent analysis.

3.2.6.2. Determination of amylose content

The amylose content of isolated millet starch (IMS) was determined as described by Kaufman *et al.,* 2015, with modifications. A 90% DMSO (Fisher chemical; D139) solution was used to prepare 12 mM iodine (Fluka, 03551), and 0.6 M urea solutions and will be referred to as IDMSO and UDMSO respectively. Methanol solution was also prepared (85%).

Subsequently, standard potato amylose (Sigma Type-III A-0512) or maize amylopectin (Sigma 10120) solution was prepared with UDMSO as described in Table 10. Firstly, isolated millet starch (5 mg) was suspended in 85% methanol solution before heating for 1 h at 60°C in a shaking water-bath (140 rpm). The millet suspension was brought to room temperature, centrifuged at 4000 rpm for 5 min. The supernatant was discarded, and a repeat process of adding methanol followed by centrifuging for 5 min was repeated 2 times. The millet sample was dried at 60°C in a water bath (Grant GLS Aqua 12 plus). Then, 1 mL of UDMSO was added to the dried isolated millet starch and heated for 1 h at 95°C with intermittent shaking in a water bath. Methanol and UDMSO were used to remove fat residues in the isolated millet starch.

Afterwards, 100 µL of millet or standard solution were transferred into 96 well-plates and 100 µL OF IDMSO was added and stirred gently for 2 min before

transferring 20 µL into new well-plates. To the each well containing 20 µL of solution, 180 µL of water was added and stirred gently for 1 min before immediately reading the absorbance at 620 and 510 nm. Amylose content was calculated using equation 9.

Amylose content (%) = $\frac{(Abs620 - Abs510) - y\ interception\ of\ regression}{Slope\ of\ regression}$ .....**Equation 9**

Table 10. Amylose standard solution preparation for standard curve.

Amylose standard (%w/v)	Amylose standard stock solution used (µL)	Amylopectin standard stock solution used (µL)
0	0	100
10	10	90
20	20	80
30	30	70
40	40	60
50	50	50
60	60	40
70	70	30
80	80	20
90	90	10
100	100	0

3.2.6.3. Determination of total starch content of millet

The total starch method by Goñi *et al.,* (1997) was optimised for millet flour and the final method is described in this section.

A 2 M solution of KOH was used in the experiment to dissolve starch before hydrolysing starch to glucose using Megazyme AMG. Millet samples (50 mg in dry weight) were weighed and suspended in 6 mL of 2 M KOH and vortexed continuously on a Scientific industries SI-0236 vortex-Genie 2 mixer (120 V) for 2 h at room temperature. The pH of the solution was adjusted to 5 with 6 M HCl and 0.2 M sodium acetate buffer was added to make the volume to 12 mL. A final concentration of 150 U of amyloglucosidase (Megazyme) was added to the solution and incubated in a 60 ºC shaking water bath (Grant GLS Aqua 12 plus) for 45 min. The enzyme activity was inactivated in a boiling water-bath (SBB14 Grant). The suspension solution was cooled to room temperature and centrifuged at 5000 rpm for 15 min at 20°C. Supernatant was collected and 4 volumes of absolute ethanol were added. The sample supernatant collected was diluted to glucose concentrations between 0.5-12 µg mL^{-1} and spiked with internal standard (fucose, final concentration of 5 µg mL^{-1}). Glucose released was analysed by HPAEC-PAD and results generated was multiplied with 0.9 to convert glucose to starch. The final result was presented as total starch of millet in dry weight of flour.

3.3. Microstructure of millet starch by scanning electron microscope (SEM)

To test the hypothesis that particle size and cooking will have an effect on *in vitro* starch digestion of millet, the microstructure of free millet starch granule of different particles sizes of millet flour and cooked gruel were observed under a scanning electron microscope (SEM). The millet samples were directly scattered on a 30mm diameter aluminium pin stubs and placed in a Cressington 208HR Platinum Sputter Coating unit for 10min to achieve vacuum for platinum coating. The coated samples were then loaded into the chamber of an FEI Quanta 200F Field Emission Gun Environmental Scanning Electron Microscope (FEG ESEM). In the FEG-ESEM, beam of electrons travelled from the electron gun through a column made of series of electromagnetic lenses to hit the samples, thereby causing electrons to react with samples under vacuum. Under vacuum signals (electrons, protons and X-rays) are produced that are transmitted through the electron detector system and viewed as images on a computer screen at a magnification of above 100000x and resolution of 1-20 nm.

3.4. Thermal properties of millet by differential scanning calorimetry (DSC)

The thermal properties of millet based on gelatinisation and retrogradation was investigated using DSC (TA Multicell DSC) as previously described (Srichuwong *et al.* 2017) with some modification. Millet flour was carefully weighed into a high pressure sample pan, and then water was added at a ratio of 1:5.5 (w/v) to replicate *ibyer* preparation. The suspension was allowed to stand for 1h at room temperature. Afterwards, sample suspensions in the high pressure pans were sealed and heated in a multicell DSC, set at temperature ranges from 10 to 150°C at 1°C/min with an empty high pressure pan as reference. The instrument

measured the onset temperature (T_o,°C), peak temperature (T_p,°C), and conclusion temperature (T_c,°C). The change in enthalpy (ΔH, J/g) was calculated using Universal TA software. The pans from the run were stored for 2 days at 4°C and then reheated using the same protocol to determine the dissociation of retrogradation. Samples were analysed in triplicate and weight of sealed pan with samples in it was recorded before and after DSC run to ensure moisture was not lost during analysis.

3.5. Moisture content

Moisture content of millet samples were determined by pre-weighing samples and drying using 2 drying methods; high temperature (incubator) for flour and low temperature (freeze dryer) for cooked samples. For high temperature method, predetermined weight of millet flour in a stainless petri dish was placed in a SANYO incubator (MIR-262) to dry at 30°C for 72 h. On the other hand, samples dried using the freeze dryer method were weighed into plastic test-tubes, placed in a -80°C freezer for 24hrs before they were removed and cover with perforated parafilm and dried in a Labconco Freezone 2.5 L benchtop free dryer set at -50°C for 48h. Both drying methods were subjected to moisture content calculations (Equation 10).

Moisture content **(%)** = $\frac{WW(g)-DW(g)}{WW\ (g)}$ X 100%.....**Equation 10**

WW = wet weight; DW = dry weight

3.6. Determination of protein by Kjeldahl method

Total protein content of millet flour and test samples were evaluated by Kjeldahl method (AOAC, 2016) with modifications. Pre-weighed dried sample (500 g) and a catalyst tablet were placed in a quartz digestion tube prior to the addition of 25 mL of concentrated sulphuric acid to initiate digestion in a fume cupboard. The solution in the flask was mounted on a Gerhardt-Turbotherm electro thermal mantle connected to an exhaust system and heated at very high temperature (around 420 °C) between 1 to 2 h to completely dissolve (by producing a clear solution) the sample. The digested sample in a solution form was allowed to cool at room temperature before transferring the content into a distillation flask. During the transfer process, the glass tube was rinsed into the flask with 400 ml of water. Afterwards, phenolphthalein indicator (approximately 10 drops), anti-bumping granules (about 1 g) and 2 mL of anti-foam agent were added to the distillation flask. The flask is placed on a stand with a burner underneath. It is also connected to an additional funnel (used for adding NaOH to digested solution and water to prevent air/gas from escaping), Kjeldahl track, and air condenser delivery tube. The condenser delivery tube was connected to inlet cold water. A solution of 4% boric acid and drops of screened methyl red indicator was made in a conical flask and connected to a condenser delivery tube. Once all connections and plugs were in place, 50% sodium hydroxide was added through the additional funnel to the digestion flask while mixing by rotating until a colour changed from light-blue to darkish brown was observed. The funnel was sealed with a plug and topped with water. Then, the Bunsen burner was ignited to generate heat for boiling of the solution in the distillation flask. The process was stopped after the distillate

collected from the condenser delivering tube into the boric acid solution exceeded 250ml. To stop the process of distillation, the plug in the additional funnel was removed and the Bunsen burner was turned off. The final step was the titration of the boric acid solution containing ammonia distillate with diluted sulphuric acid (0.25 M). Protein content was calculated by multiplying the nitrogen content (volume of sulphuric acid used during titration) with protein factor (6.25) using equation 11.

1mL of sulphuric acid 0.25N = 0.0070g nitrogen……...**Equation 11**

3.7. Statistical analysis

Results of millet experiments were presented in three to seven replicas depending on experiment. The statistical analysis was performed using GraphPad Prism Version 7.03 statistical software. The results generated were presented as replica mean ± standard deviation of mean, and the significant differences between means of the different treatments investigated were performed by one way ANOVA, which was then followed by conducting a multiple comparisons test using the Turkey test with results expressed as $p \leq 0.05$. For results of in vitro starch digested fermented samples, statistical differences were performed by one way ANOVA, then followed by the Dunett's test at $p \leq 0.05$ to make comparisons between control samples and control (250µM % digested starch of unfermented cooked millet porridge).

Chapter 4: Optimisation of protocols for carbohydrate digestibility and analysis of pearl millet

Abstract

Carbohydrate analysis protocols are not standardised and therefore previous studies that have measured carbohydrate composition and digestion are difficult to interpret and compare. In this chapter, several protocols were optimised to analyse the carbohydrate composition and digestion of pearl millet flour. The results indicated that agitation is a key requirement for the extraction of soluble sugars and effective disruption of millet starch for starch content and digestion measurement. Some commercial digestive enzymes used in the protocol were shown to contain high levels of glucose (0.03 to 73.35 mg/mL and therefore their use would have an impact on starch digestion and quantification. Two static digestion protocols were evaluated: a non-gastric method developed by Goñi and the gastrointestinal INFOGEST protocol by Minekus. The results showed that agitation was a key requirement for effective sugar extraction and starch digestion. The INFOGEST protocol digested nearly 2 times more starch in millet flour compared to the non-gastric Goñi method. This suggests that the gastric phase is important for millet starch digestion. The results presented in this chapter allowed the selection of optimal conditions for carbohydrate analysis and starch digestion for subsequent chapters.

4.1 Introduction

Methods for measuring carbohydrate composition vary widely and have not been harmonised. Common methods used are solvent extraction methods for measurement of free soluble sugars and chemical and enzymatic methods for measurement of starch (Ačanski and Vujic, 2014; Zeng *et al.,* 2017; Englyst *et al.,* 1992; Pico *et al.,* 2015; Karim *et al.,* 2016). For soluble sugars, studies reported different extraction and detection methods and in terms of starch measurement and digestibility, different enzymes, enzyme concentrations, buffers and conditions were used (Acanski and Vujic, 2014; Zeng *et al.,* 2017; Englyst *et al.,* 1992; Pico *et al.,* 2015; Karim *et al.,* 2016). Therefore, it is difficult to compare carbohydrate data between studies.

In starch measurement, enzyme concentration and extent of hydrolysis depends on the type of product being analysed (Goñi *et al.,* 1997; Chu *et al.,* 2017; Li *et al.,* 2018). For this reason, depending on the food sample being analysed, already established steps in protocols may need to be optimised. Additionally, even though digestive enzymes are used in carbohydrate analysis, information about the sugar composition of these enzymes has not been reported.

4.1.1 Aim

The aim of this study is to optimise the methods for measuring the carbohydrate content and composition, and the starch digestibility of millet flour.

4.1.2 Objectives

1. Optimise the method of soluble sugar extraction and quantification the sugars released.
2. Identify and quantify the sugars in selected commercial digestive enzymes.
3. Optimise the method for total starch content measurement of millet flour.
4. Optimise the gastrointestinal starch digestion method for millet.

4.2. Methodology

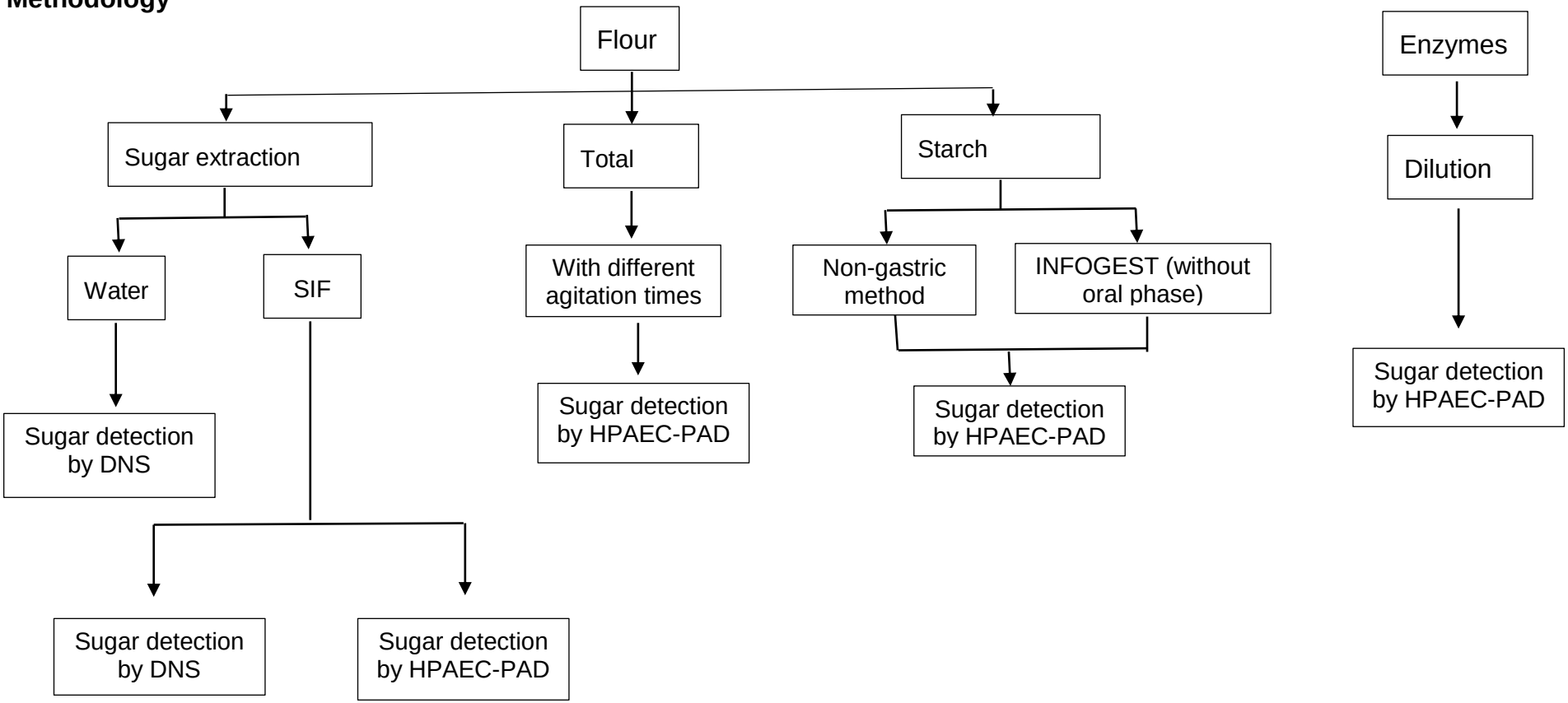

Figure 8. Summary of protocol optimisation for carbohydrate content and composition of pearl millet flour

4.3. Results

4.3.1. Carbohydrate analysis of millet flour

4.3.1.1. Optimisation of free sugar extraction in millet

Two different solvents; water and SIF were used in the extraction of soluble sugars in millet flour and total reducing sugars were detected by DNS. The result showed that 1 min vortexting (agitation) with SIF extracted more reducing sugar in millet flour than water. Therefore SIF was used for subsequent extration (Fig. 9). The extraction with SIF was prolonged over a time course up to 25 min. However, after 10 min, no increases in extractable sugars were observed.

After an optimal condition for extracting soluble sugar was attained, HPAEC-PAD was used to show sugars detected in millet flour (Figure 10). The sugars detected were glucose, sucrose, maltose, raffinose, stachyous, and fructose. This method will be used in the future for quantification and detection of soluble sugars in millet samples.

In summary, optimal conditions recommended was SIF for 10 min with vortext extraction and HPAEC-PAD for detection of sugars .

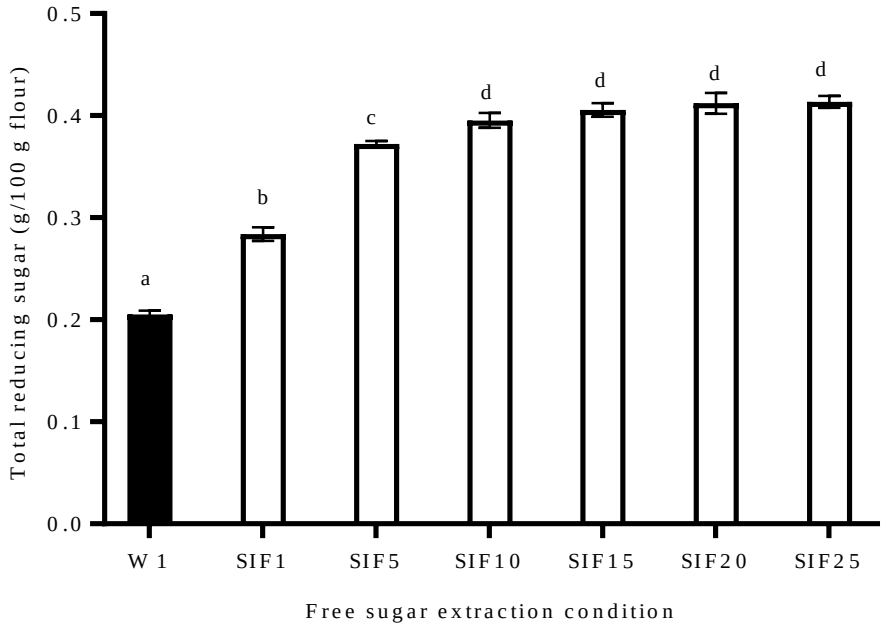

Figure 9. Total reducing sugars in millet flour after extraction with water at 1 min vortexing (W1); and vortexed in simulated intestinal fluid for 1 min (SIF1), 5 min (SIF5), 10 min (SIF10), 15 min (SIF15), 20 min (SIF20) and 25 min (SIF25) at room temperature. Results are presented as mean of 3 biological replicates and expressed in dry weight basis. Data error bars are standard deviation of the mean. Data with the same superscript are not significantly different ($p<0.05$).

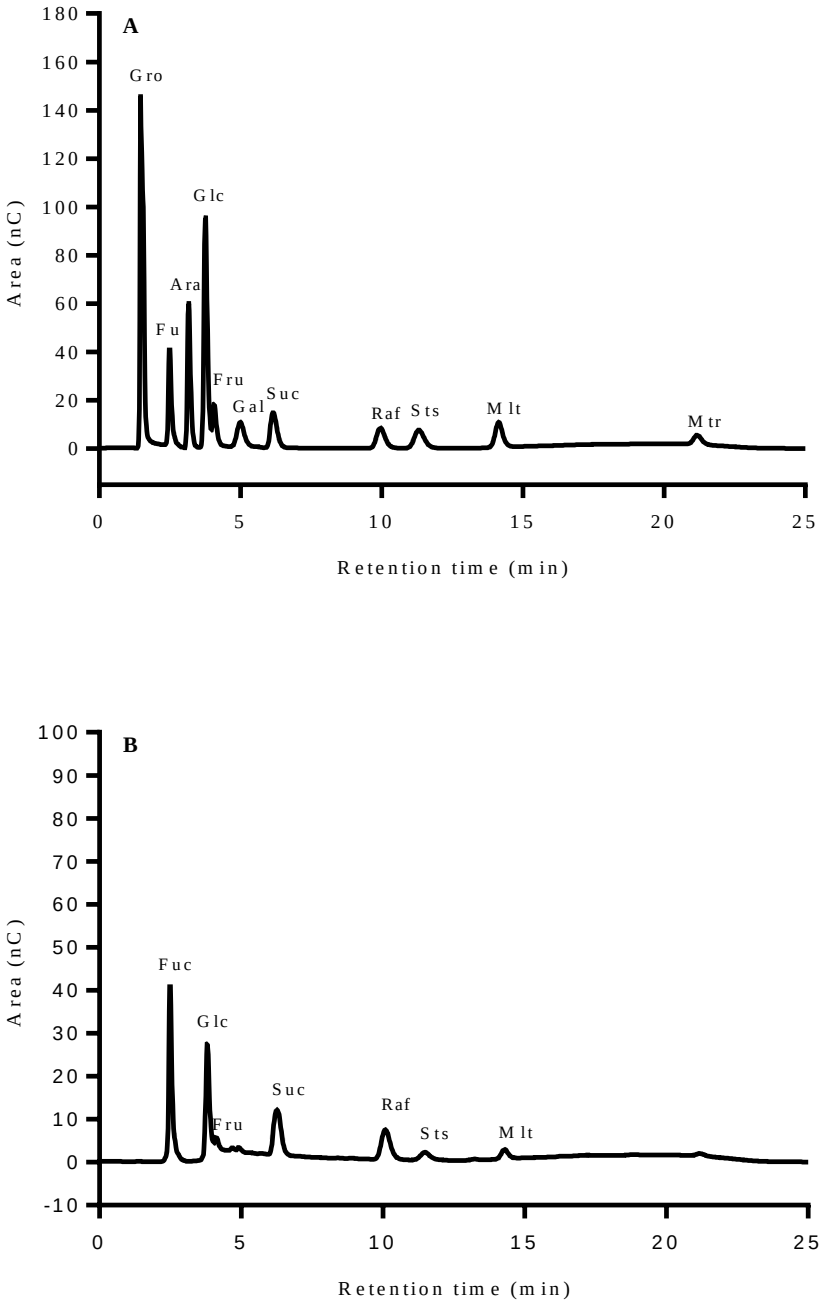

Figure 10. HPAEC-PAD chromatogram showing separation of sugars in standards (A) and the sugars detected in millet flour (B).

4.3.1.2. Identification of sugars in digestive enzymes

Identification of sugars and quantification of glucose present in different digestive enzymes was one of the initial steps taken before enzymatic analysis of millet. This was necessary following an earlier interference of sugars in Sigma AMG for the total starch estimation in millet (data not shown). Enzyme preparation for sugar detection by HPAEC-PAD and types of enzyme investigated has been described (see section 3.2.2.2 and Table 6) in the previous chapter. Sugars identified in the enzyme were arabinose, glucose, fructose, galactose, sucrose, maltose and a sugar alcohol glycerol (Fig. 11).

All enzymes analysed had varying concentration of glucose (Table 11) and contained glycerol. Apart from Megazyme AMG and Sigma pepsin that only contained glycerol and glucose, other sugars were present in other enzymes. Even though AMG from Sigma was diluted at a relatively high ratio, other sugars including a high concentration of glucose was in the enzyme blank. This suggests a strong interference may be likely when used in carbohydrate digestion. It is worth mentioning that compared to DNS, HPAEC-PAD is the best detection method for carbohydrate analysis. This is because sugars in samples and enzymes are likely to be detected and quantified, and subsequently sugars in enzymes like pepsin can be corrected when measuring starch digestion. Additionally, Megazyme AMG is the best enzyme for starch content and digestibility measurement due to its low concentration of glucose.

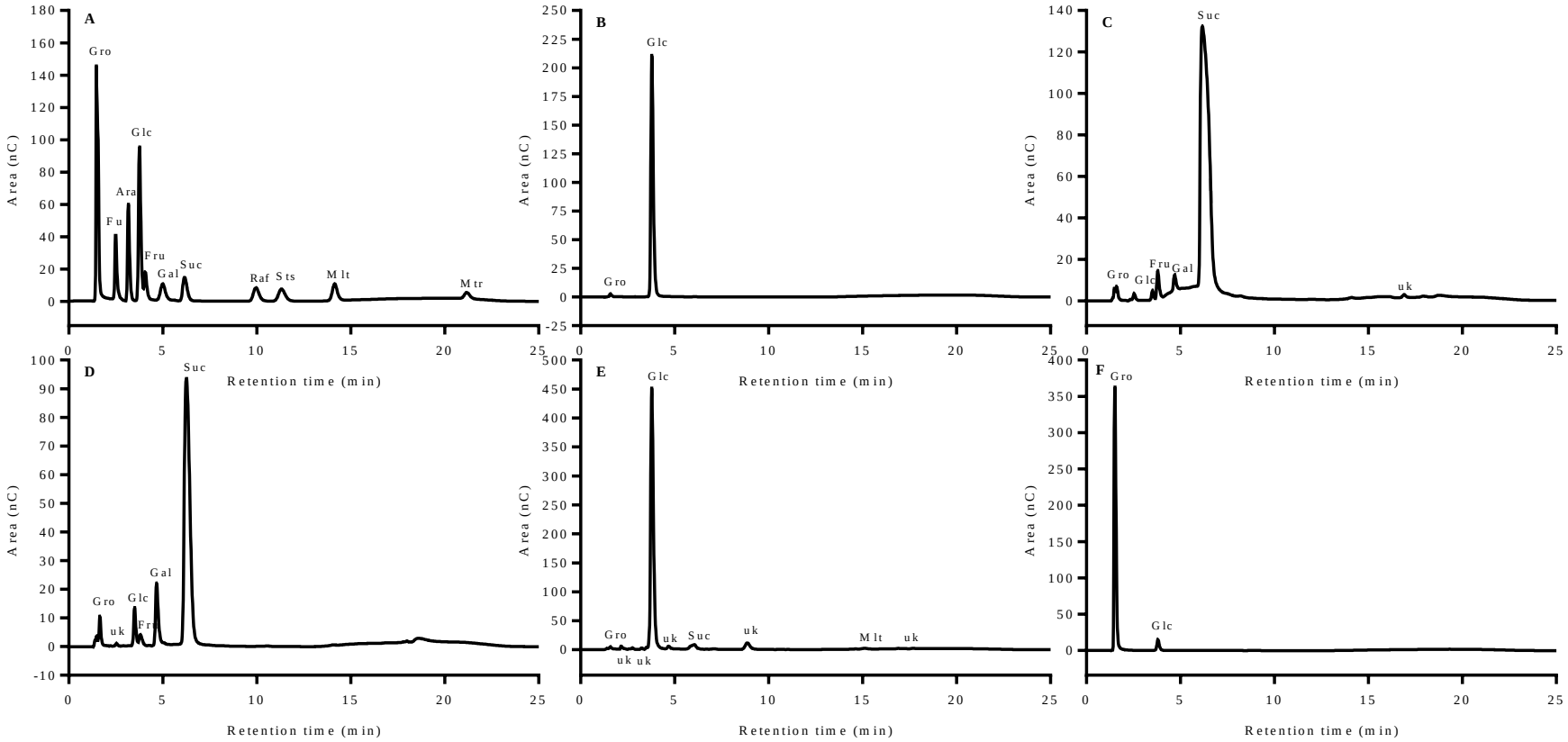

Figure 11. Chromatograph showing sugar separation and detection by HPAEC-PAD of sugar standard (A), Sigma pepsin (B),Sigma pancreatic α-amylase (C), Sigma pancreatin (D), Sigma AMG (E) and Megazyme AMG (F).

Table 11. Concentrations of glucose in some selected digestive enzymes. Glucose concentration was presented as mean±standard deviation of mean of 3 replicates.

Enzyme	Manufacturer	Code name	Enzyme stock (U/mL)	Glucose mg/mL
Pepsin	Sigma	P7000	2000	1.12±0.06
α-amylase	Sigma	A3176	15	0.03±0.00
Pancreatin	Sigma	P7545	200	0.15±0.02
Amyloglucosidase	Sigma	A7095	150	73.35±7.90
Amyloglucosidase	Megazyme	E-AMGDF	150	0.30±0.04

4.3.1.3. Optimisation of total starch determination

To determine the total starch content of millet, reducing sugar was measured after the dissolution of starch in KOH, and hydrolysis of undissolved starch to glucose was done with Megazyme AMG using the method described by Goñi *et al.,* (1997). Vigorous shaking of millet suspension in KOH for 30 min resulted in significantly lower starch content values than expected. Hence, millet suspensions were subjected to a range of shaking times (Fig. 12). The starch content of millet increased significantly with shaking time with no further increase after 120 min. The result indicated that the maximum time required to dissolve millet starch by shaking in KOH is 120 min.

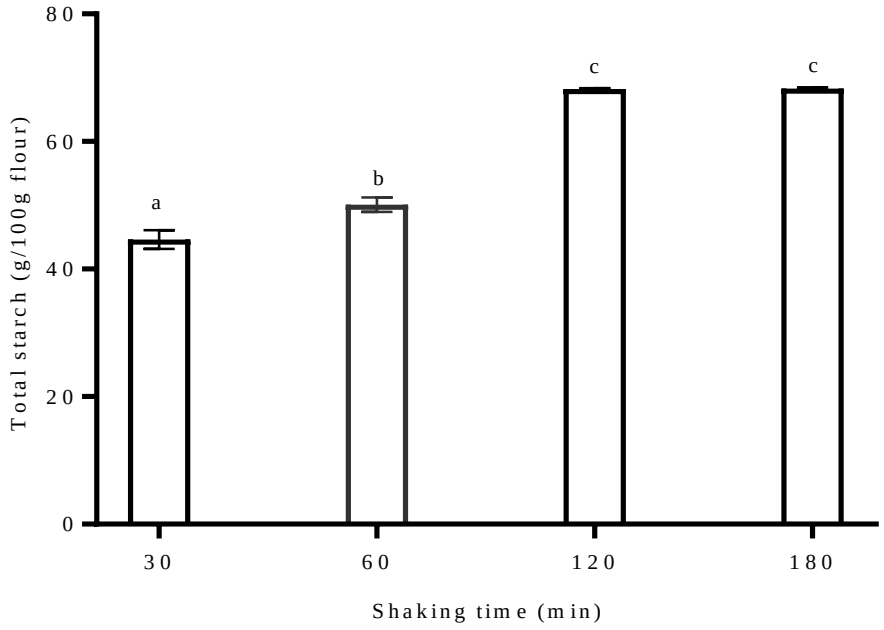

Figure 12. Total starch content of millet over different shaking periods. Results are presented as percentage mean of 3 biological replicates and expressed in dry weight basis. The error bars represent standard error of the mean. Data with the same superscript are not significantly different ($p<0.05$).

4.3.1.4. Optimisation of gastrointestinal digestion of millet flour

To determine the optimal condition for measuring starch digestion *in vitro*, gastrointestinal methods were optimised. Millet flour was digested using two different methods: without pepsin pre-incubation (as described by Goñi *et al.,* 1997) and with pepsin pre-incubation (as described by Minekus *et al.,* 2014) prior to incubation with pancreatin in SIF with intermittent shaking by inversion method while in a shaking water bath before incubation with amyloglucosidase (Megazyme) in sodium acetate buffer (Fig. 13). The supernatant was collected between 0-120 min after the intestinal phase of digestion was analysed by HPAEC-PAD. The result obtained showed irregular trends starch digested overtime. Hence, the pH of each phase of digestion was checked before incubation with digestive enzyme. Additionally, though the incubations were done in a shaking water-bath, further optimisation by intermittent mixing of digesta by inversion method was done.

Relatively low starch was digested without pepsin pre-incubation (Fig. 13A). The results obtained without (Fig. 13B) and with (Fig. 13C) intermittent mixing of digesta by inversion method suggests that the homogeneity of millet suspension can have an impact on starch digestion *in vitro*. The significant improvement in starch digestion following pepsin pre-incubation indicates that, to achieve maximum digestion, gastrointestinal digestion of millet flour suspension is necessary. In summary, starch digestion with INFOGEST and intermittent shaking of digesta by inversion method was the optimal digestion condition for millet sample and will be use in subsequent analysis.

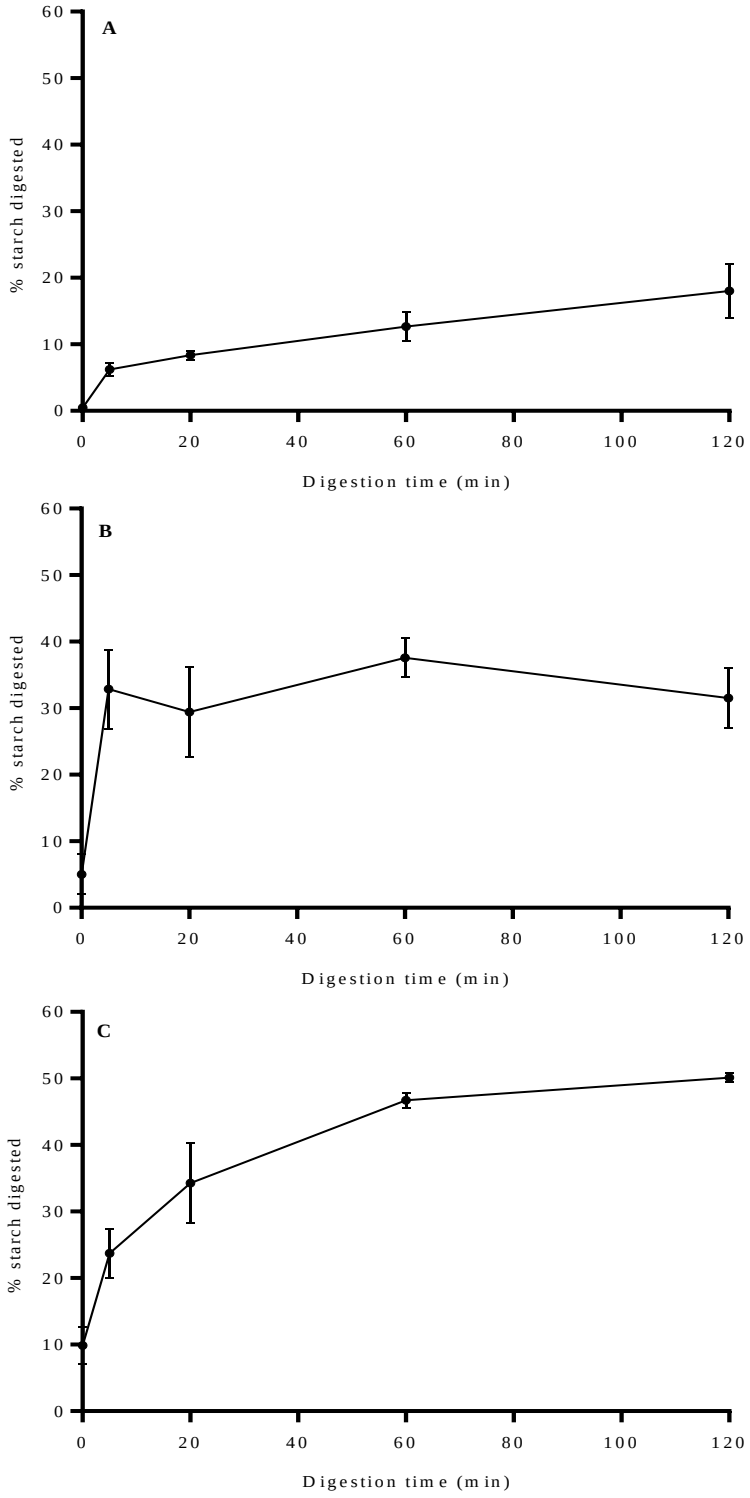

Figure 13. *In vitro* starch digestion of millet flour using non-gastric protocol according to Goñi (A) and INFOGEST (B) without intermittent mixing of digesta by inversion method and INFOGEST (C) with intermittent mixing of digesta by inversion method while in the shaking water bath. Results are presented as percentage mean of 3 biological and expressed in dry weight basis. The error bars represent standard error of the mean.

4.4 Discussion

Estimation of carbohydrate content is largely dependent on the methods used for its quantification and enzymes used for hydrolyzing starch. For this part of this current study, methods for the estimation of soluble sugar content, total starch content and *in vitro* starch digestion of millet flour were optimised. In consideration that starch content and digestion measurements require the use of enzymes, sugars present in digestive enzyme were investigated.

Reducing sugar content of millet was measured by using water and SIF extraction methods. Sugar content was relatively lower with water extraction (0.21%) and higher with SIF extraction (0.41%). In previous studies, water, ethanol or sodium acetate buffer was used for the extraction of sugars in potato and cereals (Malleshi et al., 1986, Rao and Nagasampige, 2011, Oliveira *et al.,* 2015; Karim *et al.,* 2016). Rao *et al.,* (2011) reported that extraction with water resulted in soluble sugar content ranging from 0.13 to 0.39% for finger, proso, khodo, and foxtail millet. Similarly, extraction with ethanol only yielded 0.26%, 0.23% and 0.18% of reducing sugars in pearl, finger and foxtail millet (Malleshi et al., 1986). These values are similar to what we reported in our study for water extraction method but lower than SIF extraction method. This suggests that the protocol used in this current study that included vigorous agitation is the most suitable for extracting sugars in millet flour. The free soluble sugars content of food samples, especially glucose is useful in providing true data for starch content and digestible starch fraction (Englyst *et al.,* 1992, Goñi *et al.,* 1992). Therefore, it is important to use extraction methods suitable for food samples that can result in optimum release of free sugar.

The measurement of total starch and *in vitro* starch digestion involved the use of enzymes, and our study has shown that enzymes contained sugars. In this study, compared to Sigma pancreatin and AMG from Megazyme, relatively high concentration of glucose was apparent for Sigma AMG. Apart from glucose, other identified sugars were sucrose, galactose, fructose, and arabinose and many detected in enzymes. Glycerol reported in all the enzymes investigated may have been used as enzyme stabilizer. However, from our findings in this study, it can be argued that starch content and digestion values for millet has been underestimated (Amadou *et al.,* 2014; Annor *et al.,* 2015; Ren *et al.,* 2016; Bustos *et al.,* 2017; Lemgharbi *et al.,* 2017). This is because these studies have not reported or factored the contribution of glucose in enzymes to carbohydrate data. The contribution of glucose from Sigma AMG to starch data may explain the relatively low enzyme activity (less than 0.3 U) used in the measurement of starch content and digestion previously (Lemgharbi *et al.,* 2017). The advantage of identifying sugars in enzymes is to correct for interferences and to avoid false contributions to carbohydrate data. Hence, enzymes with lower sugar concentration such as Megazyme AMG are not likely to present interferences in starch content and digestion measurements.

The most common methods of total starch content measurements are chemical dissolution of starch and enzymatic degradation of starch to glucose with AMG (Englyst *et al.,* 1992; Goñi *et al.,* 1997; Srichuwong *et al.,* 2017). Optimum starch content in this study was established when millet suspension in KOH was vigorously shaken for 2h. Conversely, the dissolution of starch in cocoyam,

potato, green pea, lentil, rice was achieved in 30 min (Goñi *et al.,* 1997; Swieca and Gawlik-Dziki, 2015; Eleazu *et al.,* 2018). Starch values reported for pearl millet were low in some cases (Lemgharbi *et al.,* 2017) which may be due to the short agitation time used to dissolve starch in flour. In this study, agitation was an important factor to consider in the measurement of pearl millet starch content. The starch content of millet if underestimated can have a big impact on the data reported for starch digestion and estimated GI.

In vitro starch digestion methods aim to mimic the gastrointestinal phases of hydrolysis (Englyst *et al.,* 1992; Goñi *et al.,* 1997; Minekus *et al.,* 2014). The food sample been investigated reflects the type of method used. For instance, while beans were boiled for 90 min prior to *in vitro* starch digestion, pasta was only boiled for 15 min (Goñi *et al.,* 1997) mainly due to their physical state. The combination of the gastric and intestinal phase is the most commonly applied *in vitro* starch digestion method. However, digestible starch is also measured with only intestinal phase of digestion or a combination of gastric and intestinal digestion (Germaine *et al.,* 2008; Minekus *et al.,* 2014; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017). Findings in this current study suggest that starch in millet flour is not well hydrolyzed when the gastric phase of digestion is omitted. Low starch digestion of millet flour (<45%) than values in this current study which has been reported (Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017) suggests that starch digestion can also be influenced by the digestion protocol used. This highlights the difficulty in comparing starch digestion data of millet between studies. Furthermore, optimal digestion of millet requires mixing by inversion method. The additional impact of intermittent mixing of digesta during hydrolysis

as reported here is likely to improve homogeneity in sample and the facilitation of enzyme access to substrate.

Generally, optimisation of protocols for measuring carbohydrate content and composition in millet was done on flour. Considering that millet is not eaten raw, optimisation using flour provides a useful starting point for understanding how domestic methods for preparing millet can contributes to its starch digestion.

4.5. Conclusion

This study demonstrates that method of analysis contributes largely to the reported carbohydrate content in millet. Carbohydrate protocols were optimised to measure soluble sugar content, starch content and digestion. For soluble sugar determination, the SIF method accompanied with constant agitation presented more extraction of sugars and the HPAEC-PAD was the best method for quantifying the sugars present in millet. This study showed that digestive enzymes contained a wide range of sugars and concentration of glucose, which has not been reported in previous studies, especially for Sigma AMG that is the most commonly used enzyme in carbohydrate analysis. Therefore quantifying the sugars in enzymes and their contribution carbohydrate data is important to correct for false results.

Initially, though vigorous agitation for starch content and intermittent mixing by inversion method for starch digestibility measurements were not contemplated, including these steps in carbohydrate analysis stabilized and improved starch content and digestion measurements. It was determined that the starch in millet was poorly digested when only the intestinal method was used as relatively higher amount of starch was digested with the optimised INFOGEST method that included gastric and intestinal phases of digestion. This suggests that previous studies may have reported underestimated values for carbohydrate composition and digestion of millet.

Therefore, having established the experimental protocols for measuring carbohydrate composition and digestion for millet flour, millet products will be

analysed using these protocols in further chapters. The analysis will include the effect of particle size of flour, cooking and fermentation on carbohydrate quality and digestion of millet.

Chapter 5: The effect of cooking and particle size of flour on carbohydrate quality and starch digestibility of pearl millet

Abstract

This study investigated the impact of particle size reduction on the carbohydrate bioaccessibility of pearl millet flour and *ibyer*, a traditional cooked porridge. Scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) were used to investigate the microstructural and thermal properties of millet. Micrographs of the flour show the tight arrangement of granules within endosperm with some undisrupted granules in cooked porridge and how pre-incubation of millet flour with pepsin before intestinal digestion resulted in more cavities in granules. DSC enthalpy curves showed presence of resistant starch in Ibyer suggesting retrogradation. Soluble sugars in both millet flour and *ibyer* were quantified by HPAEC-PAD. The results show that particle size reduction and cooking significantly increased the soluble sugar content (2.03 to 6.76%), indicating that endogenous carbohydrate hydrolases are active during the cooking process. The starch content of millet was not altered by particle size of flour or cooking (68.80%). Starch digestibility investigated using a simple non-gastric Goñi protocol and an optimised INFOGEST protocol to simulate gastric and intestinal phases of digestion showed that starch digestibility was <35% when using the Goñi protocol and 57% when using the INFOGEST method. This suggests the gastric phase has an impact on starch digestion. Using the data from the INFOGEST digestion, the RDS (41%), RS (44%) and the suggested low eGI (<55) of pearl millet porridge (*ibyer*) were calculated. In conclusion, *Ibyer* contains both rapidly digestible and resistant starch and gastric phase played a significant role in the carbohydrate digestion of millet. The

low starch digestibility and high levels of resistant starch indicates low glycemic potential that needs to be verified in vivo.

5.1. Introduction

Starch is the main component of cereal grains acting as an energy source for the developing plant during germination. The digestibility of starch is crucial in the provision of energy and regulation of blood glucose, and if low it is suggested to improve cardiovascular health (Heaton *et al.,* 1988, Ren *et al.,* 2016). The rate and extent of starch digestibility of food is influenced by processing, protein-starch-lipid interactions, microstructure, and degree of crystallinity.

In vitro digestibility studies are very popular in research and provide a practical alternative to the *in vivo* approach for measuring the digestibility of starch in food using similar conditions that imitate the human digestive system. Studies using *in vitro* methods classify starch fractions digested (i.e rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS)) and predicting glycemic response and postprandial metabolism *in vivo* (Heaton *et al.,* 1988; Englyst *et al.,* 1992; Goñi *et al.,* 1997; Annor *et al.,* 2015; Ren *et al.,* 2016; Annor *et al.,* 2017). The starch digestion method to be used is largely dependent on food samples. For instance, enzymatic digestion of starch with only intestinal phase reached more than 90% in quinoa flour and less than 40% in wheat, millet, and sorghum (Srichuwong *et al.,* 2017). Information on the starch digestibility of millet products as eaten would be useful, especially for traditional African foods.

5.1.1. Aim

The aim of this study was to investigate the factors that contribute to carbohydrate quality and digestion of unfermented pearl millet and to predict the GI *in vitro* data.

5.1.2. Objectives

1. To investigate the microstructural properties of millet.
2. To investigate the effect of particle size reduction and cooking on the soluble sugar content of millet.
3. To investigate the effect of particle size reduction and cooking on total starch content of millet.
4. To investigate the effect of particle size, cooking and pepsin pre-incubation on the starch digestibility of millet.
5. To calculate the enzymatic kinetic parameters of the starch digestibility of unfermented pearl millet products.
6. To investigate the thermal properties of millet.

5.2. Methodology

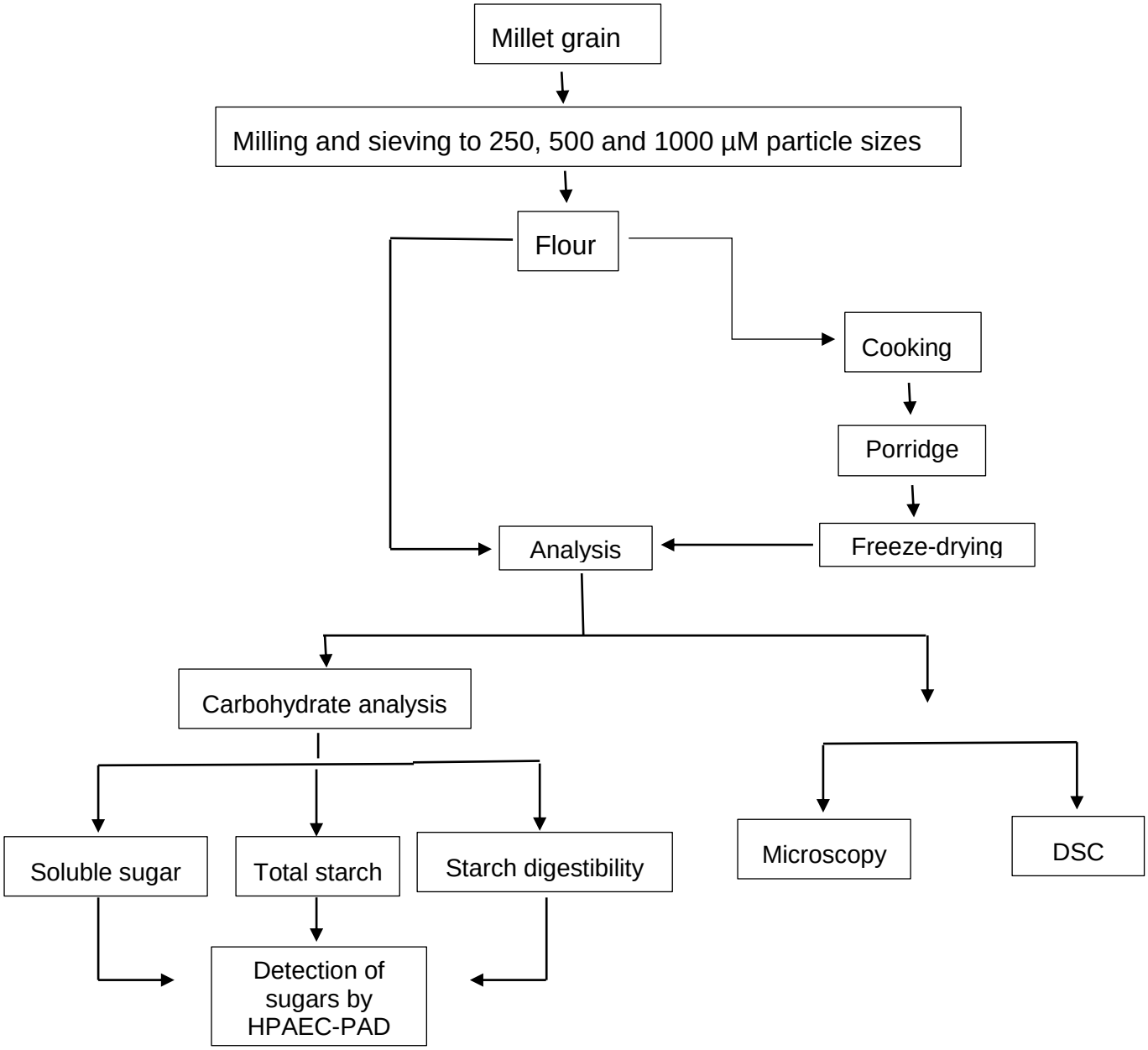

Figure 14. Summary of carbohydrate analysis for unfermented millet products.

5.3. Results

5.3.1. Preparation of *ibyer*

The first step of this study was to prepare traditional millet porridge *ibyer*. The porridge was made with flour particles of 250, 500 and 1000 µM size distribution of flour. Upon reconstituting flour with water and heating by boiling for 30 min, millet was found to swell, forming a semi-thick gel (Fig. 15). The colour of the porridge was not affected by cooking.

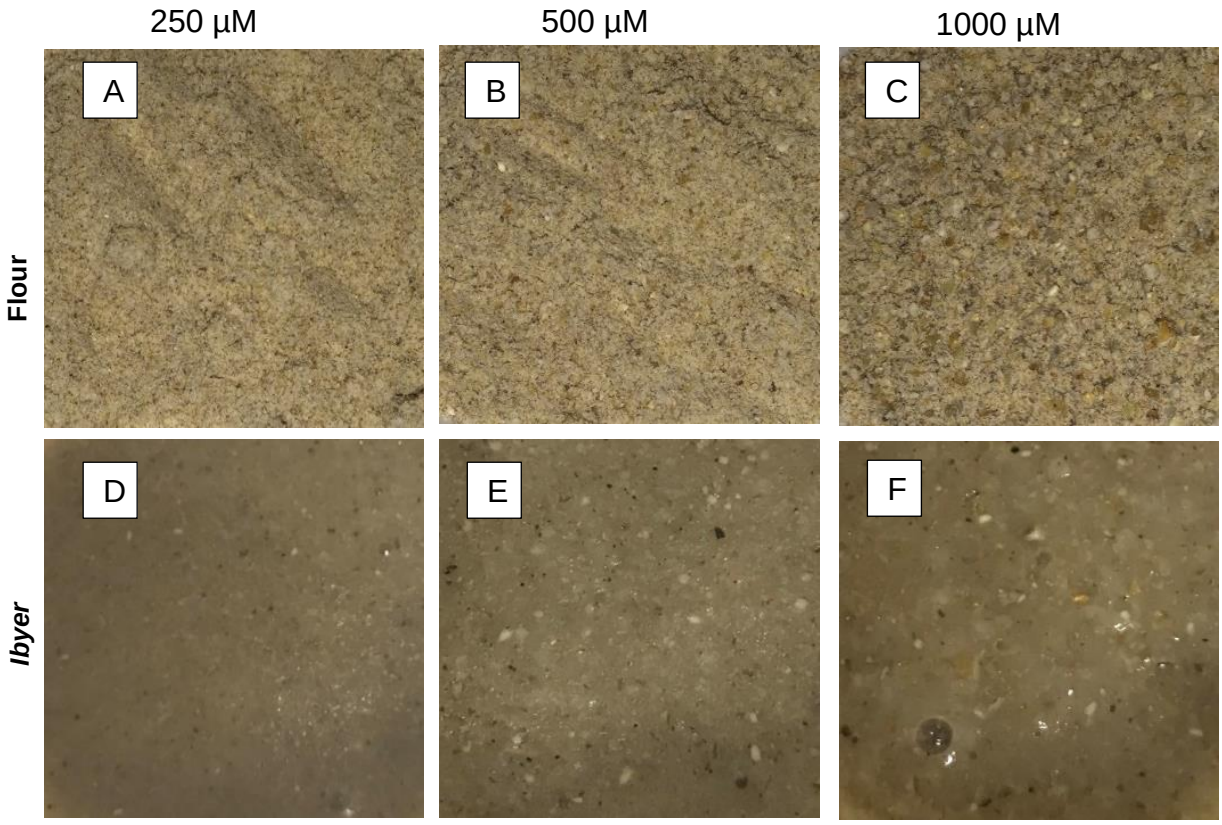

Figure 15. Images of millet flour (A-C) and *Ibyer* porridge (D-F) prepared with 250, 500 and 1000 µM of flour.

5.3.2. Microscopy

In this study, the microstructural properties of millet were investigated. Millet samples were subjected to different treatments before visualization. Millet grain was broken in half or milled to obtain three different particles sizes; 250, 500 and 1000 µM, and then subsequently cooked to make *ibyer*. In addition to milling the millet grains to flour, starch granules were isolated (IMS) by filtration. Further visualizations were conducted on the cracked grain, IMS, millet flours and porridges were using SEM to evaluate cell wall breakage and starch granule exposure. In addition, millet flour and *ibyer* were subjected to enzymatic digestion and observations were reported.

Figure 16A to F shows the SEM images of cracked millet grain. The pericarp of millet was around 25 µM thick (Fig. 16B) consisting of layers of endocarp and mesocarp. The pericarp is suggested to account for the greenish-brown colour in millet. A look at the endocarp shows dense cells folded in layers. The mesocarp of millet (Fig. 16C) was slightly further down the endocarp and comprised of thick flat layers of cell with starch granules embedded in the layers. Starch granules were not apparent in the endocarp. The millet starch granules in the endosperm were packed tightly in irregular shaped cells (Fig. 16D and E), with endosperm cells dimensions around 50 x 120 µM as delineated in Fig. 16D and E. The endosperm cells were observed to have thin cell walls (Fig. 16F) of around 1.0-1.35 µM that are unlikely to present significant barriers to digestive enzymes.

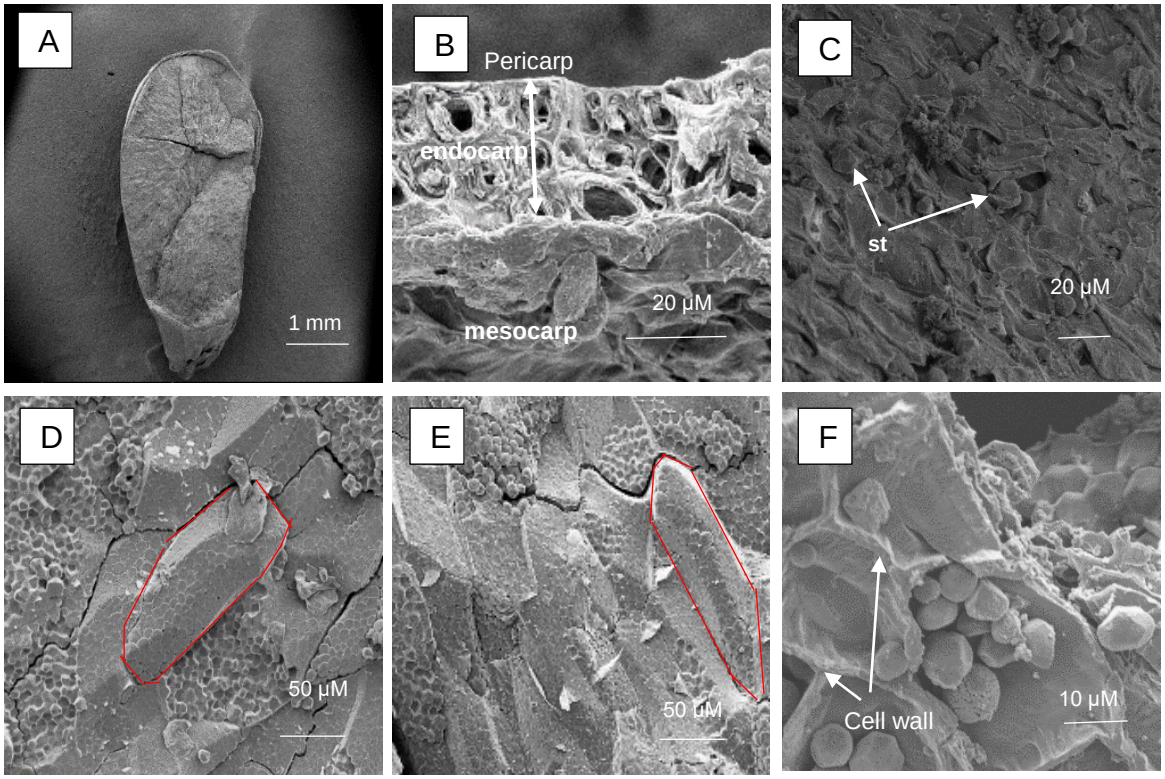

Figure 16. Micrographs of pearl millet grain showing broken grain (A), two delineated cells of the endosperm (B and C) to show the size and shape. The pericarp shows the thickness of the endocarp (D) and embedded granules in the mesocarp (E). Damaged millet grain revealed the cell walls (F).

5.3.2.1 Effect of particle size on the microscopic structure of millet

The milled millet flour particles used in this study was predominantly 250, 500 and 1000 μM in size (Fig. 17A, B and C). Milling resulted in the breakage of the endosperm cells and exposure of abundant tightly packed starch granules (Fig. 17D) with proteins on the surface and between the aggregated granules. Due to the similarities in the structure of starch in all particles of flour, only one sample was presented.

The granules observed from the isolated starch were varied in size and shape (Fig. 17D and E), and had about 4 folds difference between smaller and bigger granules (4 to 16 μM). Indentations visible on granules (Fig. 17F) may have resulted from the removal of protein or lipid bodies originally engrained in the granules. Figure 17G shows that even after the extraction of free starch, starch granules surfaces had aggregates presented by proteins. From the properties of millet starch visualized, it can be suggested that flour with the smallest particle sizes may have the largest surface area and hence, is predicted to have the fastest digestion which is likely be improved by cooking. Upon cooking flour, most of the organised structure of starch granules was lost to reveal some undisrupted granules and amorphous sheet-like structures (Fig. 17H and I) suggested being starch-protein gels. It is possible that the structure of some starch granules was not lost by cooking and may be embedded in the starch-protein gel.

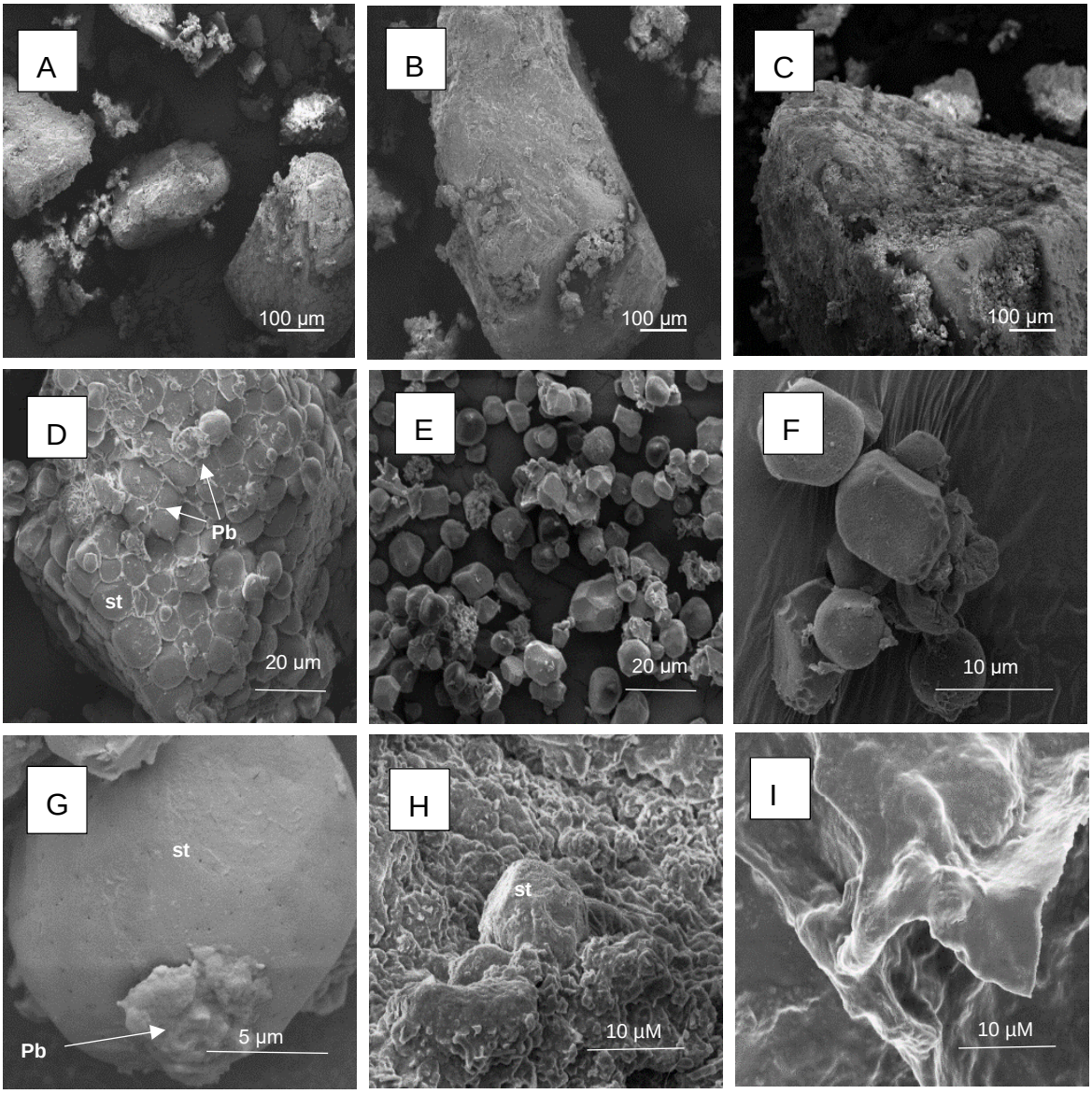

Figure 17.SEM images of millet flour of three different particle sizes 250 µM (A), 500 µM (B) and 1000 µM (C). Milling showed compactly packed abundant starch granules of different sizes from broken cells (st, visible in D). Extraction of starch granules revealed granules of different shapes and sizes (E) with indentation on granules (F) and protein bodies on surface on granule (G). Cooking of flour into porridge showed undisrupted granules (st visible in H) and the sheet-like structures formed (I).

5.3.2.2. Effect of pepsin pre-incubation on the microscopic structure of millet starch

Firstly, when millet was digested with only pepsin, indentations and pores on granules were more visible (Fig. 18A), which indicated that some proteins on the surface and in the interior part of millet granules were removed. While visualizing SEM images of digested flour, split granules due to milling revealed growth rings with layers of semi-crystalline and amorphous regions of millet (Fig 18B). These layers were thicker in the middle and thinner towards the outer part of the granule. The outer layer growth ring of millet granule was mainly the amorphous region. Further investigation of the damaged granules revealed tiny roundish protein bodies (Fig. 18C) of around 1.50 µM in size that were originally engrained in the granules.

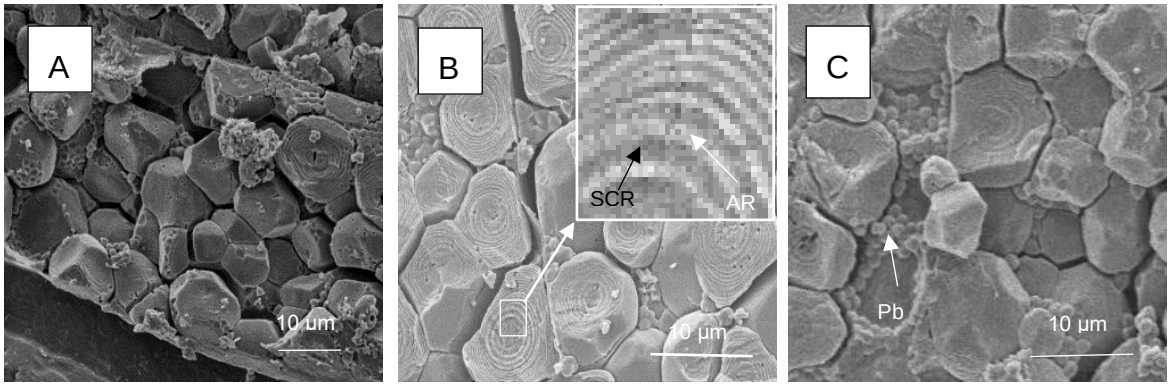

Figure 18. Digestion of milled flour with pepsin revealed indentations (A) and growth ring showing amorphous and semi-crystalline of millet (B) and tiny roundish material suspected to be protein bodies (Pb visible in C). Surface pores and remover of protein bodies from starch granules was as a result of pepsin treatment of flour. Scale bar = 10 µM.

The effect of gastrointestinal digestion on the structure of millet starch was investigated and results presented (Fig. 19). Millet flour and porridge were observed after digestion with pancreatin only (Goñi) and when intestinal phase of digestion with pancreatin was preceded by pepsin treatment (INFOGEST). Upon digestion with Goñi, many starch granules in flour remained visibly aggregated and undigested (Fig 19A and B) and protein matrices were still present. On the other hand, following INFOGEST, there was an appreciable change, as starch granules presented more cavities (Fig 19D and E) suggesting more starch hydrolysis.

Upon digestion of porridge, digestion with Goñi resulted in less apparent breakdown of the sheet-like structure (Fig 19C) compared to when INFOGEST was used (Fig 19F). We observed that, some undigested starch granules could be identified in the amorphous structure of cooked porridge even after gastrointestinal digestion. These micrographs suggest that the gastric phase has a pronounced effect on the digestion of flour and porridge. For this reason, the extent of digestion was measured analytically.

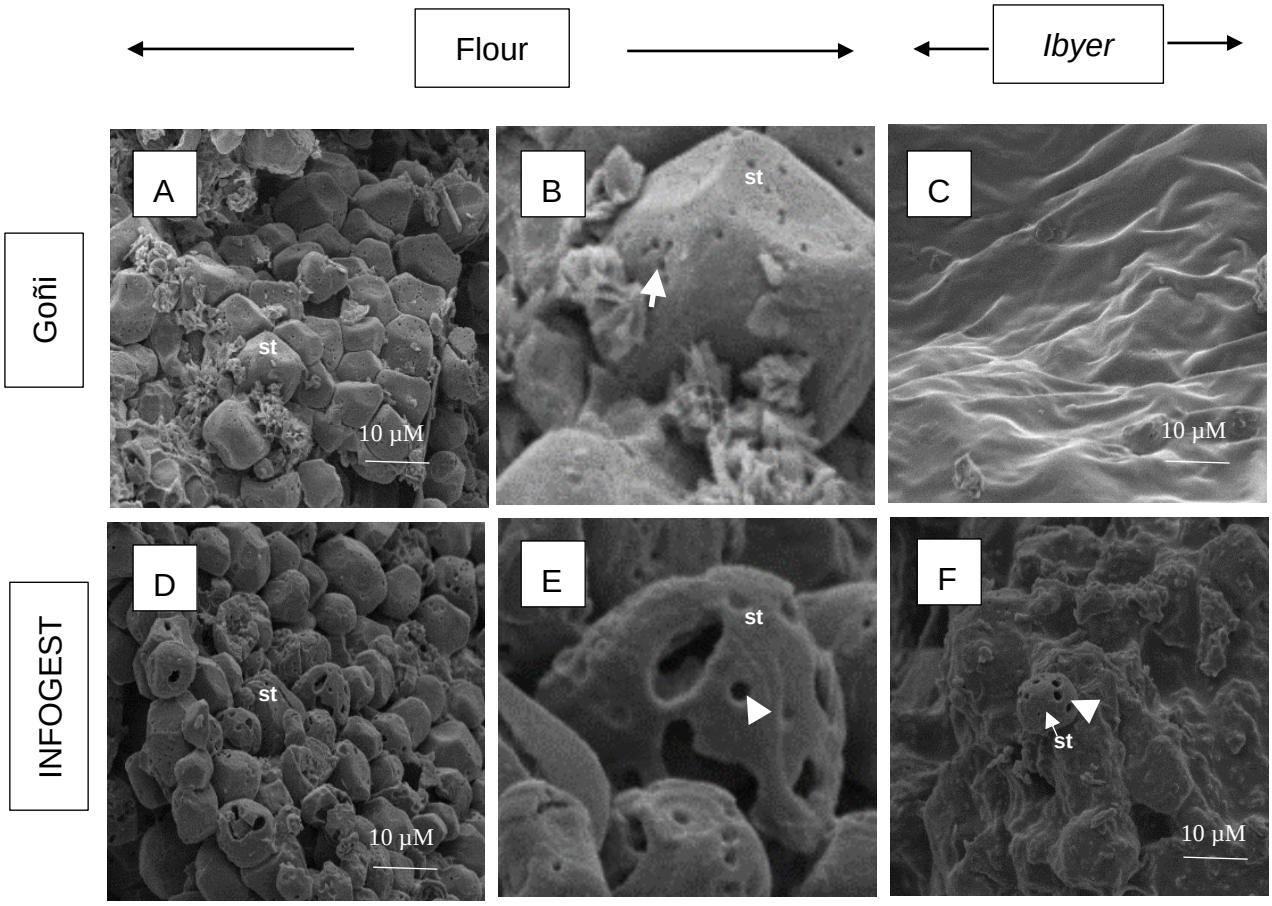

Figure 19. SEM images of intestinal digestion of millet flour without (A and B) and with (D and E) pepsin pre-incubation, and intestinal digestion of *ibyer* without (C) and with (F) pepsin pre-incubation. Digestion of starch was apparent by presence of pores (shown by arrowheads). Digestion of porridge with gastric + intestinal enzymes revealed starch granules that were trapped in the porridge gel (F). Scale bar = 10 µM.

5.3.3. Determination of soluble sugar in millet samples

The soluble sugar composition of millet was determined (Fig. 20). Comparisons were made between flour and *ibyer* prepared with different particle sizes of flour. The data presented was in dry weight of samples. Both particle size reduction of millet flour and cooking (*ibyer*) had an impact on the soluble sugar content of millet at $p<0.05$. The main sugars detected and quantified were glucose, fructose, raffinose, sucrose, maltose, and stachyose in flour (Fig. 20A) in addition to maltotriose in *ibyer* (Fig. 20B). However, trace amounts of galactose and arabinose were also detected but too low to quantify (data not shown).

Starting with flour (Fig. 20A), soluble sugars increased inversely to particle size. Meanwhile, sucrose was the most abundant sugar in millet flour, followed by the fructo-oligosacccharide raffinose. In contrast to other sugars, the fructo-oligosaccharides raffinose and stachyose remained relatively consistent in all particles sizes of flour. Millet flour presented higher content of non-reducing sugars (1.90 to 2.21%) with lower values of less than 1% for reducing sugars as shown in Table 12.

The soluble sugar content of *ibyer* prepared with different particles of flour is presented in Table 9. The trend in yield in relationship to particle sizes was similar to flour, though with significant higher values ($p<0.05$). The soluble sugar content of *ibyer* was highest for 250 µM (7.00 t0 8.82%) and lowest for 1000 µM (4.76 to 6.18%). This result shows more than 2 folds increase in values for 250, 500 and 1000 µM particle sizes compared to flour. The preparation of *ibyer* resulted in an

increase in the content of glucose, maltose and maltotriose, and decrease of sucrose (Fig. 20B). Maltotriose was the most abundant soluble sugar in *ibyer*. Conversely to the results obtained for flour, *ibyer* samples were made up of higher proportions of reducing sugars (Table 12). This observation suggests that endogenous enzymes are active during cooking, most likely during early heating stages.

The impact of particle on soluble sugar content in both flour and *ibyer* suggests that sugar solubility from flour particles were not similar for all sizes. In *ibyer*, the glucose, maltose and maltotriose contents were inversely proportional to particle size suggesting higher solubility in smaller sized particles which could be due to more enzyme activities.

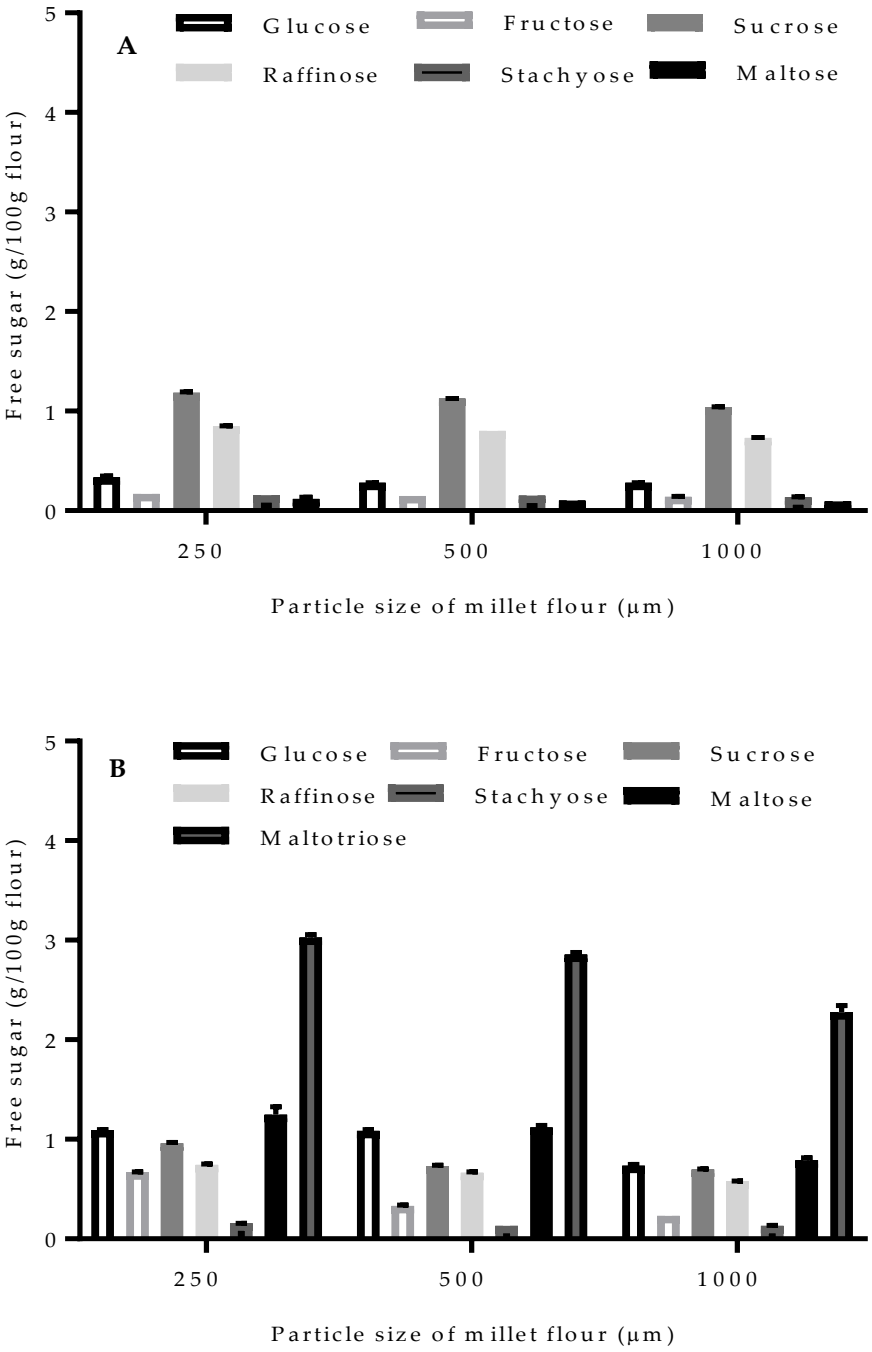

Figure 20. Free sugar content of different particles of millet flour (A) and *ibyer* (B). Results are presented as mean of 3 biological replicates and expressed in dry weight basis. Data error bars are standard deviation of the mean.

5.3.4. Total starch content of millet

The total starch content of the different particle sizes of flour and *ibyer* was determined (table 12) with more optimised chemical and enzymatic method from the previous chapter. In addition to the starch content method, millet starch was isolated (IMS) from flour using the filtration method and the total starch and amylose content were also estimated (Table 12).

The mean starch content of millet was 70.10% for flour and 68.50% for *ibyer*. The results obtained indicates that neither particle size nor cooking had an impact on starch yield at $p<0.05$. However, there was a slight increase in starch content proportionally to particle size in both flour and *ibyer*. The increase in flour was mainly due to particle size effect whilst in *ibyer*, it indicates that the amount of free glucose corrected for sample due following cooking may have had potential effect on the starch content of *ibyer*.

The starch content of isolated millet starch (IMS) was around 90%, suggesting the presence of other food components such as protein and lipid and therefore method needs to be optimised. The amylose content of millet starch was 31.98%. The amylose content may influence the crystallinity of millet when cooled and affect digestibility.

In summary, statistically neither particle size reduction nor cooking had an impact on the total starch content of millet. However, cooking resulted in lower yield of starch compared to flour primarily due to the increased content of reducing sugars in cooked millet samples.

Table 12. Moisture content and carbohydrate composition of different particle sizes of flour, *ibyer* and ungelatinised isolated millet starch (IMS). The values represent the percentage mean ± standard deviation in dry weight basis of 3 biological replicates. Data in the same column with the same superscript are not significantly different ($p<0.05$). CHO = carbohydrate.

Condition	Particle size (µM)	Moisture	Reducing sugars	Non-reducing sugars	Total soluble sugars	Total starch	Total CHO	Amylose
Flour	250	4.43±0.67^{a}	0.63±0.11^{a}	2.19±0.02^{a}	2.82±0.44^{a}	68.71±1.37^{a}	71.66±1.58^{a}	
	500	5.04±0.66^{a}	0.53±0.06^{a}	2.08±0.03^{b}	2.61±0.43^{b}	70.07±1.71^{a}	72.68±1.83^{a}	
	1000	4.93±0.03^{a}	0.51±0.10^{a}	1.91±0.01^{c}	2.42±0.39^{c}	71.40±1.90^{a}	73.83±1.77^{a}	
Ibyer	250	85.49±0.42^{b}	6.04±0.09^{b}	1.86±0.01^{d}	7.91±0.91^{d}	67.99±1.78^{a}	75.62±5.22^{a}	
	500	85.24±0.03^{b}	5.39±0.04^{c}	1.53±0.02^{e}	6.92±0.90^{e}	68.71±2.50^{a}	75.62±2.29^{a}	
	1000	84.44±0.31^{b}	4.03±0.09^{d}	1.41±0.01^{f}	5.47±0.71^{f}	68.81±1.68^{a}	74.28±1.60^{a}	
IMS	-	-				89.32±2.1^{b}		31.98±5.06

5.3.5. Protein analysis

The protein content of millet flour with a particle size of 250 µM was measured using the Kjeldhal method (Table 13). The protein yield in flour ranged from 9.20 to 11.22% and 9.62 to 11.38 in *ibyer*. Nevertheless, statistically, the protein content of millet was not altered by cooking.

Table 13. Protein content of millet flour and *ibyer* samples. The results are presented as the percentage mean± standard deviation of 3 biological replicates and expressed in dry weight basis. The data in the same column with the same superscript are not significantly different ($p<0.05$).

Sample	Protein content (%)
Flour	10.21 ± 1.01^{a}
Ibyer	10.50 ± 0.88^{a}

5.3.6. Effect of particle size on gastrointestinal starch digestion of millet

Millet samples (flour and *ibyer*) were subjected to two *in vitro* protocols; Goñi and INFOGEST. Even though it was shown in the previous chapter that the INFOGEST was the best protocol for the starch digestion of millet flour, Goñi was used again in this current study to verify if the same results could be adopted for porridge. The oral phase of digestion was omitted in this study. The result presented in this section indicates that millet starch can be hydrolyzed by both methods of enzymatic digestion.

Starting with flour, Fig. 21 shows that both particle size of flour and protocol used in starch analysis are likely to have influence on the starch digestion of millet ($p<0.05$). The starch digestibility of millet flour increased inversely to particle size, with the smallest particles of flour (250 µM) markedly higher. The results of starch digestibility following Goñi and INFOGEST were not consistent. Relatively lower values between 12.18 to 18.01% were obtained for Goñi (Fig. 21A) with more than 2 folds higher values from 44.29 to 50.21% for INFOGEST (Fig. 21B). The big variation was due largely to the impact of gastric phase in removing proteins engrained in starch and subsequently allowing amylase to hydrolyze more starch. The incubation time also had affected starch digestibility.

Cooking has been predicted to improve access of enzyme to substrate (starch). The results presented in Figure 22 suggests that disruption of starch structure by cooking, thereby forming gel significantly ($p <0.05$) improved starch hydrolysis of millet compared to flour (Figure 21). Again, our result shows that the gastric phase had the biggest impact on starch digestibility of *ibyer*, lowest within the

range of 27.48 to 32.24% for Goñi (Figure 22A) and highest in the range of 55.38 to 56.82% for INFOGEST (Figure 22B). As expected, *ibyer* made with 250 µM were more hydrolyzed compared to the 1000 µM particles of flour ($p<0.05$) between 5 and 120 min following Goñi. In contrast, an effect was only observed between 250 and 1000 µM at 5 and 20 min with INFOGEST. The digestion pattern was identical for both protocols; rapid during the initial 20 min of digestion and slower after 60 min.

Classification of starch fractions is useful in understanding digestibility. In consideration of this, we calculated the RDS, SDS and RS from data generated for both *in vitro* methods used in our study (Table 14). Our result shows very similar trends in starch fraction of millet subjected to the two *in vitro* protocols; with high RDS in *ibyer* compared to flour. The RDS content of millet flour increased inversely to particle size whilst the SDS and RS increased proportionally to particle size in both methods ($p<0.05$). For *ibyer*, the trend was similar to millet flour but the values for RDS and SDS were lower for *ibyer* following Goñi (25.74 and 4.37%) and higher for INFOGEST (41.36 and 14.65%). For RS, the values were relatively higher with Goñi (69.89%) and lower for INFOGEST (43.98%). These results suggests that millet starch following cooking to make *ibyer* is rapidly digestible and yet relatively resistant to enzymatic digestion. It is evident that gastric phase had a big effect on RSD, SDS and RS.

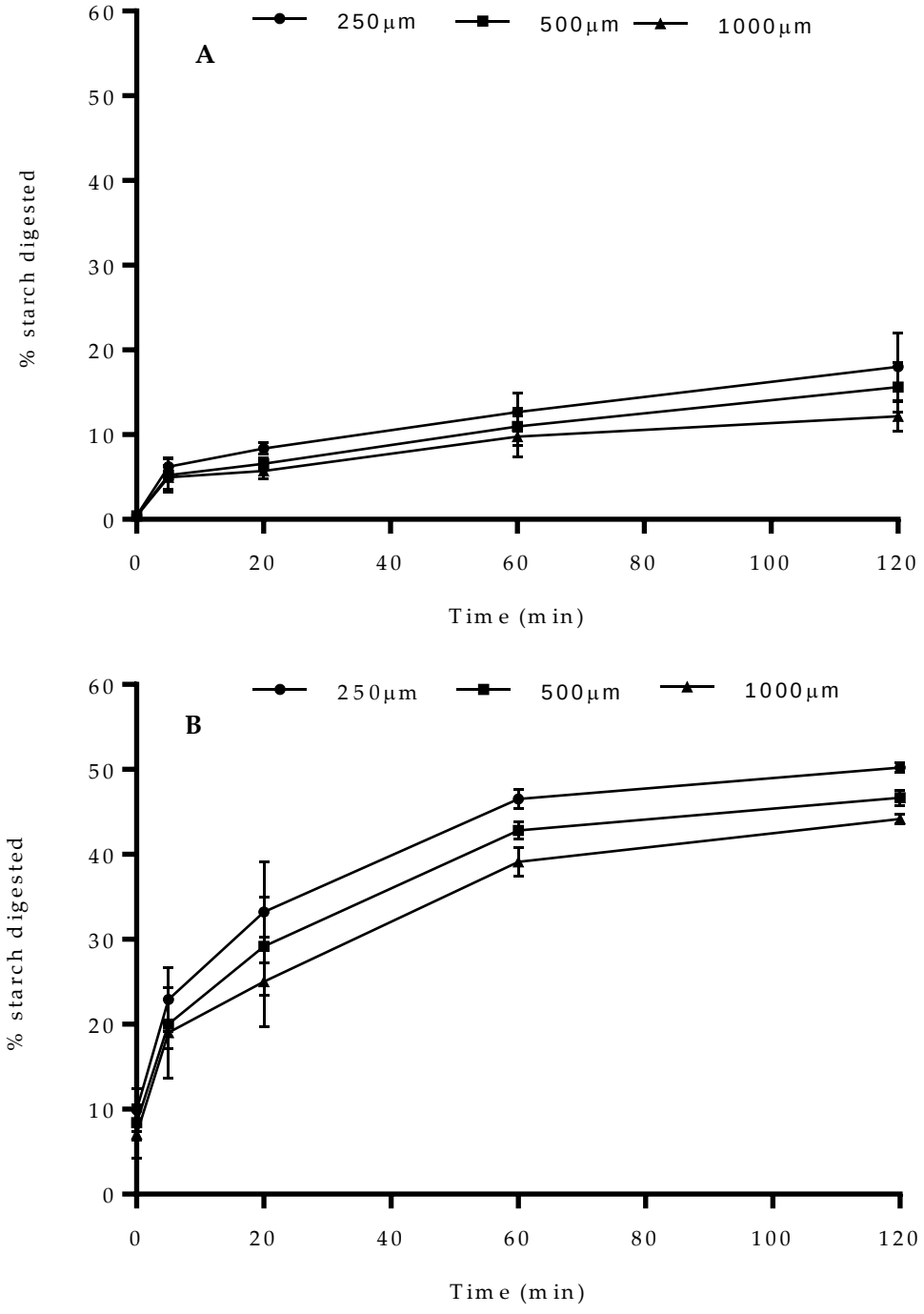

Figure 21. Starch digestion of millet flour of different particles sizes following Goñi (A) and INFOGEST (B) protocols. Results are presented as mean of 4 and 7 biological replicates for Goñi and INFOGEST respectively and expressed in dry weight basis. Data error bars are standard deviation of the mean.

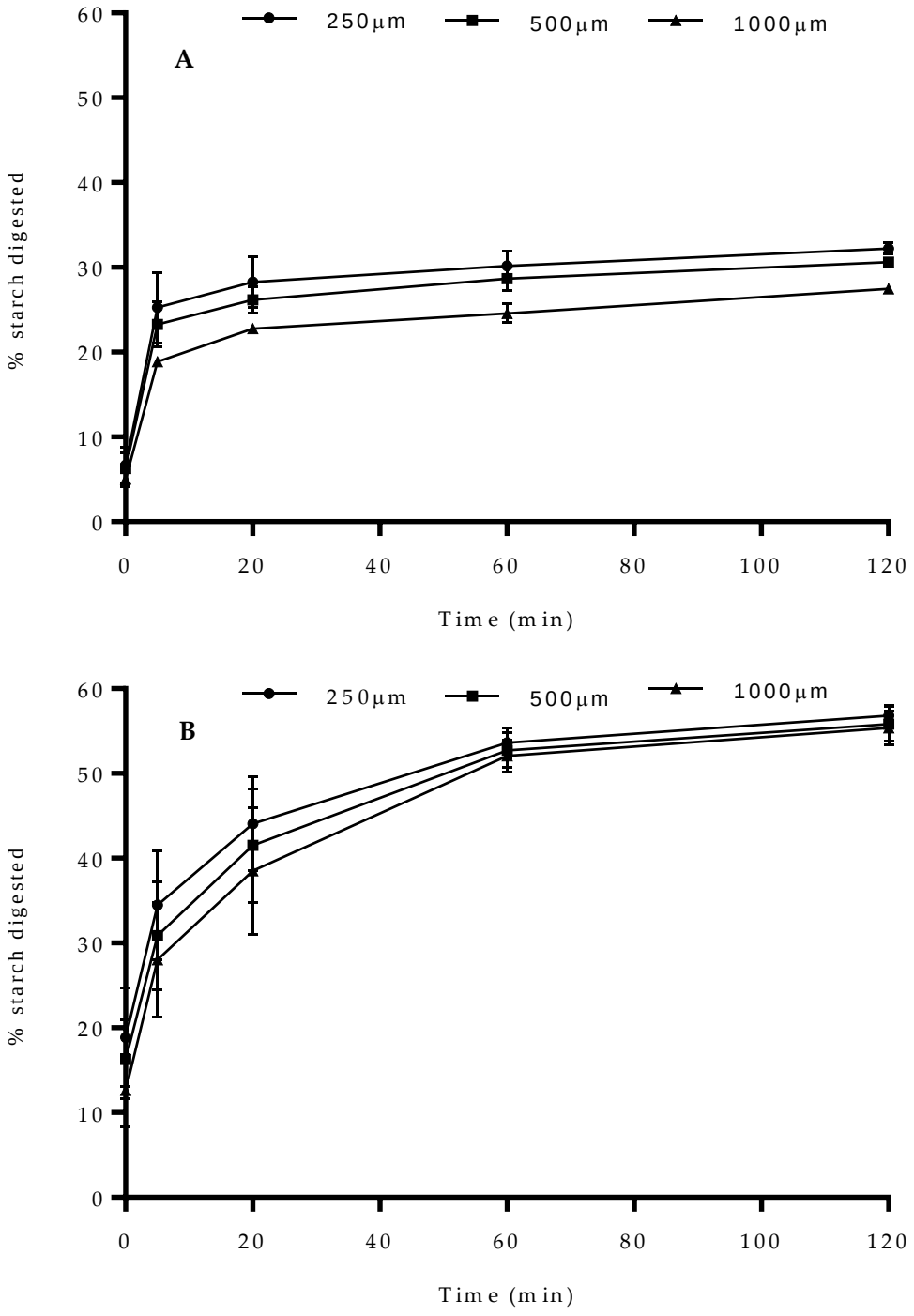

Figure 22. Starch digestion of *ibyer* of different particles sizes following Goñi (A) and INFOGEST (B) protocols. Results are presented as mean of 4 and 7 biological replicates for Goñi and INFOGEST respectively and expressed in dry weight basis. Data error bars are standard deviation of the mean.

Table 14. Results of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) of millet following Goñi and INFOGEST after 120 min. Values are presented as mean ± standard deviation of 4 and 7 biological replicates respectively and expressed in dry weight basis. Data in the same column of flour or cooked gruel with the same superscript are not significantly different ($p<0.05$).

Sample	Particle size (µM)	RDS		SDS		RS	
		Goñi	INFOGEST	Goñi	INFOGEST	Goñi	INFOGEST
Flour	250	8.39 ± 0.70^{a}	32.78 ± 5.54^{ac}	9.62 ± 4.03^{a}	17.32 ± 0.57^{a}	81.99 ± 3.49^{a}	49.81 ± 5.13^{ab}
	500	6.58 ± 0.77^{a}	28.78 ± 5.35^{a}	9.04 ± 2.93^{a}	17.93 ± 0.86^{ab}	84.38 ± 2.16^{ab}	53.29 ± 5.15^{ac}
	1000	5.71 ± 0.92^{b}	24.18 ± 5.33^{a}	6.47 ± 1.78^{ab}	20.11 ± 0.64^{b}	87.82 ± 0.87^{b}	55.71 ± 5.21^{a}
Ibyer	250	28.27 ± 3.03^{c}	44.06 ± 5.53^{b}	3.97 ± 0.67^{b}	12.78 ± 1.29^{c}	67.76 ± 3.48^{c}	43.15 ± 5.29^{b}
	500	26.16 ± 1.63^{cd}	41.53 ± 6.71^{bc}	4.46 ± 0.45^{b}	14.29 ± 2.08^{c}	69.38 ± 1.96^{c}	44.17 ± 6.37^{bc}
	1000	22.80 ± 0.32^{d}	38.50 ± 7.47^{bc}	4.68 ± 0.38^{b}	16.88 ± 1.95^{a}	72.52 ± 0.70^{c}	44.62 ± 7.51^{bc}

5.3.7. Effects of particle size and cooking on estimated glycemic index of millet

It has been known that food processing influences starch digestion with potential implications on postprandial glycemic response. The *in vitro* kinetics of the cooked *ibyer* was investigated and data obtained following INFOGEST (Table 15) and Goñi (Table 16) were presented. In this particular study, emphasis for eGI will be made on only *ibyer* samples and not flour.

The effect of particle size was more evident with the data from the kinetic constant (k) related to equation 4 to 6, which gives insight into the early stage of digestion. *Ibyer* with the smallest particle size (250 µM) had the highest k value and 1000 µM had the lowest value. These results were consistent with both protocols; however, there was no significant difference. As expected, the eGI of *ibyer* was lowest with values ranging from 39.54 to 41.57 with Goñi and highest with values from 50.58 to 51.65 following INFOGEST. The variation was largely due to method of enzymatic digestion and particle size of flour. In both protocols, *ibyer* with the largest particle size (1000 µM) revealed markedly lower eGI. The trend of k was consistent with HI and eGI, which suggests that the rate of hydrolysis in the early stage is likely to have a big impact on postprandial glycemic response.

Table 15. First order starch kinetics of millet following INFOGEST. Data represents the mean ± standard deviation of 7 biological replicate. Parameters calculated were starch hydrolysis (C_∞**)**, kinetic constant (k), hydrolysis index (HI) and estimated glycemic index (eGI) of millet. Data in the same column of flour or *ibyer* with the same superscript are not significantly different ($p<0.05$).

Condition	Particle size (μM)	C_∞ (%)	K (sec^{-1})	HI (%)	eGI
Flour	250	50.19 ± 0.57^{a}	3.10 ± 0.12^{a}	54.20 ± 0.52^{a}	48.65 ± 0.20^{ac}
	500	46.71 ± 0.86^{b}	3.02 ± 0.13^{a}	50.39 ± 0.78^{b}	47.17 ± 0.29^{a}
	1000	44.29 ± 0.64^{b}	2.98 ± 0.13^{a}	47.82 ± 0.78^{c}	46.20 ± 0.28^{a}
Ibyer	250	56.84 ± 1.29^{c}	3.33 ± 0.09^{b}	61.77 ± 1.31^{d}	51.64 ± 0.72^{b}
	500	55.82 ± 2.08^{c}	3.12 ± 0.10^{a}	60.13 ± 2.19^{d}	50.94 ± 0.78^{bc}
	1000	55.38 ± 1.95^{c}	2.98 ± 0.18^{a}	59.25 ± 2.33^{d}	50.58 ± 0.84^{bc}

Table 16. First starch kinetics of millet following Goñi. Data represents the mean ± standard deviation of 4 biological replicate. Parameters calculated were starch hydrolysis (C ∞**)**, kinetic constant (k), hydrolysis index (HI) and estimated glycemic index (eGI) of millet. Data in the same column of flour or *ibyer* with the same superscript are not significantly different ($p<0.05$).

Condition	Sample (µM)	C ∞ (%)	K (sec^{-1})	HI (%)	eGI
Flour	250	18.01 ± 4.03^{a}	3.13 ± 0.13^{a}	20.62 ± 4.31^{a}	35.73 ± 1.48^{a}
	500	15.62 ± 2.93^{ab}	3.03 ± 0.16^{a}	18.02 ± 3.13^{ab}	34.73 ± 1.08^{a}
	1000	12.18 ± 1.78^{b}	2.93 ± 0.12^{a}	14.30 ± 1.92^{b}	33.30 ± 0.66^{a}
Ibyer	250	32.24 ± 0.67^{c}	3.32 ± 0.09^{a}	35.80 ± 0.75^{c}	41.57 ± 0.24^{b}
	500	30.63 ± 0.45^{d}	3.14 ± 0.05^{a}	33.94 ± 0.49^{d}	40.85 ± 0.16^{b}
	1000	27.48 ± 0.38^{e}	3.03 ± 0.21^{a}	30.56 ± 0.35^{e}	39.54 ± 0.12^{b}

5.3.8. Thermal properties of millet

The degree of gelatinisation and retrogradation of millet were investigated by analysing whole flour by differential scanning calorimetry (DSC). The parameters analysed were the onset temperature (T_o), peak gelatinisation temperature (T_p), conclusion temperature (T_c) and change in enthalpy (ΔH) of gelatinisation and retrogradation. Emphasis in this section were made on Tp and ΔH for the gelatinisation and retrogradation of millet.

In this present study, gelatinisation of millet was observed to have three distinctive transition peaks (Figure 23) and only two transition peaks after retrogradation (Figure 24). For gelatinisation, the first transition peak had a significantly lower T_p, and enthalpy compared to the second and third transition peak ($p<0.05$). This was consistent with the transition peaks for retrogradation which had a lower T_p and ΔH for first transition peak compared to second peak. Based on our study, there was a significant difference between the gelatinisation and retrogradation of millet flour (Table 17).

The T_p, and enthalpy ranges for gelatinisation of millet flour were 72.43-99.30°C and 3.29-11.58 J/g. The lower T_p (33.96°C) and ΔH (0.34 J/g) of the first transition peak for gelatinisation suggests the melting of lipid. The second gelatinisation transition peak indicates that smaller granules in millet had inferior crystallinity and were completely disrupted at a lower temperature and energy compared to the larger sizes which are likely to have been completely disrupted at a higher temperature. The higher gelatinisation temperature peak observed in the third

transition peak indicates that millet may have starch polymers that are likely to be completely disrupted at temperatures higher than 90°C.

Interestingly, gelatinised samples allowed to retrograde resulted in only two transition peak for millet flour (Figure 24) with the first transition peak suggested to be the melting of lipid which is consistent with the gelatinization of millet flour. The T_p, and enthalpy ranges for retrogradation of millet flour starch were 93.89-95.63°C and 5.59-6.19 J/g (Table 17). This result suggests that gelatinised millet flour when cooled is likely to rearrange into a superior crystalline structure.

Treatment (retrogradation) seemed to have the biggest impact on the thermal property of millet. The result obtained demonstrates that the millet cultivar used in this present study was highly susceptible to retrogradation, which was reflected in the limitation of the gastrointestinal digestion of *ibyer*. We have also shown that gelatinisation reported here was consistent with very low variation after the experimental information was repeated 3 times.

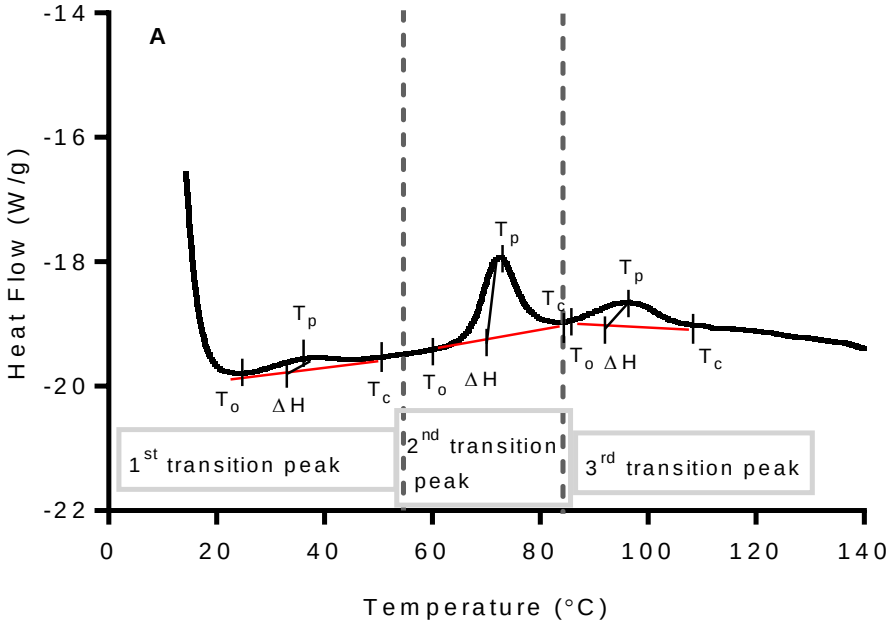

Figure 23. A representation of thermogram showing the transition peak for melting of lipid (1st) and two transitional peaks (2nd and 3rd) for the gelatinisation of millet flour.

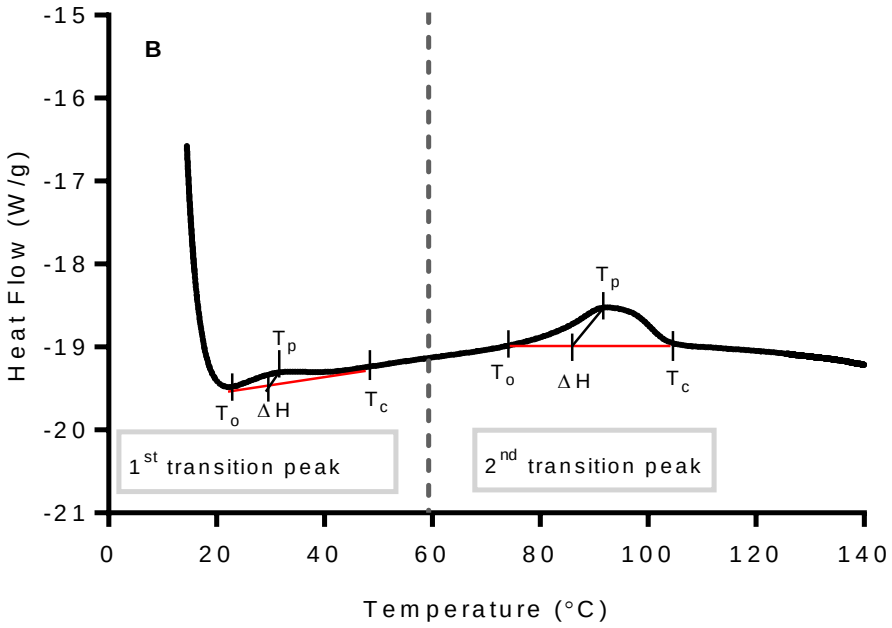

Figure 24. A representation of thermogram showing the transitional peak melting of lipid (1st) and for the degree of dissociation by retrogradation (2nd) of millet flour.

Table 17. Millet flour samples showing onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) enthalpy change (ΔH) after gelatinisation and retrogradation. Results are presented as mean ± standard deviation of 3 biological replicates. Data in the same column of gelatinisation or retrogradation with the same superscript are not significantly different ($p<0.05$).

Millet flour	T_o (°C)	T_p(°C)	T_c (°C)	ΔH (J/g)
1st transition				
Gelatinisation	22.87±1.27[a]	35.96±1.47[a]	46.48±1.95[a]	0.34±0.08[a]
Retrogradation	22.43±1.35[a]	33.00±0.65[a]	46.29±1.05[a]	0.32±0.03[a]
2nd transition				
Gelatinisation	62.21±2.13[b]	75.06±2.63[b]	83.35±1.04[b]	11.03±0.55[b]
Retrogradation	76.79±3.63[c]	94.76±0.87[c]	107.35±1.46[c]	5.89±0.30[c]
3rd transition				
Gelatinisation	85.20±0.51[d]	98.40±0.90[d]	108.61±3.19[c]	3.85±0.56[d]
Retrogradation				

5.4. Discussion

Microstructure

Considering that millet was resistant to digestion, another objective of this study was to investigate the starch structure of millet on a molecular basis. Starch structure has been widely linked to the digestion rate, bioaccessibility and is suggested to have influence on postprandial responses (Amadou *et al.,* 2014; Ren *et al.,* 2017; Srichuwong *et al.,* 2017). Generally, the starch granule size, compactness of granules within endosperm, crystallinity of starch and the degree of starch-protein-lipid interactions were some of the contributing factors attributed to carbohydrate digestion. Therefore, manipulating these factors through processing methods such as particle size reduction of flour, cooking method, fermentation, and germination may have a range of implications on starch based plant crops.

The data as presented in this study indicates Nigerian pearl millet has different sizes of granules, mostly less than 20 μM and a similar range was reported for Ivorian cultivars of millet (Srichuwong *et al.,* 2017). This study also indicates that pearl millet has proteins engrained in its starch granules. A good explanation to this was reflected in the appearance of protein bodies around granules having indentations on their surface after starch gastric digestion. Analysis of rice and barley starch by confocal laser scanning microscopy confirmed the presence of proteins and lipids in granules (Ye *et al.,* 2018; Yu *et al.,* 2018). For this reason, high amount of proteins around and in the interior of starch granules are likely to present significant barriers for enzyme access to substrate.

How granule size affects starch properties is not well understood (Srichuwong *et al.,* 2017; Ye *et al.,* 2018), especially how it correlates with enzyme diffusion. The idea that surface pores (airspaces) and granules size may facilitate water penetration and enzyme diffusion during hydrolysis may be foreshadowed by the degree of starch-protein network. For instance, starch structure of proso millet was completely lost when steamed for 15 min (Yang *et al.,* 2018) which was attributed to the extent of airspace in granules. This was not consistent with our results, as even after 30 min of cooking, not all starch structure was lost as suggested by the SEM images of *ibyer* in this study.

The internal structure of millet granule was mainly semi-crystalline and the growth rings were relatively thick compared to the amorphous region. This pattern has been reported and in combination with starch-protein complex may influence diffusion of enzyme and penetration of water in granules (Wong *et al.,* 2009; Chen *et al.,* 2016; Yu *et al.,* 2017). The limitation of hydrolysis of starch i.e size of cavities in ungelatinised structures and undisrupted structure in gelatinised granules even after extensive enzymatic hydrolysis confirms this. It is important to state that, as observed in this study, pre-incubation with pepsin removes some of the proteins wrapped around granules thereby enhances amylase diffusion (as seen in size of cavities in granule structure).

Effect of processing on the carbohydrate composition of pearl millet products

This current study provides detailed information on the carbohydrate composition of millet. The effect of particle sizes of flour and cooking on the soluble sugars and starch content of millet were studied. In addition, the amylose of millet was estimated. Though the carbohydrate composition of food crops has been investigated (Oshodi, 1999; Rao and Muralikrishna, 2001; Amadou *et al.,* 2014; Srichuwong *et al.,* 2017), the effect of processing as highlighted here has not been well reported. Majority of the data reported for millet were not conducted using optimal sugar extraction methods or starch measurements. The extent of processing and method of analysis can have impact on the carbohydrate content of foods. Data obtained for foxtail millet, finger millet, wheat in a previous study supports this (Amadou *et al.,* 2014; Rao and Muralikrishna, 2001; Malleshi et al., 1986).

The soluble sugar content of millet flour was similar to previous reports for foxtail millet (0.20-2.79%) but higher than values reported for different selected cereals (0.13 to 1.20%) such as finger millet, proso and khodo (Malleshi et al., 1986; Oshodi, 1999; Rao and Muralikrishna, 2001; Rao and Nagasampige, 2011; Shobana *et al.,* 2013; Amadou *et al.,* 2014). Generally, the estimation of soluble sugars in these studies were conducted on raw food products from fine particles and the values reported in this study were from a wide range of flour particle sizes. The soluble sugar content of *ibyer* was significantly higher (4.76 to 6.76%) in comparison to flour and increased inversely to particle size. The variation in data obtained in this current study was attributed to the impact of cooking and particle

size on millet. Cooking has been shown to activate the hydrolases activity in carbohydrates between 20 to 70°C at a pH 7 (Ashraf *et al.,* 2005). The inverse increase in sugars to particle size of millet products can be attributed to more surface area accessible to endogenous enzymes during the onset of cooking. Most of soluble sugars detected in this present study have been reported for me. However, it is also interesting that the result of millet products in this present study contains maltotriose, which has not been reported previously (Malleshi *et al.,* 1986; Henry 1989; Oshodi, 1999; Rao and Muralikrishna, 2001). The identification and quantification of these sugars are of nutritional importance as their amount can to influence the rate of absorption of carbohydrates. Henry, (2008) demonstrated that soluble sugars increased in content during drought stress when water is lost to preserve cell walls and by doing so, limiting protein denaturation due to strong interactions with sugars formed.

Previous studies have estimated the starch content in cereals and similar values to pearl millet (51.35 to 74.12%) was reported for barley (68.00%) and wheat (68.80 to 73.62%) (Yang *et al.,* 2013; Zhu, 2014; Djurle *et al.,* 2016; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017). The variation in the starch content of millet is attributed to a range of factors; method of analysis, cultivars, pre-treatment, type of grain and season of cultivation. The results obtained after the optimisation of the starch content method used in this study (Chapter 4) supports the phenomenon that method used can have a big impact on starch content quantification. Examples of varying starch content due to grain source was reported for pearl millet and wheat (Abdalla *et al.,* 1997; Chen *et al.,* 2016; Lemgharbi *et al.,* 2017) which indicates that the starch content of millet cultivars

from Nigeria as in the case with this current study may differ with those from other countries. Nevertheless, cooking did not alter the starch content in this present study, and the results are consistent with values reported for barley, wheat and other millet cultivars (Yang *et al.,* 2013; Zhu, 2014; Djurle *et al.,* 2016; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017). Furthermore, the results generated from this study demonstrates that the starch content of millet was higher than oat (54.00%) but lower than sorghum (74.67-80.96%), maize (83.75%) and rice (88.85%) (Khan *et al.,* 2013; Licata *et al.,* 2014; Souilah *et al.,* 2014; Tamura *et al.,* 2016; AlHasawi *et al.,* 2017) which could have implications on their digestion rate.

In vitro starch digestibility of pearl millet

Several *in vitro* protocols have been developed to mimic the gastric and intestinal phases for starch bioavailability studies (Englyst *et al.,* 1992; Goñi *et al.,* 1997; Germaine *et al.,* 2008; Minekus *et al.,* 2014). In this present study, the impact of particle size, cooking and *in vitro* protocol were investigated and starch fractions (RDS, SDS, RS and total starch digestibility) were calculated from *in vitro* data.

Similar results reported for millet (14.60 to 40.00%) tends to suggest that excluding the gastric results considerably lowers starch digestibility (Dhankher and Chauhan, 1987; Kheterpaul and Chauhan, 1991; Rathi *et al.,* 2004; Alka and Shruti, 2012; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017). In addition, compared to the results in this present study, more than 80% of starch from rice, wheat and sorghum flour was digested even in the absence of gastric phase

(Souilah *et al.,* 2014; Tamura *et al.,* 2016). This suggests that pearl millet starch is highly resistant to starch digestion.

The starch hydrolysis for cooked samples reported higher values for steamed bread (86.50%), porridge (93.50%), pancake (67.70%) made from foxtail millet (Ren *et al.,* 2016). Cooked rice (≥70%), whole grain wheat spaghetti (92.00%) and wheat bread (87.00%) were also relatively higher (Goñi *et al.,* 1997; Souilah *et al.,* 2014; Ren *et al.,* 2016; Tamura *et al.,* 2016; Bustos *et al.,* 2017). Interestingly, these results were obtained from studies using relatively similar *in vitro* method to this current study. On the other hand the strong physical barrier by protein matrix, and/or particle size of flour and the degree of aggregation of starch granules limited the digestibility of starch in barley, foxtail millet, sorghum (Al-Rabadi *et al.,* 2011; Ren *et al.,* 2016; Srichuwong *et al.,* 2017; Farooq *et al.,* 2018). Al-Rabadi *et al.,* (2011) and Farooq *et al.,* (2018) demonstrated that due the intact cells within the endosperm structures of larger particle sizes of barley, sorghum and rice flour, the surface area for enzyme-substrate interaction was limited. Therefore, starch was less readily digestible in larger particles of flour in comparison to smaller ones, which was eliminated by cooking at high temperature. This is consistent with the trends observed in this current study and suggests that enzyme susceptibility during starch hydrolysis of millet could be dependent on the cooking method used among many other factors including starch and protein gel forming complex. Furthermore, though a previous study demonstrated that the digestion rate of modified rice starches increased proportional to amylose content (Van Hung et al., 2016), this can be altered by the addition of bile in digesta. For instance, there is evidence that adding bile in buckwheat digesta limits amylose digestion by pancreatin (Takahama and Hirota,

2011). The amylose content in this study resulted is relatively lower starch digestion.

Starch fraction has been a focal point in understanding the effect of processing on digestion, as high content of SDS and RS are expected to confer health benefits (Birt *et al.,* 2013; Ren *et al.,* 2016; Bustos *et al.,* 2017). Food processing, especially cooking has the biggest influence on rate of starch digestion *in vitro*. Interestingly, while some studies demonstrated that manipulating the method of cooking had an impact on the starch fractions of foxtail millet and rice (Ren *et al.,* 2016; Van Hung et al., 2016), this was not the case for other rice samples (Wang *et al.,* 2017). For foxtail millet products, lower digestion rate correlated with increasing RS content (Ren *et al.,* 2016), which is consistent with our study. This study further demonstrated that manipulating the RS and not the SDS may present the biggest impact of food samples. Despite this, high content of RDS has been linked to high starch digestion rates (Yang *et al.,* 2018; Ye *et al.,* 2018). Similar pattern to our study has been reported i.e high content of rapidly digestible and resistant starch, for different cultivars of Algerian Pearl millet (Lemgharbi *et al.,* 2017). The structure of starch and high amylose content may explain the high values for RS in flour, whilst in cooked porridge (*ibyer*); it could be due to freeze-drying and the formation of retrograded starch on cooling which is evident in data presented for the thermal properties of millet in this study.

Effects of particle size and cooking on estimated glycemic index of millet

The basis for estimating the glycemic index of food samples is to predict glycemic glucose response *in vivo* (Heaton *et al.,* 1988; Van Hung et al., 2016; Ren *et al.,* 2016). However, the correlation between *in vitro* and vivo studies are not often consistent due to a number of factors; the reference food used to estimate glycemic index are varied and methods used to measure starch rate are often not standardised. Ren *et al.,* (2016) showed a correlation between eGI of foxtail millet products and postprandial glucose. On one hand, while particle size had an effect *in vitro* on the starch rate *in vitro* of oat meal, it was not consistent *in vivo* (Heaton *et al.,* 1988), which was attributed to *in vitro* experimental design. In another study, manipulating method of preparing rice correlated strongly between *in vitro* and *in vivo* findings. The glycemic index of cereals have been reported (Heaton *et al.,* 1988; Goñi *et al.,* 1997; Wolter *et al.,* 2013; De La Hera 2014; Ren *et al.,* 2016; Lemgharbi *et al.,* 2017). However, there has been insufficient information on the effect of the particle size of flour especially on millet samples as eaten. This study indicates that particle size has an influence on the eGI of millet and 1000 µM was the best choice. Compared to our results, significantly higher values (eGI = >80) were reported for bread and pancakes made from rice and foxtail millet flour (De La Hera *et al.,* 2014; Ren *et al.,* 2016). This projects millet as an important alternative for a potential low glycemic food product.

Thermal properties of millet

The gelatinisation properties of cereals has been investigated (Amadou *et al.,* 2014; Zhang *et al.,* 2014; Hsu *et al.,* 2015; Kong *et al.,* 2015; Srichuwong *et al.,* 2017; Ye *et al.,* 2018; Zhang *et al.,* 2018) and is a reflection of the complete

disruption of starch. Starch granule size correlates with gelatinisation temperature (Srichuwong *et al.,* 2017; Ye *et al.,* 2018), which was evident in rice, sorghum, wheat, amaranth, corn. These studies showed that cereal flour samples with smaller granules completely gelatinsed at lower temperature compared to those with bigger granules. Therefore, due to the gelatinisation temperatures observed in this current study, we believe that the first low transition peak was the melting of lipid present in millet whilst the second and third gelatinisation peaks were mainly from the different polymers of starch granule. However, this has not been reported for millet in the past. This may be due to the differences in experimental design of previous studies. Where the gelatinisation temperature of millet starch investigated was only up to 120°C at between 5 to 10°C/min (Amadou *et al.,* 2014; Zhang *et al.,* 2014; Hsu *et al.,* 2015; Kong *et al.,* 2015; Srichuwong 2017; Ye *et al.,* 2018; Zhang *et al.,* 2018) compared to our study which was relatively slower. Previous starch gelatinisation study on cereals including rice, wheat, corn and sorghum suggested that the removal of proteins and lipids resulted in faster disruption of starch granules at lower temperature with higher resistance to retrogradation (Srichuwong *et al.,* 2017; Ye *et al.,* 2018). In this present study, the presence of protein and lipid in whole millet flour was evident after cooling as millet starch was highly susceptible to retrogradation. It is known that the length of amylopectin branched chain can impact on crystallisation. High proportion of long chains makes starch resistant to gelatinise and whatever is gelatinised is retrograded quickly and a reverse pattern is shown for higher shorter chain proportion. The chain length in sorghum, wheat and amaranth in relationship to gelatinisation temperature of retrograded starch supports this phenomenon (Srichuwong *et al.,* 2017). In general, the range of gelatinisation temperature and degree of dissociation temperature by

retrogradation of millet in our study is relatively high. For other cereals, relatively lower values were reported, which ranged from 60.6 to 82.5°C for degree of gelatinisation and from 47.90 to 52.70°C for degree of dissociation by retrogradation (Srichuwong *et al.,* 2017; Ye *et al.,* 2018). This demonstrates that the millet cultivar used in this present study is highly susceptible to retrogradation, which is associated to its structure and may likely affect gastrointestinal digestion.

5.5. Conclusion

The potentials of millet as a low glycemic cereal with low starch digestibility is been postulated. This study demonstrated that the carbohydrate quality and digestion could be influenced by processing and protocols for carbohydrate analysis. Millet have different population of granule sizes that are densely packed with protein bodies both the surface and its internal structure, which are not completely disrupted upon cooking and may carbohydrate digestion of millet starch. Particle size reduction in flour and cooking has a big impact on the free soluble sugar content of millet due partly to surface area and endogenous enzyme activities. The starch content of millet was not altered by particle size reduction or cooking. Generally, millet starch was resistant to digestion and even more significant when pepsin pre-incubation was excluded in the digestion protocol, suggesting the importance of the gastric phase in the starch digestion of millet. The millet samples in this present study contained both RDS and RS. Nevertheless, eGI of millet products in this present study is relatively lower than what has been reported for other cereal products, which may suggest that although further research still needs to be done to validate this claim, its consumption may serve as a functional food, conferring health benefits to consuming populations, especially with type-2 diabetes. The high resistance to digestion that was reflected in the eGI of millet may be related to the crystallinity of millet upon retrogradation as indicated on how some of the starch disrupted at relatively higher gelatinisation temperature and were susceptible to retrogradation in comparison to previous studies. Also, considering that the millet product investigated in this current study is consumed with and without fermentation, understanding the impact of fermentation on carbohydrate quality

and digestion will be useful which will be the focus of the next chapter. Additionally, only the INFOGEST method will be used for starch digestibility in the next chapter.

Chapter 6: Impact of fermentation time on the carbohydrate quality and digestion of pearl millet products

Abstract

This study investigated the effect of fermentation time on the *in vitro* carbohydrate bioaccessibility and the estimated GI of traditional pearl millet porridge (*ibyer-i-angen*) was obtained. The result showed that fermentation time and not particle size of flour had the biggest impact on starch digestibility of millet. While the starch content of pearl millet products reduced, there was a significant increase in soluble sugar content and starch digestibility over a prolonged fermentation period. Furthermore, fermentation increased the RDS of millet and steadily decreased its SDS and RS content. The RDS and RS of *ibyer-i-angen* were in the following order: 0 h fermentation (41.36 and 43.98%), 12 h fermentation (52.78 and 30.93%), 24 h fermentation (64.96 and 18.35%), 48 h fermentation (87.27 and 4.19%) and 72 h fermentation (95.08 and 1.39%). The high RDS fractions in millet were reflected in the eGI values. The *ibyer-i-angen* samples were in the ranged from intermediate (<69) for 12 and 24h to high (>70) for 48 and 72h fermented samples. Therefore, pearl millet, especially fermented sample may serve as a potential beneficial food to children and adults requiring high-energy intake.

6.1. Introduction

Evidence from previous *in vitro* studies indicates that fermentation enhances nutrient availability and improves starch digestion cereal flour (Antony *et al.,* 1996; Tou *et al.,* 2006; Alka and Shruti, 2012; Amadou *et al.,* 2014; Marsh *et al.,* 2014). The low digestion of millet due to its molecular structure limiting enzyme access starch during hydrolysis has been reported (Amadou *et al.,* 2014; Ren *et al.,* 2016; Lemgharbi *et al.,* 2017). However, the impact of fermentation time on cooked ready-to-eat meals on starch digestion of pearl millet has not been reported.

This chapter provides information on the starch digestibility of fermented cooked pearl millet product (*ibyer-i-angen*) and the estimation of GI, which is an important tool for measuring food in relationship to their potential health benefit.

6.1.1 Aim

The aim of this study was to investigate the impact of fermentation time on the carbohydrate quality and digestion of pearl millet and to predict the GI from *in vitro* data.

6.1.2 Objectives

1. To investigate the effect of fermentation time and particle size on the free sugar content of millet.
2. To investigate the effect fermentation time and particle size on total starch content of millet.
3. To investigate the effect of fermentation time and particle size starch digestibility of millet.
4. To calculate the enzymatic kinetic parameters of the starch digestibility of fermented pearl millet products.

6.2. Methodology

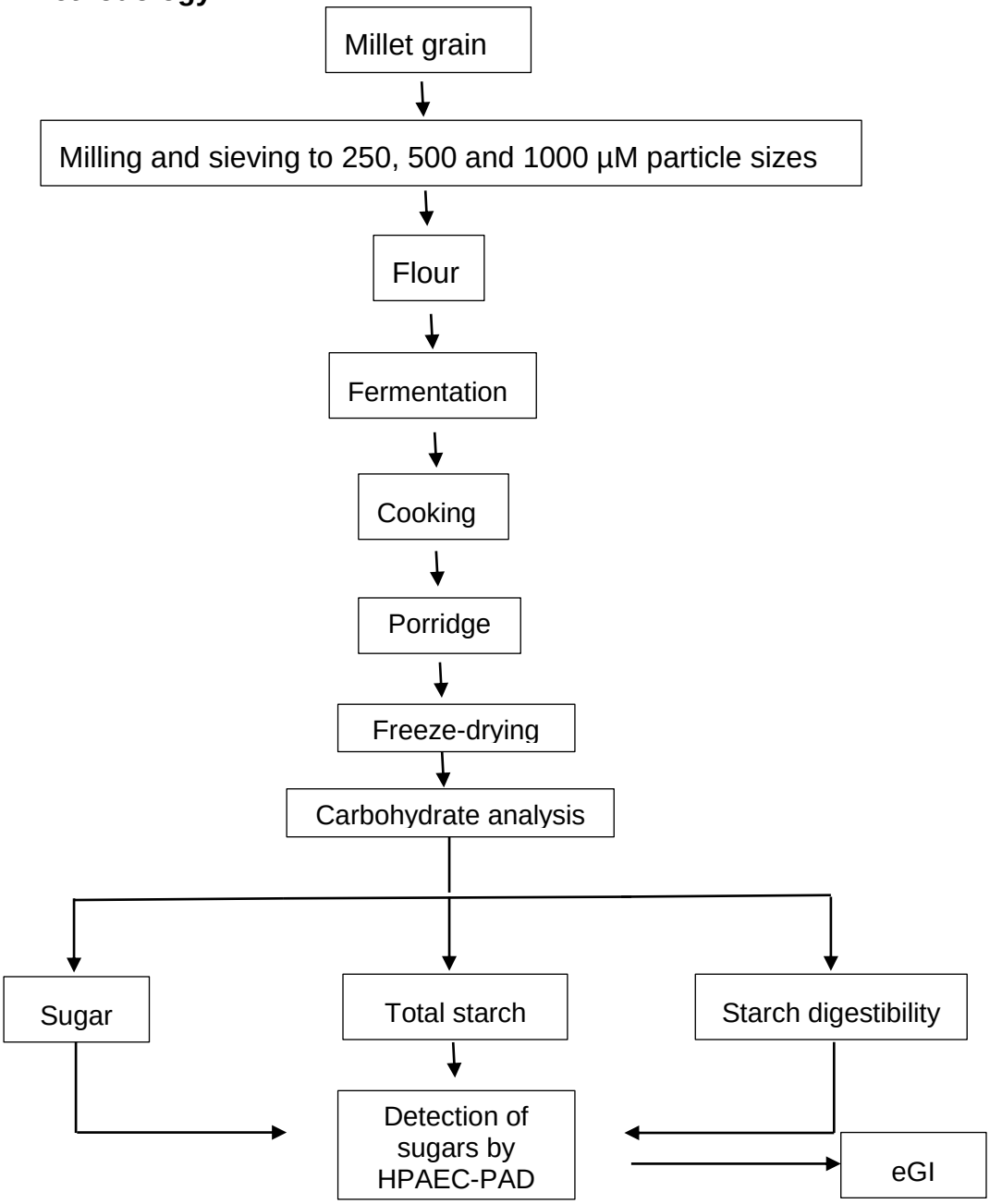

Figure 25. Summary of analysis of fermented millet products.

6.3. Results

6.3.1 Impact of natural fermentation on the appearance of millet porridge

Traditional fermented cooked millet porridge (*ibyer-i-angen*) was prepared as described in section 3.1.1. Similar observations were made for all particle sizes; hence the images presented show the overall changes in the fermentation of millet flour suspension (Fig. 26) and *ibyer-i-angen* (Fig. 27) at different time points. Starting with flour suspension, clearly, swelling and foam bubble formation increased with fermentation time. This suggests that millet flour requires longer time for water to penetrate into the interior of starch. The increase in foam bubbles formation may be indicative of the rate of conversion of carbohydrates by endogenous microorganism to carbon dioxide (CO_2) and alcohol. It is important to note that, the smell and foam colour of the fermented millet flour suspension changed from grainy and no-foam (0h), to less grainy and white (12 and 24h) and then rotten and darkish (48 and 72h).

Cooking resulted in gel formation (Fig. 27). During cooking, decrease in viscosity was observed between 0h to 72h fermentation. The porridge with the highest viscosity was 0h, and the lowest was 72h (data not shown). This trend may be related to the partial loss of molecular structure of starch following microbial degradation and gelatinisation of millet starch. Again, texture of porridge were the same for all particle sizes of millet flour used in porridge preparation. The primary greenish-brown colour of millet flour was maintained in all fermented porridge samples.

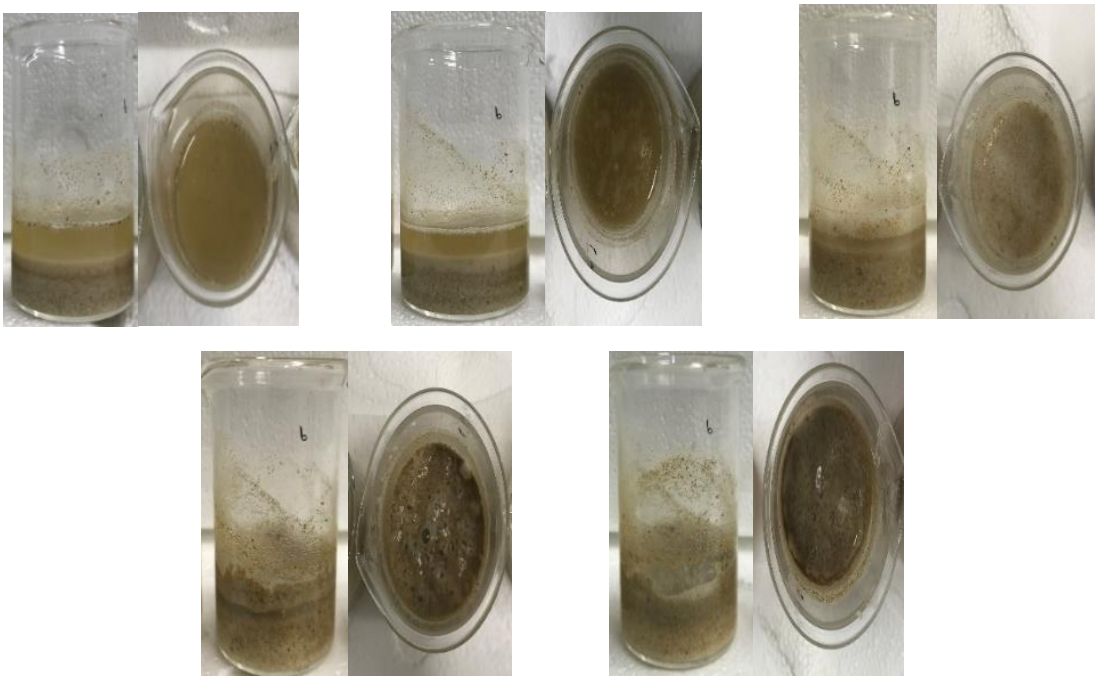

Figure 26. Images showing flour porridge after 0h (A), 12h (B) and 24h (C), 48h (D) and 72h (E) fermentation at 30°C.

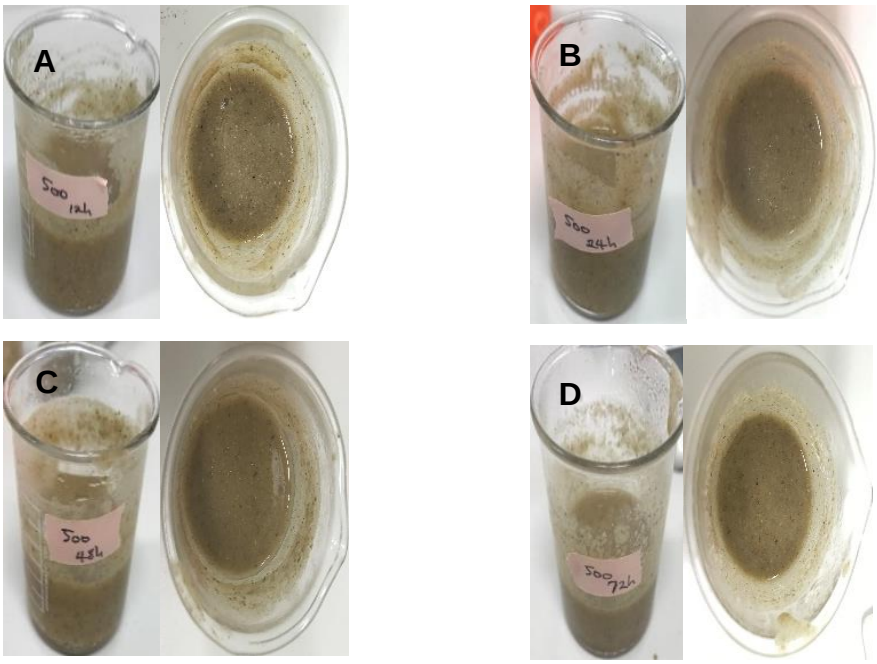

Figure 27. Images showing cooked millet porridge after 12h (A), 24h (B), 48h (C), and 72h (D) fermentation of flour at 30°C.

6.3.2. Effects of fermentation time on pH of millet products

Results for the pH change following the fermentation of different particle sizes of millet flour suspension (250, 500 and 100 µM) up to 72 h at 30°C prior to cooking is presented in Figure 28. From the results shown, particle size only had an effect on pH at the initial stage of fermentation (0 and 6 h), with 1000 µM having the biggest value (6.15±0.03) and the lowest for 250 µM (5.84±0.01). As fermentation time increased from 0 to 72 h, pH reduced from 5.97±0.14 to 3.35±0.11, thereby increasing the acidity of suspension. The increase in acidity of the suspension is likely to influence enzymatic endogenous protease digestion of millet proteins.

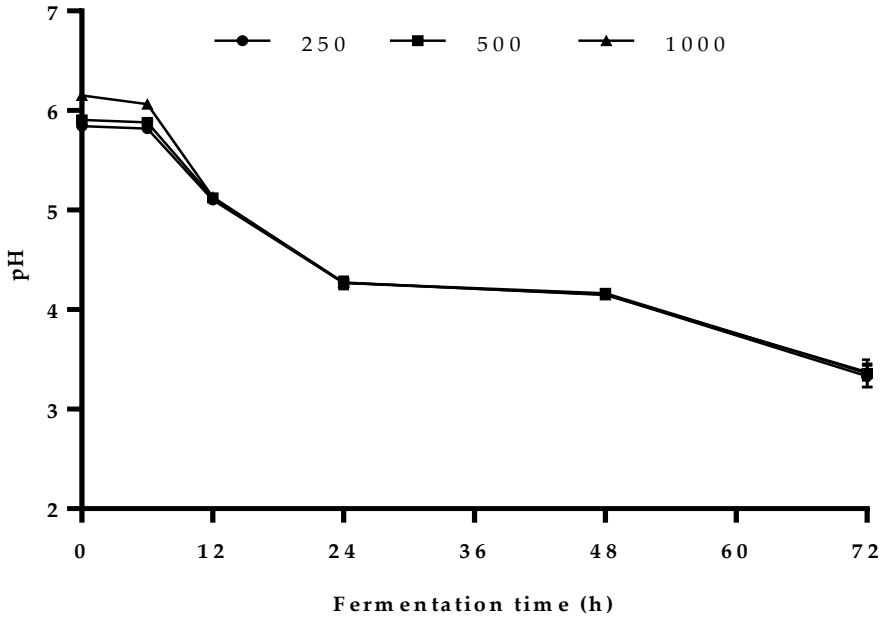

Figure 28. pH change in different particle sizes of millet flour after fermentation for 12 h, 24 h, 48 h and 72 h. Results are presented as percentage mean of 6 biological and expressed in dry weight basis. The error bars represent standard error of the mean.

6.3.3. Effect of fermentation time on carbohydrate composition of millet

The effect of fermentation time and particle size reduction on the carbohydrate content in dry weight of *ibyer-i-angen* was investigated and determined (Fig. 29 and Table 18). Comparisons were made between particle size reduction and fermentation time. Our findings show that fermentation time and not particle size reduction of *ibyer-i-angen* had an impact on the soluble sugar content of millet at $p<0.05$. The main sugars detected were glucose, fructose, arabinose, sucrose, and maltose. The mean soluble sugar content was 10.37%, 14.29%, 15.76%, and 17.02% for 12, 24, 48 and 72 h fermented *ibyer-i-angen* respectively. Glucose, maltose and arabinose increase with fermentation time. However, for sucrose, the content only increased slightly between 12 h and 24 h with a steady decrease from 24 to 72 h. Fructose content decrease as fermentation time prolonged from 12 to 24 h and was not detected after 24 h. Increases in soluble sugar content during fermentation indicates the degradation of carbohydrate by both microorganism and endogenous enzymes. We believe the reduction in sucrose between 24 to 72 h fermentation was due to its utilization by microorganisms.

The starch content of different particle sizes of fermented *ibyer-i-angen* was quantified (Table 18). Again, fermentation time and not particle size reduction had an impact on the starch content of *ibyer-i-angen* ($p<0.05$). The mean starch content ranged from 63.71, 60.04, 46.30 and 35.88% for 12, 24, 48 and 72 h fermentation respectively. The relatively decrease of starch content following the extent of fermentation may be attributed to the amount of soluble sugars released

due to microbial and endogenous enzymatic activity in millet. For protein content, only slight increase was observed between 12 h to 72 h of fermentation which was not statistically significant.

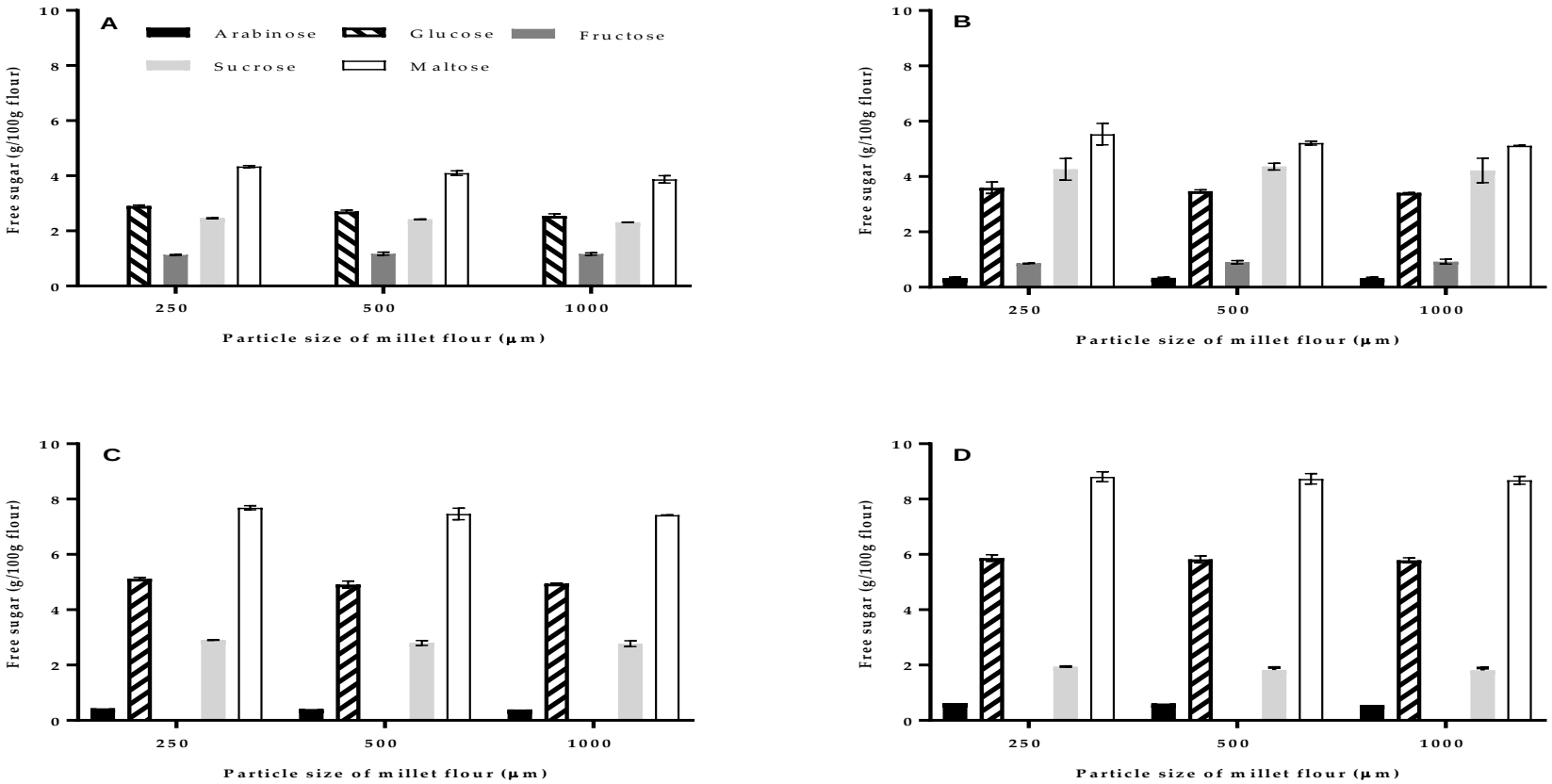

Figure 29. Free sugar content of different particles of cooked millet porridge after fermentation of flour for 12 h (A), 24 h (B), 48 h (C), 72 h (D). Data is expressed as percentage mean of 3 biological replicates expressed in dry weight basis. The error bars are standard deviation of the mean.

Table 18. The carbohydrate and protein composition of different fermentation times of *ibyer-i-angen* of different particle sizes. The results are presented as percentage mean ± standard deviation of 3 biological replicates and expressed in dry weight basis. Data in the same column with the same superscript are not significantly different ($p<0.05$). CHO = carbohydrate.

Fermentation time (h)	Particle size (µM)	Total reducing sugar	Total free sugar	Total starch	Total CHO	Total protein
12	250	7.24±0.07^{a}	10.83±0.06^{a}	63.12±1.02^{a}	73.90±1.02^{a}	11.38±1.52^{a}
	500	6.80±0.12^{a}	10.39±0.16 ^{a}	63.52±0.58^{a}	73.90±0.62^{a}	
	1000	6.42±0.19^{a}	9.89±0.21 ^{a}	64.48±1.17^{a}	74.39±1.19^{a}	
24	250	9.13±0.54^{b}	14.58±0.71 ^{b}	59.38±0.80^{b}	73.85±1.42^{a}	12.25±0.88^{a}
	500	8.69±0.12^{b}	14.28±0.15 ^{b}	60.24±0.44^{b}	74.49±0.29^{a}	
	1000	8.52±0.03^{b}	14.00±0.41 ^{b}	60.49±2.19^{b}	74.44±2.75^{a}	
48	250	12.81±0.30^{c}	16.15±0.13^{c}	45.70±0.80^{c}	61.84±0.69^{b}	12.83±0.51^{a}
	500	12.37±0.31^{c}	15.59±0.28 ^{c}	46.56±0.44^{c}	62.18±0.60^{b}	
	1000	12.37±0.01^{c}	15.54±0.11 ^{c}	46.64±2.19^{c}	62.20±2.24^{b}	
72	250	14.68±0.29^{d}	17.24±0.29 ^{d}	35.72±1.07^{d}	53.00±0.85^{c}	13.07±0.10 ^{a}
	500	14.55±0.31^{d}	16.98±0.36 ^{d}	35.94±2.15^{d}	53.00±2.05^{c}	
	1000	14.47±0.23^{d}	16.83±0.21 ^{d}	35.97±1.22^{d}	52.85±1.34^{c}	

6.3.4. Effect of fermentation time and particle size on the starch digestion of cooked millet porridge

In this present study, the impact of fermentation time and particle size reduction on the starch digestion measurement and eGI of *ibyer-i-angen* were calculated. Figure 30 shows the starch digestion pattern of *ibyer-i-angen* of different particles prepared after 12 to 72 h fermentation. Fermentation time and not particle size had an effect on the digestion rate in all samples ($p<0.05$). The initial starch available at 0 min after pepsin pre-incubation of *ibyer-i-angen* was 26.49, 30.89, 43.96, and 71.03% for 12, 24, 48 and 72 h fermented respectively. The incubation time also had an effect on rate of digestion. At 120 min, the mean range of starch digestibility was lowest for 12 h fermented (69.07%) *ibyer-i-angen* with highest values at 72 h (98.62%).

The starch parameters calculated from the starch digestion data were; RDS, SDS and RS (Table 19). Fermentation time altered the starch parameters measured ($p<0.05$). The RDS increased proportionally to fermentation time. A contrary trend was shown for SDS and RS. It is clear that starch is more readily digested with increase in fermentation time.

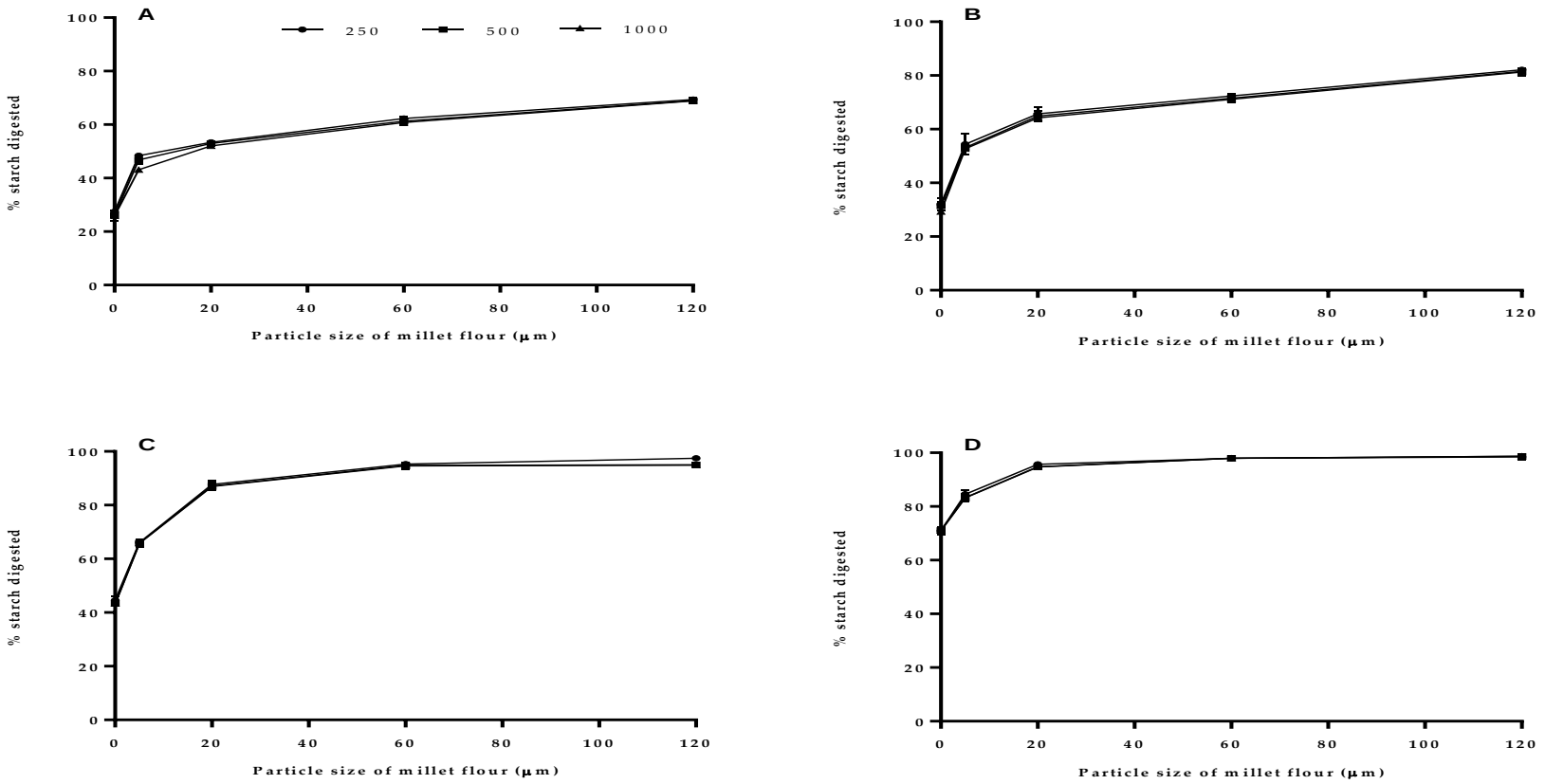

Figure 30. The *in vitro* starch digestion of different particles size of *ibyer* following 12 h (A), 24 h (B), 48 h (C) and 72 h (D) of fermentation. Results are presented as percentage mean of 6 biological replicates expressed in dry weight basis. The error bars are standard deviation of the mean.

Table 19. The values expressed as percentage mean ± SD of RDS, SDS, RS, and starch digestibility of different particle sizes of cooked millet porridge after fermentation for 12 h, 24 h, 48 h and 72 h. Data in the same column with the same superscript are not significantly different ($p<0.05$).

Fermentation time (h)	Particle size (µM)	RDS	SDS	RS	Starch digestibility
12	250	53.36±1.44^{a}	16.03±1.39^{a}	30.62±2.36^{a}	69.39±1.39^{a}
	500	52.94±0.75^{a}	15.99±1.19^{a}	31.07±1.26^{a}	68.93±1.19^{a}
	1000	52.03±0.96^{a}	16.88±0.87^{a}	31.10±1.74^{a}	68.91±0.87^{a}
24	250	65.73±2.48	16.36±0.65^{a}	17.91±2.67^{b}	82.10±0.65^{b}
	500	64.91±1.84^{b}	16.63±0.59^{a}	18.46±1.75^{b}	81.54±0.59^{b}
	1000	64.23±0.61^{b}	17.08±1.25^{a}	18.69±1.46^{b}	81.31±1.25^{b}
48	250	87.71±1.59^{c}	9.73±1.54^{b}	2.56±0.64^{c}	97.44±0.64^{c}
	500	87.13±1.57^{c}	7.94±0.67^{b}	4.93±2.06^{c}	95.07±0.67^{c}
	1000	86.98±1.56^{c}	7.92±0.67^{b}	5.09±2.05^{c}	94.91±0.67^{c}
72	250	95.68±0.38^{d}	2.75±1.04^{c}	1.58±1.10^{d}	98.43±1.04^{c}
	500	94.83±0.83^{d}	3.91±0.51^{c}	1.26±0.92^{d}	98.74±0.51^{c}
	1000	94.73±0.83^{d}	3.96±0.55^{c}	1.32±0.84^{d}	98.69±0.55^{c}

6.3.5. Effect of fermentation time on the estimated glycemic index of cooked millet porridge

For the early stage of digestion i.e first order starch kinetics of *ibyer-i-angen*, we only focused on the effect of fermentation time (Table 20) and not particle size of flour used to prepare fermented millet samples. This was because, fermentation time and not particle size of flour used to prepared porridge altered the starch digestibility of millet ($p<0.05$). The kinetic constant (k), HI and eGI of different fermentation time points of *ibyer-i-angen* was compared to 250 µM particle size of *ibyer* (unfermented millet porridge). *Ibyer* had the lowest k value and *ibyer-i-angen* at 72 h fermentation had the highest value. Similarly, the eGI increased significantly relatively to fermentation time from 51.64 at 0 h to 73.38 at 72 h. This result suggest that prolong fermentation had a significant impact on eGI for millet product.

Table 20. Data showing mean±SD of starch hydrolysis (C_∞), kinetic constant (k), half time of hydrolysis (T1/2), hydrolysis index (HI) and estimated glycemic index (eGI) of millet calculated with kinetic equation after different fermentation times of *ibyer*. Data in the same column with the same superscript are not significantly different ($p<0.05$).

Fermentation time (h)	C_∞ (%)	K (sec^{-1})	HI (%)	eGI
0	56.84±1.29^a	3.33±0.09^a	61.77±1.31^a	51.64±0.72^a
12	69.08±0.27^b	4.67±0.03^b	78.34±0.35^b	57.96±0.13^b
24	81.65±0.40^c	4.99±0.10^b	93.23±0.31^c	63.71±0.12^c
48	95.80±1.42^d	7.25±0.09^c	113.22±1.57^d	71.39±0.61^d
72	98.62±0.17^e	9.23±0.14^d	118.40±0.16^e	73.38±0.06^d

6.4. Discussion

Fermentation is one of the oldest methods used in food preparation, especially in Africa, aimed to improve palatability and enhance food quality and nutrition (Antony *et al.,* 1996; Tou *et al.,* 2006; Alka and Shruti, 2012; Marsh *et al.,* 2014). This present study focused on the effect of fermentation time on the carbohydrate quality and digestion *in vitro* of millet products and its potential implication on human nutrition. However, from physical observation, only natural fermentation of millet up to 24 h was within the recommendation range for consumption. Fermentation time and not particle size of flour altered the carbohydrate quality and starch digestion of millet porridge (*ibyer-i-angen*).

Effect of fermentation time on soluble sugars

Glucose, maltose, fructose and xylose were main sugars detected in fermented millet products (Antony *et al.,* 1996; Osman, 2011). In our study, glucose was the predominant sugar in fermented product, and increased as fermentation was prolonged from 0 to 72 h. The soluble sugar content of fermented *ibyer-i-angen* at 12 h was slightly lower than the value (11.89%) reported in a previous study (Osman, 2011). The study by Osman, (2011) showed a decrease in soluble sugar content after 12 h, which was attributed to microbial utilization of reducing sugars. In this study, soluble sugar content increased with fermentation time, especially glucose content. The decrease of sucrose and fructose content in millet with fermentation has been reported for maize and sorghum (Mugula *et al.,* 2003), which may suggests that microbial utilization of soluble sugars is dependent on sample type. Furthermore, in comparison to the soluble sugars released in *ibyer*

(Table 12 of Chapter 5), the combined effect of microbial and endogenous enzyme activities may have a significant effect on freeing the sugars' bond to the cell walls and degrading carbohydrates in the endosperm of millet.

Effect of fermentation time on total starch

Considering the content of starch in unfermented millet (*ibyer* in Table 12), pretreatment by fermenting over a prolonged time reflected a steep decrease in total starch. Our observation was consistent with previous studies on fermented samples (Antony *et al.,* 1996). Another study on Saudi Arabian cultivar of pearl millet showed a contrary trend (Osman, 2011), starch content only reduced between 0 to 8h of fermentation and gradually increased thereafter, which was attributed to activity of β- and α-amylase and resulting acidity. The steady increase of starch in our study may be due to the increase in the activity of both microbial and endogenous enzyme, considering the amount of soluble sugars presented. We also showed that the overall carbohydrate content (soluble sugar plus starch content) decreased with fermentation time, which is in agreement with studies on foxtail millet (Amadou *et al.,* 2014). The decrease in carbohydrate content reflects the reducing effect of starch content over prolonged fermentation time.

Effect of fermentation time on starch digestibility

Processing treatments, especially the combination of fermentation and cooking have been shown to enhance nutrient availability in food samples (Alka and Shruti, 2012; Marsh *et al.,* 2014; Amadou *et al.,* 2014). However, there is limited data on the *in vitro* starch digestibility of fermented foods (Alka and Shruti, 2012; Amadou *et al.,* 2014) as the majority of these studies focused on the proximate composition, sensory attributes and physical changes in products (Tou *et al.,* 2006; Kure and Wyasu, 2013; Akintola *et al.,* 2017). Interestingly, this is the first study to investigate the starch digestibility of pearl millet up to 72 h of fermentation treatment. It was with that consideration that in this study, we subjected millet to different fermentation time points to give a detailed evaluation on its *in vitro* starch digestion. The digestibility of millet was highest at 72 h fermentation, followed by 48 h and lowest at 12 h, and even lower when unfermented (Figure 22). Considering that starch structure was a key factor in the limitation of digestion in unfermented sample in this study (Chapter 5), the increased digestibility of fermented product may be due to the removal of most of the proteins engrained in starch granules. A possible explanation to this may be the increase in activity of endogenous enzymes and loss of crystallinity following microbial degradation of starch before even enzymatic hydrolysis of starch. This phenomenon was consistent for millets, sorghum and maize starches (Kheterpaul and Chauhan, 1989; Kheterpaul and Chauhan, 1991; Alka and Shruti, 2012; Amadou *et al.,* 2014). Alka *et al.,* (2012) showed that proteins in sorghum, millet and maize flour was more readily digestible at 36 h compared to 0 h of fermentation and was reflected in the rate of starch hydrolysis. We have shown in this study that fermentation had no effect on protein content, which was consistent with previous

studies on pearl millet (Kheterpaul and Chauhan, 1989) but protein digestibility was improved by degree of fermentation (Kheterpaul and Chauhan 1991). This may explain the less resistance of millet starch to digestion as fermentation prolonged.

Effect of fermentation time on starch fractions

The starch fractions of unfermented millet samples (*ibyer*) was previously highlighted (Chapter 5) and showed a relatively lower RDS and higher SDS and RS to fermented millet samples (*ibyer-i-angen*). Though manipulating the cooking method of foxtail millet was found to influence starch fraction rates (Ren *et al.,* 2016), the data generated from this current study suggests that the application of pretreatment methods like fermentation has additional impact. For instance, the RDS of millet increased from 41.36% at 0 h (Chapter 5) to 95.08% at 72 h of fermentation. It is important to point out that though >80% of starch in millet was readily available between 48 and 72 h, from our observations for *ibyer-i-angen*, only fermentation up to 24 h was suitable for consumption. In general, fermentation and cooking resulted in the conversion of RS to RDS. Nevertheless, the RS content of millet between 12 and 24 h in this study was still relatively higher than values reported for unfermented rice, sorghum and maize samples (Goñi *et al.,* 1997; Souilah *et al.,* 2014; Tamura *et al.,* 2016; Bustos *et al.,* 2017). The starch fraction of millet in this study was hugely affected by degree of fermentation and likely to influence the amount of glucose available for glycemic response.

Effect of fermentation time on eGI

This current study is the first to provide information on the eGI of pearl millet products up to 72 h fermentation. Previously, a study by Lemgharbi *et al.,* (2017) conducted on pearl millet flour showed a low estimated GI ranging from 27.41 to 38.82. In this study, *ibyer-i-angen* samples were suggested to be within intermediate (<69) for 12 and 24h and high (>70) for 48 and 72h of fermentation.. Nevertheless, our findings were lower than the results presented by Ren *et al.,* (2016) for unfermented foxtail millet products (77.60 to 86.80). The extent of processing and method of carbohydrate measurement are likely to present variation in the digestion of starch, which subsequently influences the GI of foods. A good example of how different processing methods impact on GI was evident in published data for rice products (54.5 to 132.00) in the international tables of GI (Foster-Powell and Miller, 1995). In the above study, the GI of rice boiled for 1 min was relatively lower than when boiled for 6 min.

The GI and starch digestibility are positively linked (Ren *et al.,* 2016). Previous *in vivo* studies suggested that high bioavailability and accessibility of carbohydrate based products may not be suitable for type-2 diabetes population (Heaton *et al.,* 1988; Edwards *et al.,* 2015a; Mlotha *et al.,* 2015; Van Hung et al., 2016; Ren *et al.,* 2016). Hence, the resulting GI form of millet products as presented in this present study needs to be verified *in vivo*.

6.5. Conclusion

This present study demonstrates that the extent of fermentation as a pre-treatment method has a huge impact on the carbohydrate composition, starch digestibility and fraction, and subsequently the estimated glycemic index of millet samples. Particle size of millet did not alter the starch digestion or soluble sugar content of fermented millet sample. Glucose was the predominant soluble sugar, and though soluble sugars increased proportional to fermentation time, a decrease was reflected in the starch content of samples, suggesting microbial and endogenous enzyme degradation of carbohydrate.

This study provided information for the first time on the starch digestibility of cooked millet products after fermentation for up to 72 h. The results indicate that fermentation enhanced the starch digestibility and parameters measured for millet products. The rate of digestion was attributed to the removal of proteins by endogenous enzymes under acidic condition that may have resulted in loss of crystallinity of millet starch. Fermentation for 12 and 24 h resulted in less than 65% of starch digestibility in the first 20 min of hydrolysis (RDS) compared to >80% between 48 and 72 h of fermentation. The fermented millet products in this study were classified as either intermediate or high-GI foods. Nevertheless, the RS of millet up to 24 h of fermentation was still higher than values reported for other cereals. Millet samples in this study may be beneficial to children and adults requiring high-energy intake.

Chapter 7: Summary of study and future prospects utilization of pearl millet

7.1. General discussion

Nigeria depends on carbohydrate rich foods (rice, millet, maize, sorghum, yam, and cassava) for the survival of its growing population. Pearl millet is one of the most common traditional cereals cultivated and eaten as part of breakfast, lunch and dinner. Common use of pearl millet in Nigeria is in the preparation of breakfast porridges known such as *ogi*, *ibyer, ibyer-i-angen*, and in the preparation of *fufu* eaten with soup as lunch or dinner. Recently, the utilization of pearl millet has gained the spotlight as an important cereal due to its rich nutrition profile and low starch digestion (Annor *et al.,* 2015; Annor *et al.,* 2017; Lemgharbi *et al.,* 2017; Dias-Martins *et al.,* 2018). Interestingly, while these postulations have been made, and low starch digestible food products appear to have been supported in a number of in vivo studies on their potentials in managing type-2 diabetics, its carbohydrate digestion is yet to be fully investigated.

In fact, to date, there is scarce information on the digestibility *in vitro* or *in vivo* of pearl millet products as eaten. Considering how *in vitro* studies are good alternative to *in vivo* studies, easy to manipulate, cheap and reproducible, the analysis of carbohydrate for pearl millet as eaten was optimised to test its quality and digestion. The optimisation of method for carbohydrate analysis was necessary because, though several developed methods appear to have been used in many studies, variations exist in conditions of digestion such as enzyme quality, buffer, incubation time, and how carbohydrate is measured, making it

difficult to compare results between studies. Considering that millet is not eaten raw (flour), this section of study will focus on millet as eaten.

7.1.1. Main findings and novelty of study

For the first time, the effect of processing on sugar and starch content, starch digestibility and GI of pearl millet as eaten was investigated (Chapter 4, 5 and 6). From our study, the choice of method for measuring starch digestibility is a key consideration in experimental design as it can have a big impact on overall carbohydrate result. The first step of this study was the optimisation of carbohydrate protocol using millet flour (Chapter 4). The best method for measuring carbohydrate composition and digestion was reproducible (CV < 5%) and included agitation steps to enhance the release of sugars and dissolution of starch, and the homogenization of millet suspension during enzymatic digestion and detection of sugars by HPAEC-PAD. Therefore, the quality of carbohydrate and its digestion in this study were obtained under optimal conditions of analysis.

As shown in Chapter 5, the gastric phase had a big impact on the digestion of millet as eaten. This gave a new insight to the digestibility of millet as majority of previous studies were focused on the nutritional attributes of its flour or the proximate composition of its products as eaten (Kheterpaul and Chauhan, 1989; Alka and Shruti, 2012; Adebiyi *et al.,* 2016; Lemgharbi *et al.,* 2017; Srichuwong *et al.,* 2017). For this reason, studies on the starch digestibility of pearl millet products will be very useful in understanding their contribution in human nutrition. Based on this study, the particle size reduction of millet flour before the

preparation of *ibyer* increased its eGI. The eGI of *ibyer* was in the low range (Table 21, <55), with samples having both fast and slow digestible starch with about 44% of starch resistant to digestion. The gelatinisation and microscopic study presented in Chapter 5 demonstrated the interesting factors that may be responsible for the resistance of millet to digestion. The factors identified were the higher gelatinisation temperatures (as measured with DSC) than previously investigated required to completely disrupt millet starch granules during gelatinisation and following retrogradation, and the presence of undisrupted granules in *ibyer* as viewed under SEM.

Since the porridge investigated in this study is not only eaten in its unfermented form, the effect of traditional fermentation was studied. Fermentation was conducted on different particle sizes of millet flour at 30°C for up to 72 h before cooking the porridge (*ibyer-i-angen*). However, as a result of the time frame of this research, it was unfeasible to conduct microbial analysis and only the impact of fermentation on carbohydrate quality and digestion were studied (Chapter 6). While particle size did not alter the carbohydrate content, starch digestibility and eGI of *ibyer-i-angen*, fermentation time had the biggest contribution (Table 21). The results for carbohydrate content were consistent for both *ibyer* (unfermented) and *ibyer-i-angen* (fermented) porridges. Nevertheless, compared to the starch digestibility results of *ibyer*, it can be argued that fermentation converted most of the resistant starch to rapidly digestible starch due to the combine impact of microbes and enzymatic hydrolysis of starch. In addition, *ibyer-i-angen* samples were within the intermediate (<69) and high (>70) range of eGI. From a food nutrition point of view, the information presented for *ibyer* and *ibyer-i-angen* could

be useful in industrial processing to meet consumer preferences and the health needs of different population.

Table 21. Summary of results of carbohydrate quality and digestion in vitro of pearl millet. Results are presented as percentage total soluble sugar, total starch, total carbohydrate, starch digestibility, rapidly digestible starch (RDS) and resistant starch (RS).

CHO=Carbohydrate

Sample	Total soluble sugar	Total starch	Total CHO	Starch digestibility	RDS	RS	eGI
Ibyer	6.76±1.07[a]	68.80±2.94[a]	75.17±3.04[a]	56.01±1.83[a]	41.37±6.69[a]	43.98±6.15[a]	51.64±0.72[a]
Ibyer-i-angen 12 h fermentation	10.37±0.47[b]	63.71±1.03[b]	74.06±0.87[a]	69.08±1.12[b]	52.78±1.17[b]	30.93±1.75[b]	57.96±0.13[b]
Ibyer-i-angen 24 h fermentation	14.29±0.29[c]	60.04±1.29[c]	74.26±1.58[a]	81.65±0.90[c]	64.96±1.82[c]	18.35±1.93[c]	63.71±0.12[c]

7.1.2. Millet as a nutrient source for diabetes and malnourished population

The result from this study indicates that utilizing pearl millet as a food can provide opportunities to develop millet as a more general source of carbohydrate for a large population, because of its interesting carbohydrate properties. The main nutrient in millet (carbohydrate) is important in providing the needed energy for daily activities, and varied starch fraction (RDS, SDS, and RS) can be beneficial to different population of people. Studies have shown potential beneficial effects of starchy food consumption on diabetes, especially those containing resistant starch and low content of sugar (Heaton *et al.,* 1989; Thathola et al., 2011; Ren *et al.,* 2016; Fayet-Moore *et al.,* 2017). In Nigeria, the prevalence of type-2 diabetes as published by WHO, (2016) was 4.4 and 4.3% for males and females between the ages of 30 and above. The prevalence of type-2 diabetes in males and females were higher for countries such as the United Kingdom (8.4 and 6.9%), South Africa (7.7 and 11.80%), Ghana (4.6 and 5.0%) and the United States of America (9.8 and 8.3%) (WHO, 2016). However, a systematic review by Guariguata *et al.,* (2014) and Uloko *et al.,* (2018) reported 5.8% for Nigeria. Since the recent population of Nigeria is speculated to be 185 900 000 to 190 000 000, this puts the prevalence of diabetes at 7 993 700 to 11 020 000. This number is expected to be larger because the data reported does not include people from rural communities who have no financial means to access the health care of Nigeria due to poverty and low education. Therefore, while the high RS products (*ibyer*) could benefit the diabetic population in Nigeria and its introduction into the diets of other developing and even developed countries may have potentials in improving health and managing diabetes. Furthermore, all millet products (*ibyer*

and *ibyer-i-angen*) in this study could benefit both malnourished people in developing countries.

7.1.3. Sustainability and economic importance of millet products

There is growing dryness in the global vegetative areas due to climate change (Huang *et al.,* 2016). If actions are not taken, this could have a huge impact on the food security and economic growth of countries that provide food for the world. Interestingly, pearl millet is one of the very few food crops cultivated under very harsh weather conditions, therefore making it an important crop, providing nutrient to the world's growing population. In Nigeria, there is a high demand for pearl millet as people from different region of the country adopt it as their healthy option as part of breakfast, lunch and dinner. Approximately five years ago, the production of pearl millet was 1 384 900 tones (FAO, 2015) and 1 kg is sold for less than 2 USD or 1 pound sterling. Therefore, with more spotlight and published data on the potentials of pearl millet to health and its importance as a future food crop, more investments could be made to improve its production, which may contribute to the economy of Nigeria and influence its global industrial utilization.

7.2. Limitation of study and future prospect of pearl millet products

In this study, we have produced protocols that are robust and reproducible. Data for carbohydrate digestion was obtained after only two years of *in vitro* trials, resulting in the limited information provided here. Nevertheless, the results from our findings have highlighted the relevance of pearl millet products as a functional food. This current study has provided data on the influence of processing

methods such as particle size reduction, cooking and fermentation on carbohydrate quality and digestion of pearl millet products. The key limitations in this study are listed below;

7.2.1. *In vivo* trials

A number of *in vivo* studies have shown that low starch digestion and resistant starch were strongly correlated with GI and blood glucose response (Heaton *et al.,* 1989; Thathola et al., 2011; Edwards *et al.,* 2015a; Ren *et al.,* 2016). *In vivo* studies such as acute or chronic human trials to validate the *in vitro* starch digestion and estimated GI results of pearl millet products could be useful to correlate result and make assertions on health claims.

7.2.2. Comparative studies of ibyer using different varieties of pearl millet and food preparation methods

Domestic and industrial methods of food preparation such as pancake, bread pasta and spaghetti making resulted in a wide range of starch fractions (Bustos *et al.,* 2017; Ren *et al.,* 2017). Furthermore, it is evident that different cultivars of pearl millet differ greatly in their nutrient availability (Lemgharbi *et al.,* 2017). However, this study only investigated a single variety of pearl millet using very limited food preparation methods (size reduction, cooking and fermentation) which were mainly domestic. Investigating industrial methods of preparation and storage of millet products (fermented and unfermented) will bring the benefit of millet to a wider population.

7.2.3. Identification of microorganisms involved in *ibyer-i-angen* fermentation

During the preparation of traditional fermented foods from cereals such as maize pearl millet and sorghum, lactic acid bacteria, yeasts and fungi were the main microorganisms identified (Owusu-Kwarteng *et al.,* 2012; Izah *et al.,* 2015). The fermentation of food is thought to improve the shelf life, palatability, flavor and even nutrient availability. However, the extent of fermentation and food preparation revealed fungal metabolites, which can have adverse effect on health (Ezekiel *et al.,* 2015). In this study, the microbial analysis of *ibyer* and *ibyer-i-angen* was not studied and information on their contribution to human nutrition would be useful.

7.2.4. Sensory analysis

The reintroduction of pearl millet as part of a diet has been studied (Ndiku *et al.,* 2014) and accepted by children and adults in Kenya. In Africa, traditional fermented and unfermented foods are tied to specific cultures. In Nigeria, ibyer and *ibyer-i-angen* are only popular amongst the Tiv ethnic group of the Middle Belt, *kunu* (a thin non-alcoholic beverage) is common in the Northern and Middle Belt region, *abacha* (cooked grated cassava) is common in the Eastern region and *amala* (*fufu* made from dried yam flour) is popular in the South West region. Sensory analysis to test the acceptability of the millet products in this present study among a wide range of people from different culture and race will be useful and a future direction for global recognition.

7.3. Conclusion

This study is the first to investigate and report the impact of processing on the carbohydrate quality, starch digestibility and eGI up to 72 h fermentation of pearl millet as eaten, using optimal condition of analysis. In addition, at the time of optimisation this study was the first to conduct an in vitro starch digestion of millet using the INFOGEST protocol. The potentials of pearl millet products as a universal food consisting of both rapidly digestible and resistant starch have been highlighted and could benefit different populations. This study was intended to stimulate interest in the study of pearl millet and its products, which will be beneficial to science, food industry, plant-based food consumers and the farmers.

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Appendices

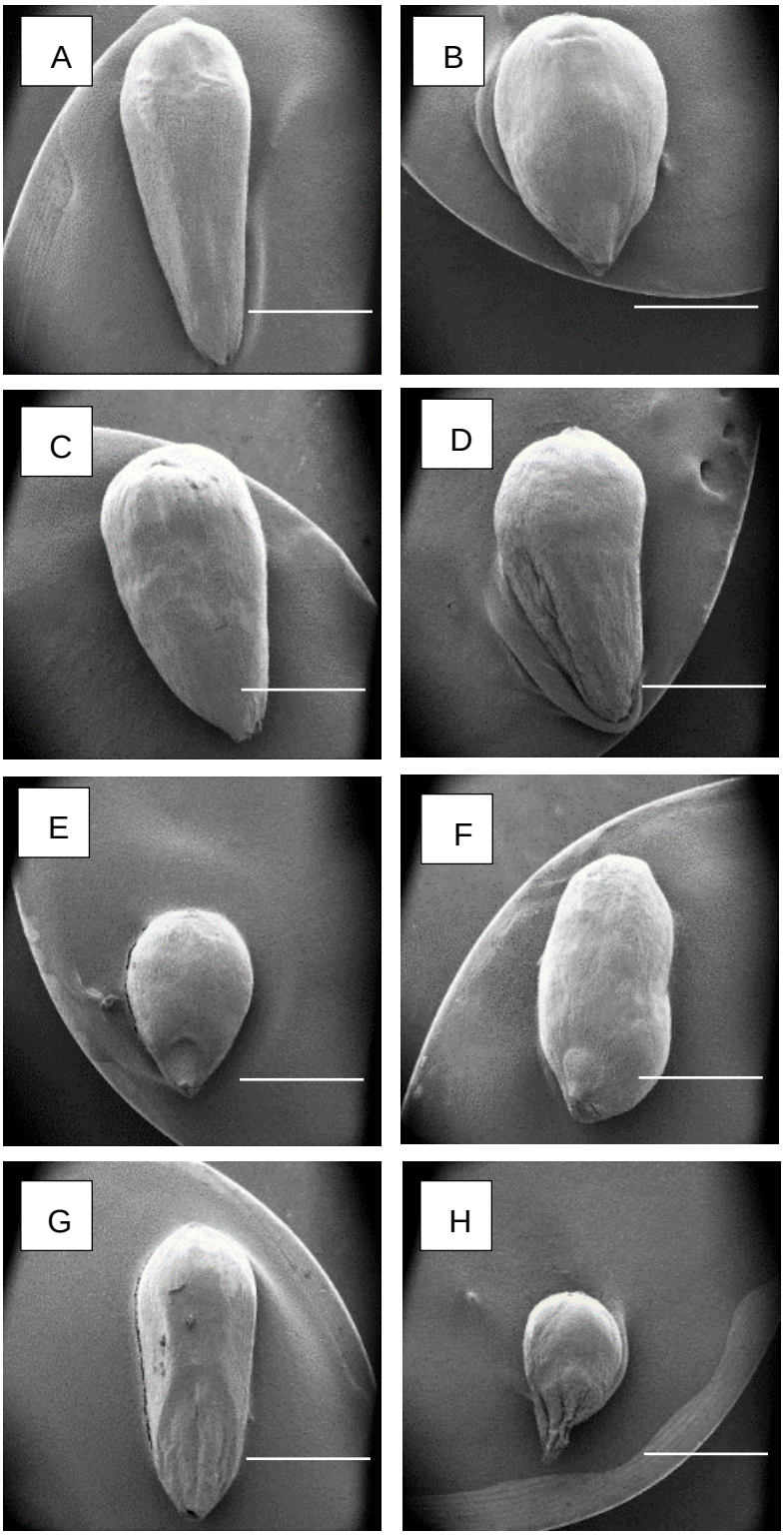

Figure S1. SEM images showing a selection of the different shapes of pearl millet grains. Scale bar = 2mm

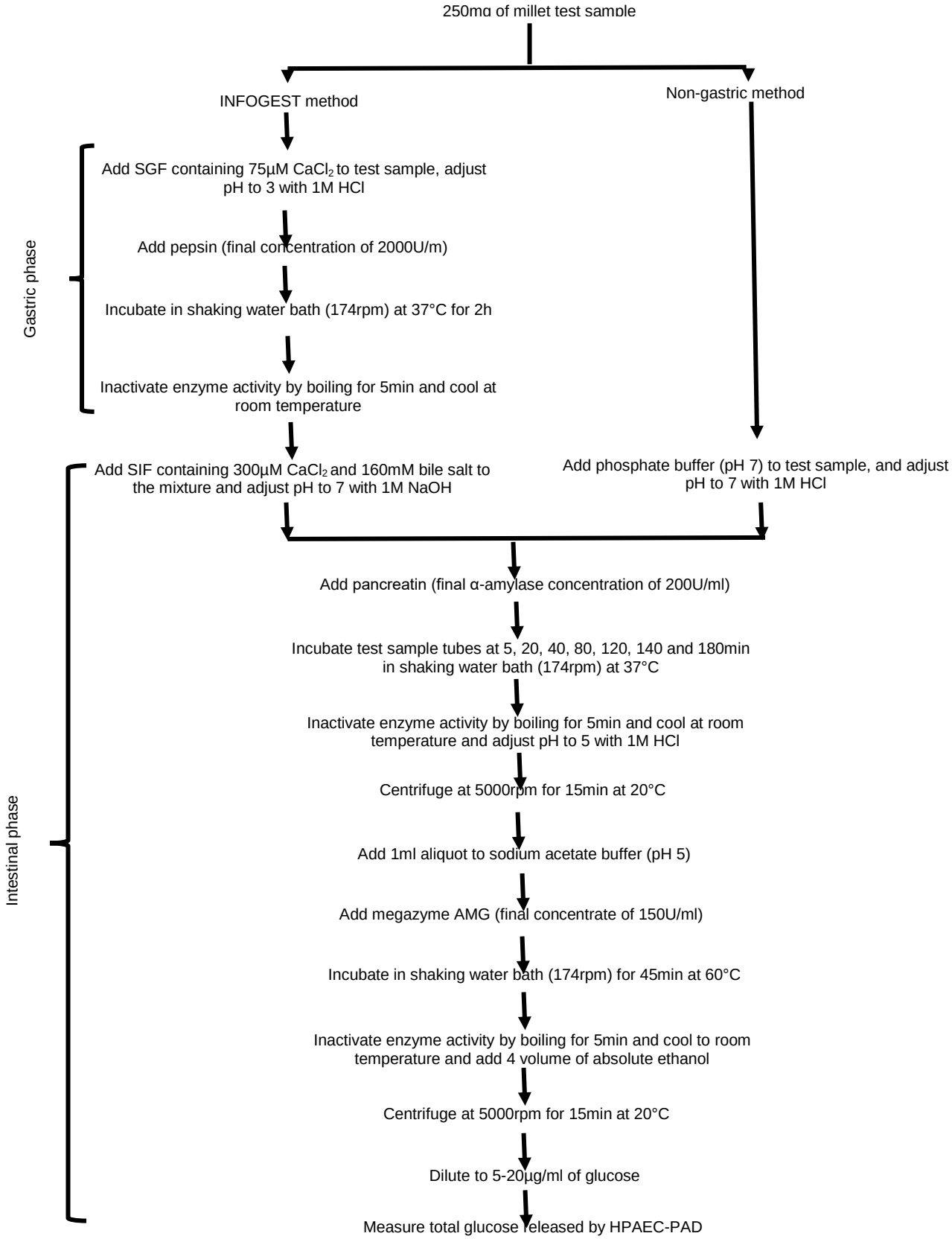

Figure S2. Summary of *in vitro* starch digestion by INFOGEST and non-gastric phase Goñi protocol.

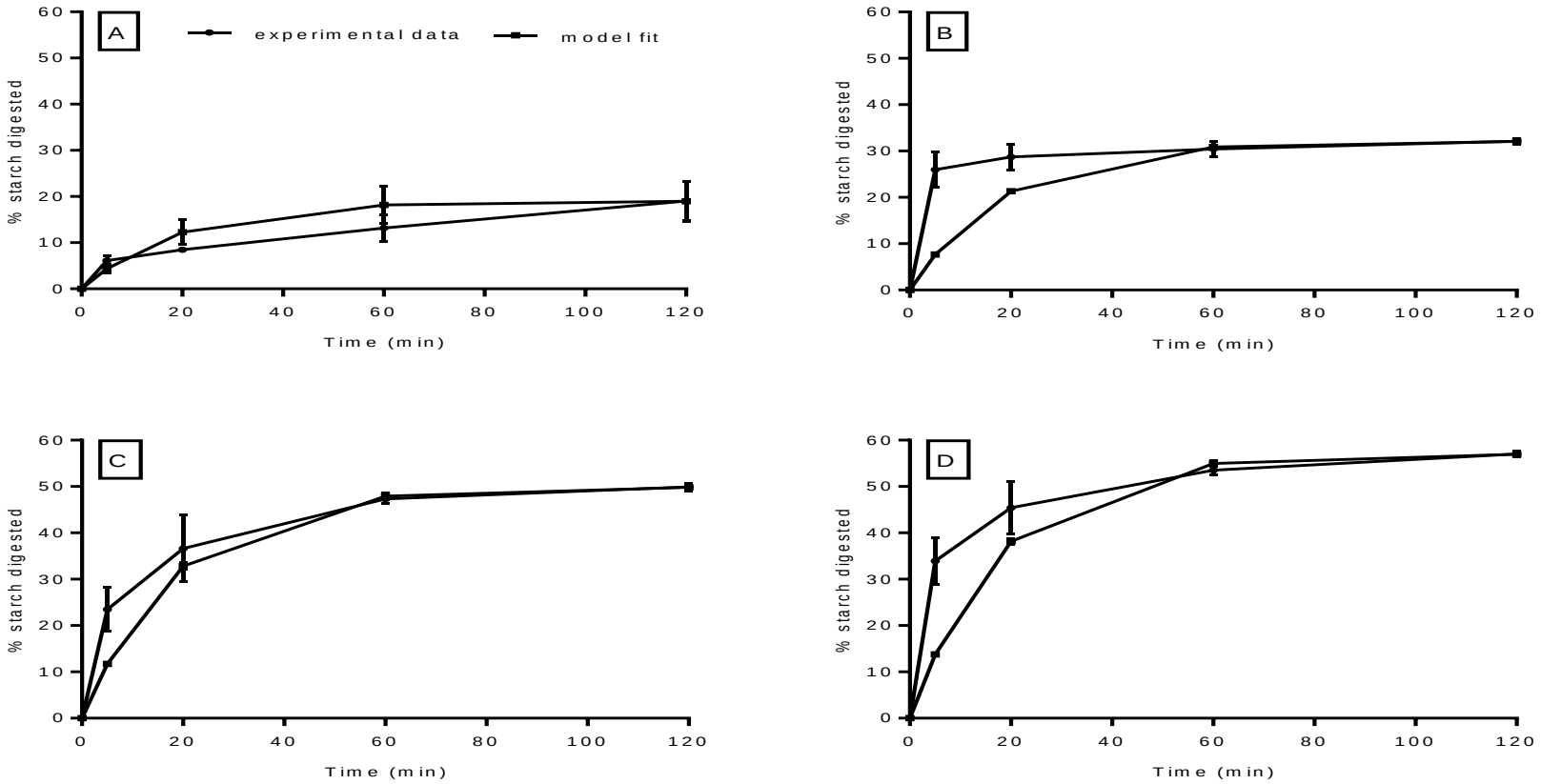

Figure S3. Starch digestion and model fit of millet flour (A) and cooked porridge (B) following Goñi (A), and millet flour (C) and cooked porridge (D) following INFOGEST protocols. Results are presented as mean of 3 biological replicates for Goñi and INFOGEST respectively and expressed in dry weight basis. Data error bars are standard deviation of the mean.

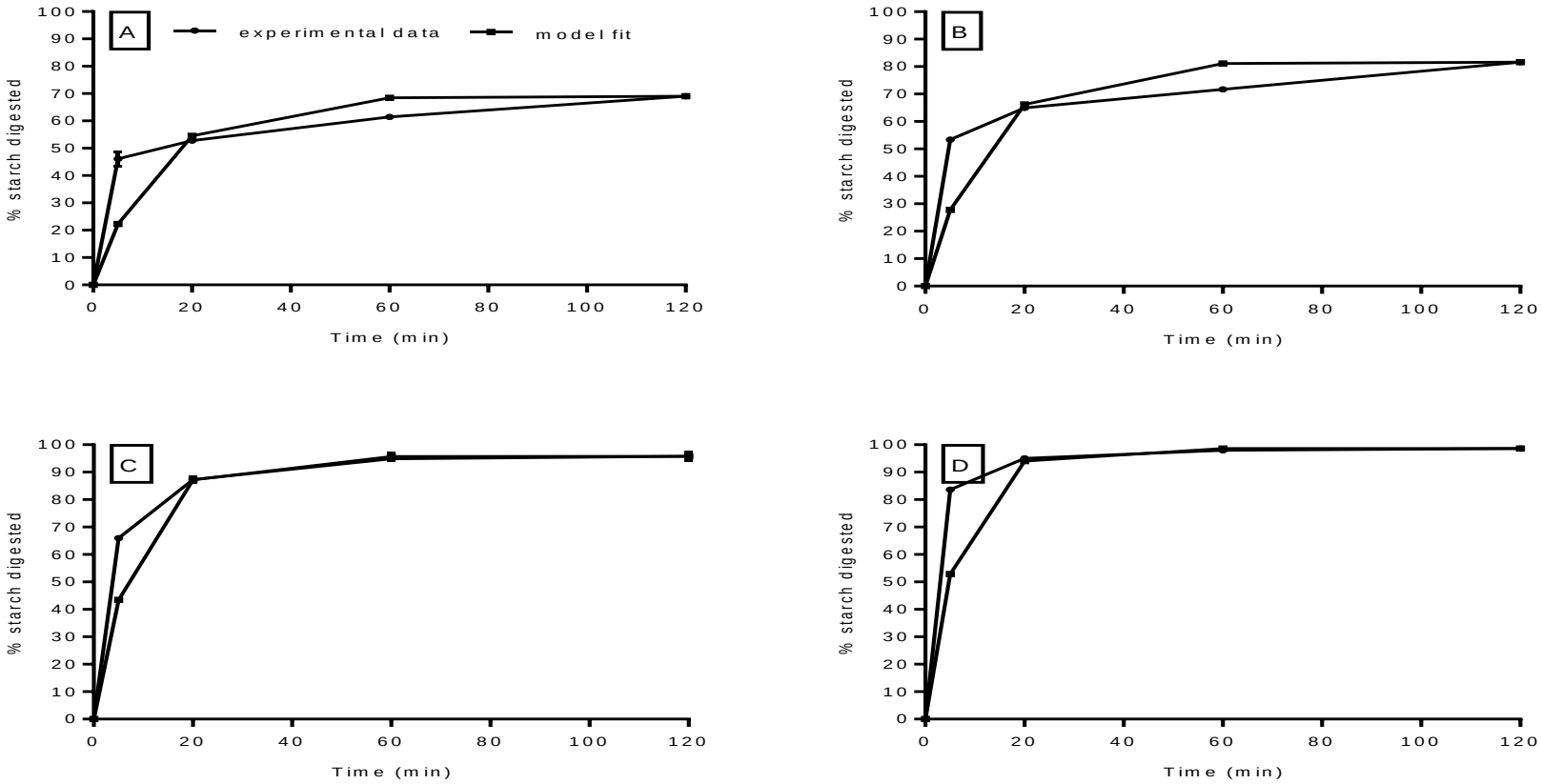

Figure S4. Starch digestion and model fit of fermented millet porridge (*ibyer-i-angen*) after 12 h (A), 24 h (B), 48 h (C) and 72 h (D) fermentation. Results are presented as mean of 3 biological replicates for Goñi and INFOGEST respectively and expressed in dry weight basis. Data error bars are standard deviation of the mean.