Technologies for improving the quality of bread doughs made with barley spent grain and sorghum

by

Itumeleng Evidence Magabane

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DECLARATION

I declare that the dissertation herewith submitted for the degree MSc (Agric) Food Science and Technology at the University of Pretoria has not previously been submitted for a degree at any other university or institution of higher education.

Itumeleng Evidence Magabane

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ABSTRACT

Technologies for improving the quality of bread doughs made with barley spent grain and sorghum

Student: Itumeleng E. Magabane

Supervisor: Prof J.R.N. Taylor

Co-supervisor: Dr J. Taylor

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Expenditure on wheat importation in sub-Saharan African countries is increasing greatly arising from the region's rapidly expanding human population, urbanisation, and unfavourable conditions for wheat cultivation. Adoption of composite flours is encouraged to reduce wheat importation and promote local agriculture. Barley brewer's spent grain (BSG), a high-fibre by-product of the brewing industry, is relatively inexpensive and available at large quantities. Sorghum, which is well-adapted to cultivation in sub-Saharan Africa, is an underutilized grain-crop. BSG and sorghum are potential vehicles for producing less expensive bread of improved nutritional properties. However, both lack functional gluten, which is responsible for good viscoelastic dough and high bread volume.

BSG particle size reduction in combination with a sourdough fermentation were investigated as BSG pre-treatment technologies to improve wheat-BSG composite dough and bread quality. Fractionation of dried BSG through roller milling enriched the protein of BSG flour, but gave poor loaf volume and denser crumb. Additionally, the much lower flour extraction yields compared to hammer milling which gives a 100 % extraction rate flour would impact negatively on the product economic viability. Mixolab dough rheology showed that a 15 % BSG substitution significantly increased dough development time and flour water absorption. However, application of a short (3 h) 'sponge and dough' sourdough process improved the gas-holding properties of composite, increased loaf volume and crumb softness compared to a straight dough method. At 20 % BSG substitution, the composite wheat bread had 71.4 % more dietary fibre as well as higher protein and mineral contents than a commercial wheat brown bread.

The effects of chemical (using glacial acetic acid) and physical treatment (through sheeting) on the functionality of sorghum doughs from normal and transgenic high protein digestibility (TG-HD) lines with supressed γ-kafirin expression were investigated. Normal sorghum flour doughs were subjected to sheeting in combination with sourdough addition. Partial flour pre-gelatinization, by cooking, was a pre-requisite for formation of a cohesive dough and was hence applied throughout this study. Upon baking, the combination of sheeting (15 passes) and sourdough addition (50% w/w of total flour) produced bread with a more aerated crumb and greater volume compared to the untreated control.

Tensile tests of TG-HD doughs showed 38 and 42 % higher extensibility, compared to their null control doughs. These effects were attributed to the greater accessibility of α-kafirins in the invaginated protein bodies of these high protein digestibility lines. Shear forces applied by manual sheeting and glacial acetic acid treatment were used in attempt to free the protein body-encapsulated kafirins and hence functionalise them in sorghum dough. Transmission electron microscopy (TEM) of these doughs revealed successful disruption of protein bodies by the respective treatments. Starch granules observed by scanning electron microscopy (SEM) seemed to remain intact, indicating the effects to be protein-related. However, the elevated temperature ($>50^{o}$C), glacial acetic acid treatment and combination thereof, reduced dough extensibility. This was possibly due to the presence of other components in the dough system apart from the kafirins, mainly the starch granules, as well as insufficient plasticisation.

The study shows that a combination of physico-chemical treatments, with emphasis on functionalising inert components such as fibre and protein, can substantially improve the dough functionality and consequent bread quality of gluten-void cereal grain materials.

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1 INTRODUCTION

Expenditure on wheat imports in sub-Saharan Africa (SSA) is estimated to increase by 38 % within the next 10 years (Macauley, 2015). This, together with the escalating bread consumption and adverse conditions for wheat cultivation in the developing countries of SSA poses a major economic problem. Consequently, food price increases are most detrimental to the poor populations (Wodon and Zaman, 2008) and compromises diet quality and ultimately, child growth and development (Meerman and Aphane, 2012). As a solution, the use of composite flours to reduce wheat importation and promote local underutilized crops is encouraged (Noorfarahzilah *et al.*, 2014). Sorghum, for example, is a locally grown crop that is adapted to the harsh conditions of growth in Africa (Belton and Taylor, 2004). Another cereal material which is also available in large in large quantity and is inexpensive is barley brewer's spent grain (BSG) - a major by-product of the brewing industry (Mussatto, 2014).

However, both sorghum and BSG lack gluten, which possesses unique viscoelastic properties that enable dough gas retention during fermentation of wheat dough (Brites *et al*., 2010). Hence, the gluten-free doughs have much poorer elasticity and cohesiveness, resulting in lower loaf volume, poor texture and crumb characteristics (Houben *et al*., 2012). Also of great importance in bread is its nutritional quality. An alternative to normal sorghum types, which have poor quality (low lysine content and protein digestibility) are protein biofortified sorghums which have higher protein digestibility and high lysine (HDHL) lines. Such sorghum lines have been developed by the Africa Biofortified Sorghum project through suppressing the expression of specific kafirin subclasses (Biosorghum, 2010). With regard to BSG, it has the potential to improve the nutritional value of bread by increasing both the protein and dietary fibre content (Ozturk *et al*., 2002).

Achieving acceptable bread quality characteristics with these non-wheat cereal materials however requires much development. Sourdough fermentation is a traditional cereal processing technology that can improve the volume and texture of gluten-free bread bread due to modifications in the starch granules, which in turn affect dough strength and gas-holding ability (Falade *et al*., 2014) and non-gluten storage proteins (Schober *et al*., 2010). Sourdough is a mixture of flour and water fermented with lactic acid bacteria (LAB) and yeasts (Moroni *et al*., 2009). Another common practice is the particle size reduction of fibrous cereal ingredients prior to incorporation in baked products. For example, the particle

size of BSG flour has been found to affect the quality of wheat biscuits (Guo *et al*., 2014). BSG of reduced particle size produced biscuits of increased fluffy texture and high sensory scores compared to unmodified BSG.

It has been shown that cohesive maize doughs of improved extensibility can be obtained through mechanical sheeting of the doughs between rollers however, starch pre-gelatinization is a prerequisite (Khuzwayo, 2016). Pre-gelatinized starch acts as a binder in gluten-free systems (Sozer, 2009). Sheeting of dough through a set of rollers produces a dough of reduced resistance and increased extensibility (Engmann *et al*., 2005). Application of shear forces to dough has been suggested as a means to free kafirins from their confinement in protein bodies to improve their ability to form functional structures in foods (Hamaker and Bugusu, 2003). Further, it has been found that stable viscoelastic masses can be formed by dissolving kafirin in glacial acetic acid followed by simple coacervation by addition of water (Elhassan *et al*., 2018). The (elastic and viscous flow balance of these fresh kafirin masses resembled that of wheat gluten.

It is proposed that application of these technologies will induce physical and or chemical modifications to these gluten-void cereal ingredients and hence improve their functional role to produce dough and bread of acceptable quality.

2 LITERATURE REVIEW

Brewer's spent grain (BSG) and sorghum are examples of highly available, relatively inexpensive non-wheat ingredients that can be exploited for the production of cereal-based staples such as bread. This literature review looks at the physiochemical properties, composition, as well as functionality of BSG and sorghum flour, respectively. The various research techniques aimed at the improvement of the dough and bread making properties of these cereal materials is also discussed.

2.1 Brewer's spent grain

2.1.1 Physicochemical properties and chemical composition of brewer's spent grain

BSG is commonly obtained from malted barley grain that has been through the mashing process for wort extraction in the brewing process (Fillaudeau *et al*., 2006). It is considered a lignocellulosic material because the major components are the barley husk-pericarp-seed coat layers, which are rich in cellulose, non-cellulosic, non-starch polysaccharides and lignin (Mussatto *et al*., 2006). The non-starch polysaccharides constitute between 30 and 50 % of BSG dry weight (Xiros and Christakopoulos, 2012), with high content of arabinoxylans and some residual (1-3, 1-4) β-glucan (Gupta *et al*., 2010). There is considerable variation in the chemical composition of BSG; which is attributed to barley variety used, harvest time, malting and mashing conditions as well as the adjuncts used, in terms of quality and type (Robertson *et al*., 2010).

Table 2.1.1: Chemical composition of brewer's spent grain (BSG) in % dry weight, as reported in literature

Components (% dw)	Russ *et al.* (2005)	Niemi *et al.* (2012)	Farcas *et al.* (2014)	Mussatto and Roberto (2005)
Cellulose	23- 25	-	-	16.8
Hemicellulose	30- 35	22.9	-	28.4
Lignin	7- 8	19.4	-	27.8
Protein	19- 23	23.3	18.00	15.3
Lipid	9- 12	7.8	6.61	-
Ash	4.0- 5.5	4.9	3.82	4.6

- = No data

Although fibre constitutes the highest proportion of BSG, there is also approximately 23 % protein, 8 % lipid and 5 % ash (minerals) *(Table 2.1.1)*. Vitamins are also present in BSG. The vitamin fraction consists of mg/kg (dry basis); biotin (0.1), choline (1800), niacin (44), riboflavin (1.5), thiamin (0.7), folic acid (0.2), pantothenic acid (8.5) and pyridoxine (0.7) (Huige, 1994; Mussatto and Roberto, 2006). The minerals present in high concentrations are calcium, silicon, magnesium and phosphorus (Aliyu and Bala, 2011). Silica in BSG is also present due to the fact that 25% of the minerals in barley are in the form of silicates (Kunze, 1996) and considerable amounts are located in the husk (Macleod, 1979). Using scanning electron microscopy (SEM), Mussatto *et al*. (2006) showed the appearance of silicates which appear as bright points on the surface of BSG husk *(Figure 2.1.1)*.
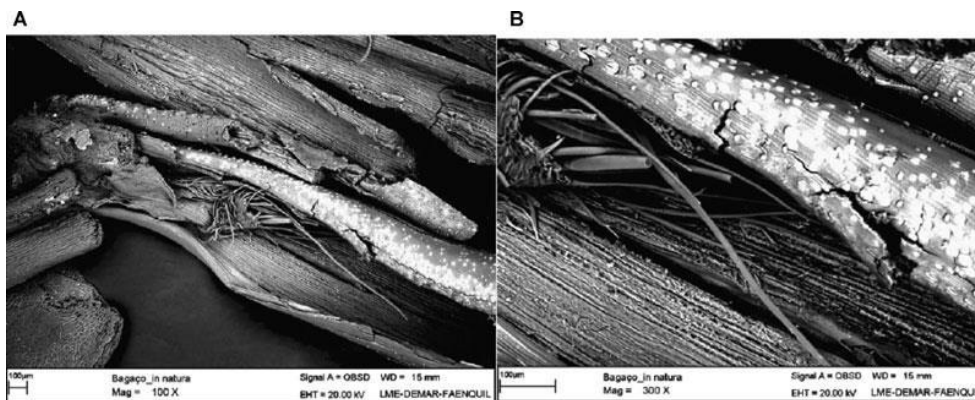
Figure 2.1.1: Scanning electron microscopy of BSG particles. **(A)** X 100; **(B)** X 300 (Mussatto *et al.,* 2006).

The structure of BSG is considered as being highly heterogeneous (Forssell *et al*., 2008). Analysis of BSG flour by SEM reveals mainly husks, fibre filaments and starchy endosperm remains (Ktenioudaki *et al*., 2012). Remnants of other grains (non-malt sources of fermentable sugars) may also be present in addition to the malted barley remnants (Reinold, 1997).

2.1.2 Brewer's spent grain as a bread ingredient

2.1.2.1 Effect of BSG on bread quality

Following the 'no-waste' ethos, utilization of BSG as a food ingredient is becoming more common (Stojceska, 2011; Burningham, 2012). BSG is not only high in protein and fibre but importantly, it is derived from constituents suitable for human consumption (Aliyu and Bala, 2010), thus making it suitable for incorporation in food products such as cereal flakes, whole-wheat bread, biscuits and saltine snacks (Mussatto *et al.*, 2006). Nevertheless, there are some limitations regarding the use of this brewing by-product as a partial replacement for currently used flours (Mussatto *et al.*, 2006).

Substitution of wheat flour utilising such a high fibre non-wheat material not only creates a gluten dilution effect but also interferes with the viscoelastic gluten network (Waters *et al.*, 2012). The gluten-fibre interactions in the dough weaken the gluten matrix and reduce dough elasticity. The dough's ability to expand is also physically restricted due to the higher complex modulus (G^*) of spent grain incorporated dough. Furthermore, an increase in water absorption is reported with BSG inclusion in wheat flour. According to Rosell *et al.* (2001), the increase is due to higher number of hydroxyl groups in the fibre structure which increases water interaction through hydrogen bonding. This intervention reduces the amount of water available for gluten hydration.

As a material that is so rich in dietary fibre, negative effects on end-product quality such as texture, appearance and taste are anticipated when added to bakery foods (Ktenioudaki *et al.*, 2012). Firstly, BSG is dark in colour and therefore noticeably affects the colour of the end-products (Ktenioudaki *et al.*, 2012). These authors noted this effect in the production of bread-sticks. Stojceska and Ainsworth (2008) found that loaf specific volume was inversely related to the level of BSG addition in wheat bread.

Increase in crumb firmness is a major concern as it represents one of the major signals to the consumer of bread staling (Gray and BeMiller, 2003). The increased firmness associated with BSG inclusion is likely due to the presence of arabinoxylans, glucans and xylo-oligosaccharides (Waters *et al.*, 2012). Courtin *et al.* (1999) reported the potential of insoluble arabinoxylans to induce disruptions in the viscoelastic network in wheat bread dough. In addition, because the fibre fraction binds high amounts of moisture, water

availability in the bread is diminished, thus increasing the rate of starch retrogradation (Waters *et al*., 2012).

2.1.2.2 Effect on human nutrition

Because of the high levels of dietary fibre, protein and essential amino acids present in BSG (Waters *et al*., 2012), it is anticipated that its ingestion and that of derived products should provide benefits to human health. Non-communicable diseases (NCDs) are currently a major contributor to global burden of disease and mortality, claiming over 14 million lives between the ages of 30 and 70 (WHO, 2014). The burden of these diseases has been predicted to increase over the years. However, they can be prevented or controlled by focusing on the associated contributing risk factors such as and unhealthy diet (Boutayeb and Boutayeb, 2005). The fibre, protein and mineral fortification benefit that comes with BSG inclusion thus makes their formulated foods potentially beneficial to human health.

Huige (1994), found that compared to conventional wheat bread, inclusion 10 % of BSG led to an increase in overall protein and essential amino acid content by 50 and 10 %, respectively. Because the calorific density of BSG is only half that of most cereals, the energy content of BSG-containing breads is less.

BSG polysaccharides consist mainly of cellulose, arabinoxylans and at much lower levels, (1–3, 1–4)-β-D-glucan as well as traces of starch (Forssell *et al*., 2008). The β-glucans are of great interest because they have prebiotic effects associated with soluble dietary fibre (Waters *et al*., 2012) and lower blood serum cholesterol (Hecker *et al*., 1998) as well as glycaemic response (Venn and Mann, 2004). Also, the high content of non-cellulosic polysaccharides contributes benefits to intestinal digestion associated with alleviation of constipation (Mussatto *et al*., 2006). In this respect, the levels of insoluble fibre is particularly very high in BSG (48 % total fibre) (Waters *et al*., 2012). The implications are delayed transit time and gastric emptying as well as increased faecal weight, resulting in slower rate of nutrient absorption (Blackwood *et al*., 2000).

The minerals calcium, magnesium and phosphorus minerals are present in relatively high levels in BSG. Calcium in particular, may help in reducing the risks of osteoporosis and colon cancer when increased in the diet (Newmark *et al*., 2004).

2.1.3 Pre-treatment of BSG for bread making

2.1.3.1 Size reduction

A number of researchers have reported that BSG cannot be directly added to food, as it is too granular, and must therefore first be reduced to flour (Hassona, 1993; Miranda *et al*., 1994; Ozturk *et al*., 2002). Whole unmilled BSG contains particles as large as 5 mm (Niemi *et al*., 2012). Attempts at BSG direct inclusion in biscuits, bread and baked snacks was found to result in poor flavour, texture and sensory quality (Waters *et al*., 2012). Also, BSG flour particle size has been found to affect the quality of wheat biscuits (Guo *et al*., 2014). Smaller particle sized BSG gave lower bulk density- an indication of fluffier texture and mouthfeel of biscuits. With smaller particle size BSG, biscuits also had higher sensory scores in respect of high perception of colour, crispiness, texture, mouthfeel and general acceptability. It is therefore vital for BSG to be modified prior to its application as a bakery ingredient.

Unlike in biscuits, the impact of fibre or bran particle size on bread loaf volume remains unclear because of opposing results from various researchers (Hemdane *et al*., 2015). Zhang and Moore (1999) reported that bread made with medium sized bran (415 μm) had higher specific volume than breads made with coarse (609 μm) and fine (278 μm) bran, thus suggesting that an optimum bran particle size may exist for the production of bran-rich bread. Finer particle size, however resulted in a better crust appearance and less gritty mouthfeel in bread.

Importantly, milling induces degradation of cell walls, thus increasing the surface area of particles and carbohydrate solubility (Niemi *et al*., 2012). The solubility of arabinoxylan, in particular, was increased in BSG that was milled prior to enzymatic treatment. Zhao *et al*. (2006) reported that this effect was due to reduction in cellulose crystallinity and hence an increase in amorphous regions.

2.1.3.2 Pre-fermentation of BSG

The adverse effects of fibre on the quality of baked products has led to various approaches being investigated with the aim of improving quality and hence the acceptability of these products with added fibre (Ktenioudaki and Gallagher, 2012; Hemdane *et al*., 2015). These are mainly through the use of enzymes and processes such as fermentation and extrusion cooking.

The application of sourdough fermentation in bread making is a common practice, especially in rye bread (Lorenz and Brummer, 2003). Katina *et al*. (2006) studied the effect of different bran fermentations (instant yeast and a *Lactobacillus brevis* starter) in combination with commercial enzymes (α-amylase, xylanase and lipase) on the quality of high-fibre breads. Fermentation of bran significantly increased loaf volume and shelf life compared to regular bran wheat bread, the improvement was more pronounced with the inclusion of enzymes. The authors reported that sourdough fermentation of bran improves the gluten network, and hence gas retention as well as possibly improving the solubility of cell wall components. The improved protein network is thought to be as a result of proteolytic activity which modifies the physical properties of gluten (Corsetti *et al.,* 1998). Furthermore, acidification by sourdough is known to increase protein solubility and encourage proteolysis (Katina *et al.,* 2006).

Salmenkallio-Marttila *et al*. (2001) observed an improvement in uniformity of bread crumb structure and in flavour with sourdough fermentation. Acid aromas and flavours were found to be enhanced when lactic acid bacteria (LAB) sourdough was incorporated in bread, and sweetness subsequently reduced (Waters *et al*., 2012). Crust colour is also affected. A lightening effect of BSG sourdough on crust colour was observed due to a reduction of polyphenols and fatty acids (Corsetti and Settanni, 2007). Production of a dark colour as a result of polymerisation of endogenous phenolic compounds and enzymatic (polyphenol oxidase) discoloration is thus diminished (Waters *et al*., 2012). Furthermore, the reduction of free sugars by LAB fermentation possibly also reduces the occurrence of maillard reactions.

Apart from textural improvement, sourdough fermentation is known for its role in improving the nutritional properties of bread. Lactic acid fermentation of cereals induces an optimum pH for phytase activity (Larsson and Sandberg, 1991). As a result of the decreased phytate content, minerals such as magnesium and phosphorus have greater bio-accessibility (Lopez *et al*., 2001).

2.2 Non-wheat dough systems with sorghum

2.2.1 Chemistry, structure and functionality of cereal prolamin proteins in dough

2.2.1.1 Gluten

Gliadin and glutenin proteins are the major classes of wheat storage proteins and are localized in the endosperm (Veraverbeke and Delcour, 2002). The gluten protein complex can be isolated by simple gentle washing of wheat dough under running water (Shewry *et al.*, 2002). Gluten is formed from the monomers, gliadins and glutenins (Shewry *et al.*, 2002). Glutenins have molecular weights (MW) ranging from about 80 000 to several millions while monomeric gliadins have MWs between 30 000 and 80 000 (Goesaert *et al.*, 2005). The Gliadins are readily soluble in aqueous alcohols and although this property is not shared with glutenin polymers, their building blocks (called 'subunits') have similar solubility (Veraverbeke and Delcour, 2002). Glutenin proteins are further distinguished into high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS). The uniqueness of the gluten proteins is primarily attributed to the amino acid compositions. Gliadins and GS both have high levels of proline and glutamine and low amounts of the charged amino acids (Wieser, 2007). Cysteine residues are crucial in the structure of both. These cysteine residues either facilitate disulphide bonding within the same polypeptide (intra-chain disulphide bonds) (gliadins) or between different polypeptides (inter-chain disulphide bonds) (glutenins) (Veraverbeke and Delcour, 2002).

Glutenins and gliadins provide the elastic and cohesive properties of wheat dough respectively (Wieser, 2007). Thus both have important roles in the rheological properties of the dough. The HMW glutenin subunits are the major determinants of dough and gluten elasticity (Shewry *et al.*, 2002). For good quality bread making, an optimal balance of dough viscosity and elasticity is needed. Too low gluten elasticity results in low bread loaf volume whilst too high elasticity hinders gas cell expansion and therefore also leads to lower volume (Veraverbeke and Delcour, 2002). Glutenin elasticity is hypothesized to be mediated by non-covalent interactions, mainly hydrogen bonds, between and within glutenin chains (Belton, 1999). This class of chemical bonds is implicated for gluten protein aggregation and dough structure (Wieser, 2007). On the other hand, gliadins are the plasticizers that weaken the interactions between glutenin chains (Khatkar *et al.*, 1995), thereby causing increased dough viscosity (Belton, 1999).

2.2.1.2 Zein and kafirin

In order to mimic the functional properties of wheat gluten in cereal dough systems that lack gluten, it is critical to study the differences in the structure and composition of their prolamin proteins (Taylor *et al*., 2016).

Many studies have shown sorghum kafirin to be analogous to maize zein; with both being encapsulated in protein bodies within the endosperm and also having close similarities in chemical composition and properties (Belton *et al*., 2006). Kafirins are classified into a number of major subclasses based on their solubility, structure and amino acid sequence (Shull *et al*., 1991). Alpha-kafirin represents the major subclass, making up 65- 85 % of the total kafirins, whilst β and γ-kafirins represent 7- 8 % and 9- 12 % (Hamaker *et al*., 1995). The same classification holds for maize zein subunits (Shull *et al*., 1991). Protein bodies consisting of kafirin/zein show α-kafirins/zeins to be mainly localized in the centre of the protein bodies whilst the β and γ subclasses are located at the periphery (Oria *et al*., 2000).

In comparison to other cereal prolamins, the sorghum kafirins are less digestible (Duodu *et al.*, 2003). Kafirins are more hydrophobic and form extensive cross-links which are compounded when the kafirin is wet-heated. The polypeptide monomers of both zein and kafirin are much smaller in size compared to wheat HMW-GS, but due to the high cysteine content of the β- and γ-sub-classes, they are capable of polymerization through disulphide cross-linking like the HMW-GS (Taylor *et al*., 2016).

Kafirin and zein also have a high proportion of α-helical secondary structure conformation (Belton *et al*., 2006). Predictions of the structure of zein suggest that high proportion of the α-helix conformation stems from the repetitive sequences found in the α-zein protein core. According to Argos *et al*. (1982), the cylindrical structure *(Figure 2.2.1 A)* stems from nine of these repeats clustering together, whereby each forms an α-helix separated by a turn region. The more recent model by Bugs *et al.* (2004) indicates an extended hairpin-type structure *(Figure 2.2.1 C)*, comprising of elements of α-helix, α-sheet and turns folded back on itself (Belton *et al*., 2006).
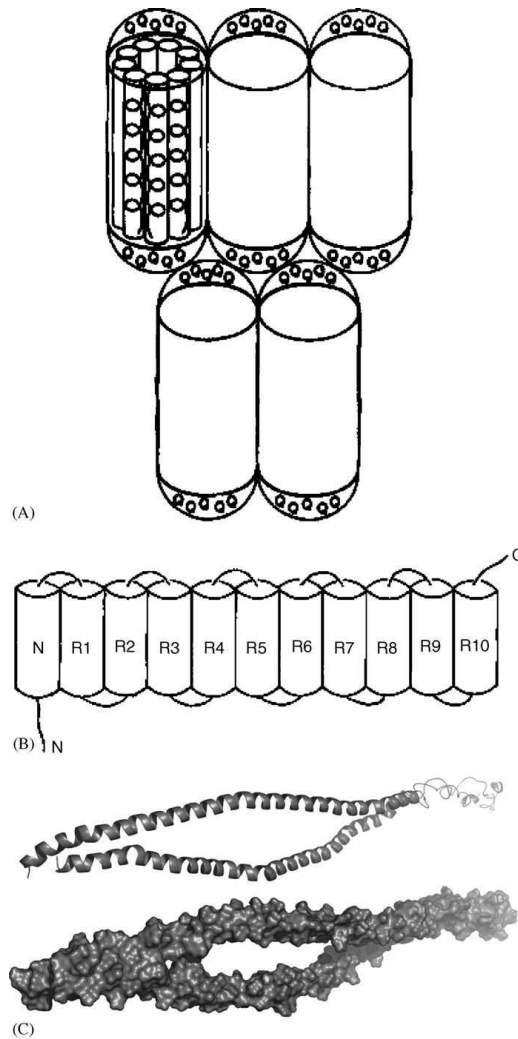
Figure 2.2.1: Proposed structural models for α-zeins of maize. **(A)** Alpha helices arranged antiparallel to form a distorted cylinder. The glutamine-rich turn regions allow hydrogen bonding to molecules in neighbouring planes. **(B)** Alpha helices arranged in antiparallel to form an extended structure. **(C)** A hairpin comprising elements of α-helix, β-sheet and turns (Belton *et al*., 2006).

The functionality of kafirin and zein in dough systems has only been realized with proteins in the isolated form, this is in part due to their natural encapsulation within protein bodies in the starchy endosperm that inhibits functional behaviour of the proteins (Goodall *et al*., 2012). This arrangement is unlike in wheat where the glutenin and gliadin proteins form a continuous matrix around the starch granules (Shewry and Halford, 2002).

Although zein and kafirin share similar type storage proteins which have a similar composition to glutenin, in that they exhibit extensive disulphide bonded polymerisation, differences in

amino acid composition and sequence, as well as tertiary and quaternary structure still exist (Taylor *et al*., 2016). With these dissimilarities set aside, recent studies have shown that isolated zein protein, analogous to kafirin, can be made viscoelastic to positively impact on bread dough strength and loaf volume (Schober *et al*., 2010; Khuzwayo, 2016). Recently, Khuzwayo (2016) found that addition of zein (mixed above its glass transition temperature in water) formed a more elastic maize flour dough. The dough's viscoelasticity was further improved by sheeting, which evenly distributed the zein dough throughout the maize dough. Intermingling of zein fibrils within the dough was seen to be responsible for the improved dough properties.

2.2.2 Non-wheat cereals of improved protein functionality

2.2.2.1 High protein digestibility, high lysine sorghum

A discovery of highly digestible sorghum mutants that have abnormal shaped protein bodies has been documented (Oria *et al*., 2000). There lies some promise that these changes might affect the functionality of sorghum flour made from these mutant lines, including bread (Elhassan *et al*., 2015).

Sorghum lines with high protein digestibility and high lysine (HDHL) were found within a high-lysine population developed from crosses of the high-lysine mutant P721 opaque (Q) and normal cultivars (Oria *et al*., 2000). In vitro protein digestibility in these HDHL lines was higher with both uncooked (about 85%) and cooked (about 80%) flour. SDS-PAGE and ELISA time-course analysis of undigested proteins from HDHL lines showed that the digestion of α-kafirin was more rapid compared to normal sorghums. This is due to more of the easy-to-digest α-kafirin protein being exposed in the protein bodies (Goodall *et al.*, 2012). In the HDHL sorghum, the normal spherical protein body shape has been altered *(Figure 2.2.2)* to assume a folded morphology (with deep invaginations) due to a shift of γ-kafirins from the outer parts of the protein body to the interior (Oria *et al.,* 2000). Therefore, it is generally considered that the improved accessibility of proteases to the α-kafirins, and the increased protein body surface area due to the irregularly shaped protein bodies are linked to the HDHL sorghum increased digestibility (Duodu *et al.,* 2003).
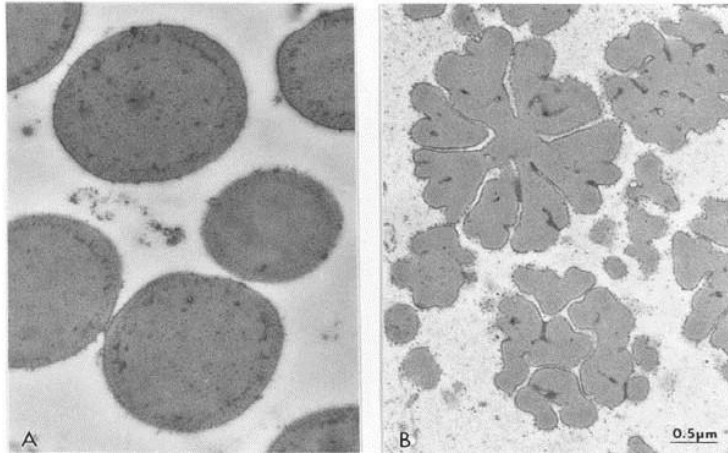
Figure 2.2.2: Transmission electron micrographs of protein bodies from normal (left) and high protein digestibility mutant (right) sorghum genotypes (Hamaker and Bugusu, 2003).

Sorghum lines with high lysine content and improved protein digestibility, which also have similar altered protein bodies, have been developed through genetic engineering whereby the synthesis of γ-kafirin, in particular, has been inhibited (Da Silva *et al*., 2011).

Goodall *et al*. (2012) used conventionally bred HDHL sorghum composited with wheat flour to produce bread. HDHL sorghum resulted in doughs of much improved viscoelasticity when the dough was treated above its glass transition temperature (T_g) compared normal sorghum-wheat composite dough. The bread crumb texture and loaf volume was also improved. This indicates that isolated, protein body-free kafirins can be mobilized, like wheat gluten, at temperatures above their T_g to affect their functionality in viscoelastic dough development and therefore good quality bread making.

Elhassan *et al.* (2015) investigated novel biofortified sorghum lines with combined waxy and high protein digestibility traits for their endosperm and flour properties. These sorghums have a modified endosperm texture with loosely packed starch granules. The floury endosperm texture is a result of an incomplete protein matrix surrounding the outer floury endosperm because of the altered protein body structure. The authors furthered the work by studying transgenic lines from Africa Biofortied Sorghum (ABS) consortium. The sorghum mutants had higher water flour solubility at 30 °C, higher paste viscosity, and produced stronger doughs that are more elastic compared their null controls (Elhassan *et al*., 2017). The improved flour and dough properties were attributed to the reduced endosperm compactness and improved protein-starch interactions due to reduction of hydrophobic γ-kafirins content.

2.2.3 Viscoelastic zein and kafirin

2.2.3.1 Glass transition temperature

All amorphous macromolecules, and thus proteins, are capable of undergoing reversible physical change of states from glassy to rubbery which the application of heat and uptake of plasticizer, this phenomenon is termed 'glass transition' (Bugusu *et al*., 2001). The temperature at which the transition occurs is the glass transition temperature (T_g); an important parameter in dough rheology that explains the behaviour of proteins during mixing.

Hoseney *et al.* (1986) showed that gluten, like any other amorphous polymer, has a glass transition temperature (T_g) that can be lowered by increasing the water content. They reported that at 13 % moisture, the T_g of gluten occurred at 21 ^{o}C. They explained that upon hydration of flour, and as water is absorbed during mixing, gluten undergoes a glass transition that promotes interaction with other gluten polymers to form a dough (Faubion and Hoseney, 1989). Gluten viscoelasticity upon hydration has therefore been attributed to its polymeric nature. Maize zein requires higher temperatures than wheat gluten to form viscoelastic fibrils (Lawton, 1992).

The correlation between protein glass transition and dough properties has been applied to a zein-starch synthetic dough system (Lawton, 1992). Because no dough was developed below 25 ^{o}C, the dough forming ability of zein-starch doughs is clearly dependent on the mixing temperature. As the temperature was raised and held at 35 ^{o}C, where the T_g of zein was 28 ^{o}C at 15 % moisture, a viscoelastic dough was formed. Thus indicating that an extensible dough, similar to that of wheat, can be formed due to formation of extensive protein fibre networks.

Mejia *et al.* (2007) examined the secondary structure of viscoelastic polymers of wheat gluten and α-zein proteins using Fourier-transform infrared (FT-IR) spectroscopy. Differences and similarities of zein-starch and gluten-starch doughs prepared at 25 and 35 ^{o}C were analysed. The results showed a lower amide II region of the zein-starch dough spectra in the viscoelastic state compared to gluten-starch and native zein systems at 25 and 35 ^{o}C. This pointed towards conformational changes having occurred due to protein–protein hydrophobic interactions as opposed to protein–water interactions, as would be seen in the viscoelastic polymers of gluten and soluble protein. The amide I region from the FT-IR, being more reliable, was used for analysing secondary structure of the viscoelastic dough systems.

Hydrated viscoelastic zein at 35 °C showed a 48 % increase of β-sheet structures accompanied by a 30 % decrease in α-helical structures. However, when the temperature of the zein polymer dropped from 35 to 25 °C, the content of β-sheet structures dropped to 30 % and the polymer viscoelasticity was lost. These findings suggest that when shear is applied above T_g, zein loses its native structure due to protein rearrangement and displays viscoelastic properties. Furthermore, the secondary structures in the viscoelastic state are similar to those of gluten, but only if mixed and held at 35 °C. Thus, β-sheet content is a fundamental part of and determinant of viscoelasticity in the zein-starch dough.

The discovery of viscoelastic zein sparked more investigations with a focus on other gluten-free cereals such as sorghum, with an aim of gaining more insight on kafirin behaviour, which has similarities with zein. Bugusu *et al*. (2001) utilized commercial (protein body-free) zein in a sorghum-wheat composite flour system to study its effects on dough rheology and loaf volume. When mixed above zein T_g, both 5 and 10 % levels of zein substitutions resulted in improved dough development time, mixing time, extensibility and loaf volume. These results were attributed to two main reasons: the use of protein body-free zein, that is available for participation in the formation of fibrils, and secondly, the mixing of dough above the T_g of zein, which results in enhanced reactivity of the protein.

2.2.3.2 Plasticization

Plasticisers can be defined as significantly non-volatile, non-separating substances with high boiling point that have the ability to alter the physical and mechanical properties of another material (Banker, 1966). They are therefore considered adjuncts to polymeric materials for the reduction of brittleness, improvement of flow properties, flexibility and increased strength of films.

Hoseney *et al*. (1986) found that zein, without a plasticiser, produced hard, brittle-like solids. The T_g of a macromolecule can be lowered through addition of a plasticiser (Ferry, 1980). Plasticisers are therefore used in functionalising zein, as they can, by lowering the T_g of the polymer, yield films of improved flexibility and processing ability (Vieira *et al*., 2011). One of the criteria for a plasticizer to be effective is a balance of polar and non-polar groups, which determines its solubulisation effect. Some of the effective zein plasticisers include lactic acid, dibutyl tartrate, oleic acid.

The mechanism of plasticizer action on polymeric substances is explained by three theories. Firstly, the changes are thought to be due to a decrease in the overall intermolecular forces and hence cohesion along the polymer chains (Banker, 1966). This has been termed as 'The Lubrication Theory'. The small molecular size nature the plasticizer allows it to diffuse into the polymer and interfere with polymer-polymer interactions (Sears and Darby, 1982). An extension of this theory is the 'Free Volume Theory', which states that as the free volume (internal space available) of a polymer is increased, there more room there is for molecular chain movement. The introduction of thermal energy and molecular vibrations to a polymer, together with plasticisers, increases the free volume, allowing molecules or chains to move across each other more freely. The 'Gel Theory' considers the plasticized polymer as an intermediate state held together by loose attachments occurring along the polymer. These weaker forces allow the plasticised polymer to move and elongate easily.

Lawton (1992) used dibutyl tartrate as a second plasticiser along with water in order to achieve viscoelasticity in zein-starch composite doughs at temperatures below 60 ℃ *(Figure. 1)*. The T_g of zein decreased rapidly with water addition, whereas addition of up to 20 % dibutyl tartrate could not lower the T_g to below 50 ℃. However, extended doughs with and without dibutyl tartrate differed. The latter had low extensibility just after mixing and tended to lose its extensibility after resting, regardless of the temperature.
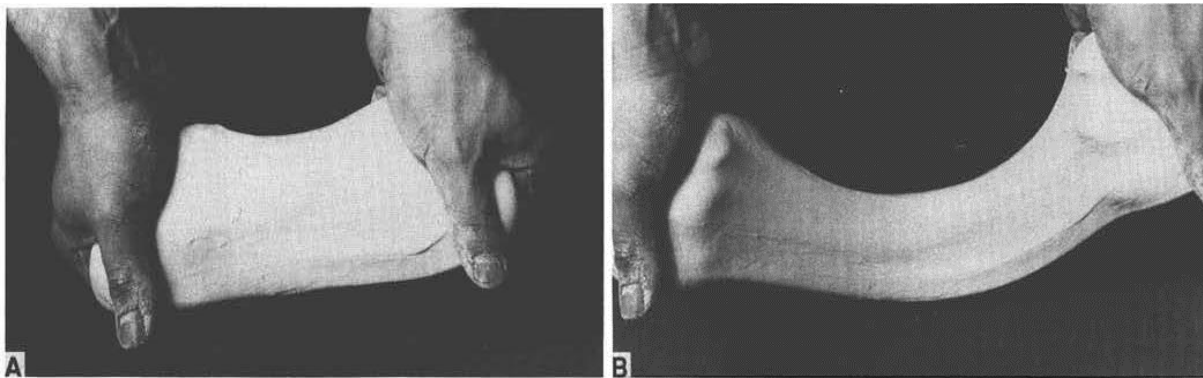
Figure 2.2.3: Photographic appearance of zein-starch dough plasticised with dibutyl tartrate; **(A)** Relaxed, **(B)** extended (Lawton, 1992).

Cast films and resin films from zein have been made with oleic acid as a plasticiser (Lai and Padua, 1997). Effectiveness of the use and choice of plasticiser was determined by tensile measurements, and hence the low Young's modulus obtained was a positive indicator.

Furthermore, oleic acid as a plasticizer was found to be more effective in stretched resin zein films than in cast films. Dynamic Mechanical Analysis (DMA) scans of zein and kafirin resins, plasticised with oleic acid, identified T_g in the range -4 and -3 ºC (Oom *et al.,* 2008). This is lower than the suggested T_g of zein plasticized with only water; which is at normal ambient temperature at high water content (25 %).

2.2.3.3 Defatting

The importance of lipids in dough is more complex than that of proteins (Schober *et al*., 2010). In wheat dough, lipoproteins may contribute to the softness and plasticity of gluten through the formation of slip planes within the gluten matrix (Grosskreutz, 1961). Other researchers suggest that lipids in wheat dough, at their natural levels, do not affect the rheological properties (Gan *et al*., 1995). However, polar lipids stabilize gas cells and ensure a greater loaf volume.

The HMW-GS is unique to wheat gluten and there exists no protein class analogous to it (Hamaker and Bugusu, 2003). Therefore, zein is incapable of forming the large, linear, disulphide-linked polymers that are responsible for wheat gluten viscoelasticity. The mechanism for viscoelastic dough formation in zein has instead been proposed to be due to aggregation of zein monomers via non-covalent interactions (Smith *et al*., 2014). Zein has relatively high hydrophobicity compared to that of gluten. This indicates hydrophobic interactions and components that affect these, such as lipids, are highly important (Schober *et al*., 2010).

It has been discovered that defatting or removal of surface lipids can improve the viscoelastic properties of zein (Schober *et al*., 2010). Furthermore, removal of polar lipid compounds such as β-carotene and ferulic acid through chloroform extraction promotes protein-protein

interactions and hence improved chances of zein aggregation (Erickson, 2014). For zein defatting, Schober *et al*. (2010) used chloroform and hexane in a bench-scale study, as well as accelerated solvent extraction with the combination of both solvents in conditions of high temperature and pressure. Light microscopy showed that zein particles were coated with a lipid film, which by preventing protein-protein interactions and water uptake, apparently hampered aggregation of zein particles into strands above zein's T_g in an aqueous system.

Defatted zein formed more cohesive, extensible and smooth strands. The more efficient the defatting of zein surfaces, the easier and therefore at lower temperatures protein crosslinking occured. As a result, the stability of 'hearth-type' rolls was improved during baking. Sly (2013) obtained similar results after defatting commercial zein with n-hexane. Defatting the zein allowed for formation of smoother and softer aggregates. Thus, improving dough cohesiveness and extensibility, which ultimately means better dough-forming properties of zein.

With the aim of verifying the work of Schober *et al*. (2010), Johansson *et al*. (2012) investigated the influence of lipids found in commercial zein on the rheological and microstructure of zein-starch doughs containing hydroxypropyl methylcellulose (HPMC). However, the authors reported that no difference in dough properties was observed when mixing with a mixograph between defatted versus non-defatted zein doughs. However, slightly faster dough development was observed with defatted zein. This was attributed to finer particle size of defatted zein, which led to more rapid protein network formation. Rheological analyses showed defatted zein doughs to have a higher modulus of elasticity. The authors went on to conclude, after observing no differences in the microstructures of the zein networks of both zein doughs, that the differences in rheological properties were probably not due to protein network related. Instead, the lipids present in the zein could have had a plasticizing effect, hence the lower modulus.

Due to the dough mixing process being extremely different between the work of Johansson *et al.* (2012) and Schober *et al*. (2010), the extent of dough development was probably not controlled. This shows that the conditions of zein mixing are crucial.

2.2.4 Chemical improvement of gluten-free dough functionality

2.2.4.1 Acidification

Sourdough fermentation

Sourdough is a mixture of flour and water fermented with lactic acid bacteria (LAB) and yeasts (Moroni *et al*., 2009) whose colonisation of natural dough affects the rheology, flavour and nutritional properties of baked goods (Gobetti *et al*., 2005). Typical representative genera of

sourdough are Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, and Weissella (Corsetti and Settanni, 2007; Moroni *et al*., 2009: Gobetti *et al.*, 2008).

The technology of sourdough fermentation has, for long, been used to improve volume, texture, flavour, nutritional value of bread as well as shelf-life by retarding the staling process (Arendt *et al.,* 2007). The positive attributes associated with sourdough are due to the metabolic activities of naturally occurring microorganisms such as lactic acid fermentation, proteolysis and exopolysaccharides (EPS) production (reviewed by Moroni *et al*., 2009). Acidification of sourdough and of the bread dough directly influences the structure forming components such as gluten, starch and arabinoxylans (Clarke and Arendt, 2005). According to Gänzle *et al*. (2008), protein degradation that occurs during sourdough fermentation is among the key phenomena that affect the overall quality of sourdough bread. Proteolysis affects dough rheology and overall texture of bread (Arendt *et al*., 2007). Hydrolysis of water-soluble proteins, which are activated by the acidic conditions (Wu *et al*., 2012), and extracellular peptidases of LAB prevents protein aggregation in the bread crumb upon baking.

Sourdough fermentation has also been shown to have beneficial effects in gluten-free dough systems. Edema *et al.* (2013) used sourdough fermentation to improve properties of fonio dough. Improvements in the fonio dough and final bread quality were due to slight changes in the starch granules, which probably increased water absorption and consequently, improved the dough's strength and gas-holding capacity. Falade *et al.* (2014) showed that sourdough had a beneficial increase in loaf volume and specific volume of maize breads with *L. plantarum* starter or multiple strains starter culture maize sourdough (*Figure 2.2.4*). The effect of sourdough on volume was greater than is beyond dough acidification as sourdough breads were superior to chemical acidification. Sourdough fermented breads had a more open crumb structure with distinct gas cells.
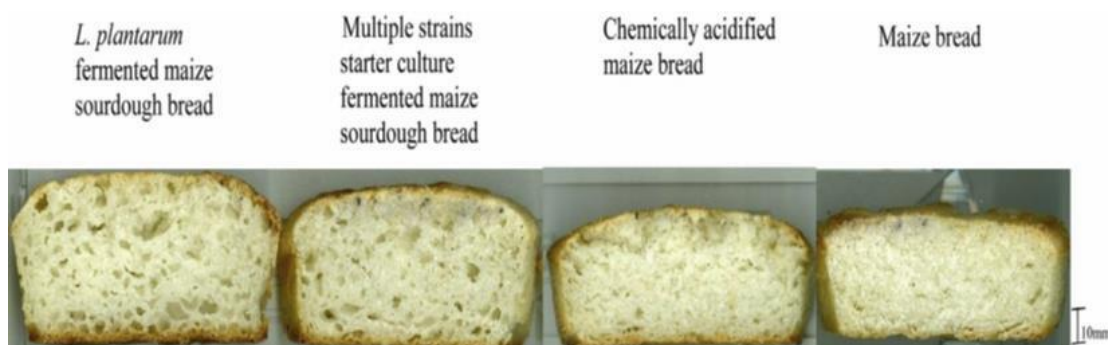
Figure 2.2.4: Effect of *L. plantarum* or multiple strains starter culture fermented maize sourdough on the crumb structure of maize bread. (Falade *et al.,* 2014).

Acid treatment

Acidification of dough is not only achievable by sourdough fermentation but also by lactic acid addition, which is one of the major products in sourdough (Houben *et al*., 2010). The effects of chemical acidification on the rheological parameters of dough has therefore been investigated by researchers, more-so in gluten-free dough systems, where there is not much systematic studies that have been reported.

Blanco *et al*. (2011) studied the effect of four acids commonly used as food additives: acetic acid, lactic acid, citric acid and monosodium phosphate (an inorganic salt that was expected to give similar acidic behaviour in gluten-free dough). Acetic acid increased loaf volume by 10 % at a low concentration of 0.2 %, which diminished as the acid concentration increased. The authors attributed this to the action of acetic acid against yeast activity in the dough.

Zhang *et al*. (2011) used mild acid treatment (0.0005-0.002 N) with hydrochloric acid to cause structural changes and therefore affect the rheological behaviour of commercial zein. The reported structural changes included reduction of ordered α-helix, β-sheet and β-turn contents likely due to glutamine deamidation. These conformational changes accounted for a decrease in zein viscosity and more specifically the viscoelastic property of the acidic zein doughs. The authors explained that surface hydrophobicity of zein, due to partial unfolding, would result in increased hydrophobic interactions with the solvent and less polymerisation of zein molecules. The reduced content of ordered structures in the acid-treated zein caused more liquid-like behaviour of the dough.

More research on mild acid treatment of zein doughs was conducted by Sly *et al.* (2014), with the aim of affecting the functional properties of the prolamins. Increasing the concentration of acetic acid and lactic acid, from 0.7 % to 5.4 %, increased zein dough extensibility and reduced the dough strength, whilst still maintaining cohesion. In agreement, King (2015) found that α-zein dough with 1.3 % acetic acid had a lower young's modulus than that of wheat gluten dough. A slight increase in α-helix proportion compared to zein mixed with water indicated that preparation of zein doughs above T_g with dilute organic acids improved dough properties

by reversing changes of α-helical conformations into β-sheets. It was hypothesised that deamidation of zein molecules was responsible for the increased dough structure uniformity.

2.2.4.2 Application of reducing agents/ reduction of disulphide bonds

Sorghum is noted for its lower protein digestibility compared to other cereals, which is further compounded upon cooking (Duodu *et al*., 2003). This is also indicative of lower protein availability that not only is a nutritional constraint but affects protein functionality in food systems. In fact, the sorghum prolamin proteins have been considered as being incapable of interaction to form structures that ultimately play a role in textures in foods (Hamaker and Bugusu, 2003). One of the main reasons was suggested to be the organizational structure of sorghum protein bodies, which encapsulate the kafirins (Hicks *et al*., 2001). However, Hamakar and Bugusu (2003), in their work, further concluded that if released from their confinement, kafirins have the potential to contribute viscoelastic properties in food systems, as has now been demonstrated by Elhassan *et al.* (2018).

Kafirin proteins are organized in such a way that the α-kafirins located in the core of the discrete spherical protein body, whilst the β- and γ-kafirins form an outer layer of protection around the periphery (Shull *et al*., 1992; Duodu *et al*., 2003). The relative crosslinking behaviour of each protein class is directly related to the number of cysteine residues per monomer; an indication of potential to form disulphide crosslinks. Beta-kafirins contain 10 cysteine residues (Belton *et al*., 2006) and can assist in formation of large polymers by acting as a bridge between oligomers of α-kafirin (26.6 kDa, 2 cysteine residues) and γ-kafirins (El Nour *et al*., 1998). The latter have monomers consisting appreciably more cysteine residues (15 residues) and are naturally present as polymers stabilised through disulphide bonds (Belton *et al*., 2006).

In trying to alter the digestibility and functionalize kafirins in sorghum flour, it is vital to cause a disturbance in the architecture of the PBs through disruption, by reduction, of disulphide bonds located at the periphery (Kumar *et al*., 2012). This is because disulphide cross-linkages formed act as barriers to block access to the more digestible α-kafirins (Hamaker *et al*., 1994). Furthermore, formation of polymeric structures exaggerates the already low protein digestibility, as suggested by Hamaker *et al.* (1987). These polymeric structures may be less susceptible to digestion compared to lower molecular weight protein units.

In vitro studies on the use reducing agents to improve sorghum proteins digestibility have been mainly focused on preventing the drastic lowering of protein digestibility after cooking due to formation of disulphide linkages (Hamaker *et al*., 1987; Oria *et al*., 1995). The mechanism behind the increase in digestibility with reducing agents is due to these compounds targeting disulphide linkages in both the kafirins and the protein matrix. Protein bodies are located between starch granules, embedded in a protein matrix made up of mainly glutelins held together by intermolecular disulphide linkages (Taylor *et al*., 1984). By cleaving the disulphide bonds, reducing agents are thus capable of possibly opening up this protein matrix, potentially making the protein bodies more accessible to be functionalized (Hamaker *et al*., 1987).

The reducing agents ascorbic acid, sodium meta-bisulphide, glutathione, L-cysteine are suitable for some food use (de Mesa-Stonestreet *et al*., 2010) and therefore could be exploited in inducing changes in protein digestibility and protein body structure.

2.3 Conclusions

Over the years, non-wheat cereal grains have been receiving much attention in the development of bread, with particular emphasis being on getting their doughs to mimic the viscoelastic dough obtained from wheat flour. There is sufficient research that highlights the possibility of modifying non-gluten proteins in order to improve their functionality in dough formation. The functionality of both BSG and sorghum can be improved by applying technologies aimed at enhancing dough viscoelastic properties and inducing physico-chemical modifications of the cereal components. The literature discussed on chemical modification of gluten-free dough systems shows that investigating kafirin functionalization by acidification is a likely route to get closer to improving its role in bread making. Coupling chemical treaments with physical dough treatment by sheeting holds further potential. With regard to BSG, the alterations imparted on the physical properties and flavour profile of the final product limits the quantities that can be incorporated. Emphasis therefore needs to be placed on converting BSG into a value-added ingredient. The documented benefits associated with particle size reduction and pre-fermentation technology of bran, and BSG in particular, make it a viable bio-process that could break the stereotype of poor quality characteristics of high-fibre baked products.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Hypothesis 1

Pre-conditioning/ pre-fermenting barley brewer's spent grain (BSG) flour using a 'sponge and dough' process, in combination with particle size reduction, will improve the crumb structure and texture of BSG-wheat composite bread and improve loaf volume compared to utilizing a 'straight dough' method of bread making. Particle size reduction through milling induces degradation of cell walls, thus increasing the surface area of particles and carbohydrate solubility (Niemi *et al*., 2012). It has been found that bread made with medium sized bran (415 μm) had higher specific volume than breads made with coarse (609 μm) and fine (278 μm) bran, indicating that an optimum bran particle size exists for the production of bran-rich bread (Zhang and Moore, 1999). Sourdough fermentation of bran improves the gluten network, and hence gas retention as well as possibly improving the solubility of cell wall components (Katina *et al*., 2006). The proteolytic activities during fermentation and acidification also modify the physical properties of gluten (Corsetti *et al.,* 1998). The increased surface area of fibre particles available for modification by the fermentation process will lead to increased dough medications and improved bread characteristics.

Hypothesis 2

Glacial acetic acid treatment of doughs made from high protein digestibility sorghum followed by addition of water and raising the dough temperature above 50 $^{\circ}$C will result in sorghum doughs of improved rheological properties, by freeing the kafirin proteins from the protein bodies so that they functionalise in the dough. In sorghum, kafirins are encapsulated in protein bodies in the endosperm (Belton *et al.,* 2006). Sorghum lines with high lysine and high protein digestibility traits have much higher flour water solubility, high pasting viscosity and form softer, less sticky pastes compared to normal sorghum (Elhassan *et al*., 2015). These mutant cultivars have an altered protein body shape with increased surface area, thus increasing accessibility of the kafirins (Oria *et al*., 2000). This would mean increased availability of the kafirins for modification by acid treatment.

The high temperature of 50 $^{\circ}$C keeps the kafirin above its glass transition temperature, an important parameter in dough rheology that explains the behaviour of proteins during mixing as a polymer changes state from glassy (brittle) to rubbery (viscoelastic) (Levine and Slade, 1989). Improved viscoelasticity in HDHL-wheat composite sorghum doughs was reported by

Goodall *et al.* (2012) when doughs were treated above the glass transition temperature (T_g) compared to normal sorghum-wheat composite dough. Viscoelastic masses have been formed from kafirin by dissolving it in glacial acetic acid followed by addition of water to precipitate out the protein as a viscoelastic mass (Elhassan *et al*., 2018). Dissolving kafirin in glacial acetic acid causes dissociation of the molecules and hence increased ordered α-helical conformation. Consequently, water binding and fibril formation is enhanced upon the coacervation process with water addition.

Hypothesis 3

Gluten-free breads prepared from sorghum flours with the aid of combined treatments of dough sheeting, flour pre-gelatinization and sourdough fermentation will result in improved loaf volume and crumb structure compared to sorghum control breads. Starch pre-gelatinization has been shown to mimic hydrocolloids when added to gluten-free batters. It improves dough handling properties by acting as a binder and allowing formation of a cohesive dough, a property that gluten-free flours lack (Sozer, 2009). Sheeting of maize dough in combination with pre-gelatinized starch has been found to improve dough cohesiveness, extensibility and strength (Khuzwayo, 2016). These improvements in rheological dough properties may lead to improved gas-holding properties and therefore the loaf volume and crumb porosity. Sourdough fermentation has been found beneficial in improving non-wheat dough and bread quality. Houben *et al.* (2010) used *L. plantarum* sourdough in the modification of amaranth dough rheological properties and found that sourdough fermentation was able to produce doughs with viscoelasticity similar to pure wheat flours. The effects were attributed to the metabolic activity (carbohydrate, peptide and lipid metabolism) of the starter culture. Fonio dough strength and stability as well as bread quality was also improved due to starch granule modifications and increased water absorption occurring as consequence of natural sourdough fermentation (Edema *et al.*, 2013).

3.2 Objectives

Objective 1

To determine the effects of particle size reduction in combination with pre-conditioning/pre-fermentation of BSG, on wheat composite dough and ultimately bread quality characteristics; i.e. loaf volume, crumb texture and appearance.

Objective 2

To determine the effects of subjecting transgenic high protein digestibility sorghum flours (with modified kafirin expression), to glacial acetic acid treatment followed by water addition on the sorghum dough rheolological properties.

Objective 3

To determine the effects of utilizing sheeting, flour pre-gelatinization, and sourdough fermentation in combination on the dough properties of sorghum flour.

4 RESEARCH

4.1 RESEARCH CHAPTER 1: FUNCTIONALIZATION OF BREWER'S SPENT GRAIN FOR INCORPORATION IN WHEAT BREAD

4.1.1 Abstract

There is a need to reduce wheat imports expenditure in African developing countries. Brewer's spent grain (BSG) - a major by-product of the brewing process, is available in very high quantities and is relatively inexpensive. The particle size of fibre materials such as bran and BSG has been shown to affect the quality characteristics of baked products from wheat. The use of sourdough fermentation has been successful in the improvement of loaf volume, crumb structure and texture of non-wheat and composite breads. Therefore, particle size reduction in combination with a sourdough process were applied to study the effects of modifications of BSG inclusion on its dough and ultimately bread making properties. Fractionation of dried BSG through roller milling enriched the protein of BSG flour, but seemed less economically viable due to lower extraction yields compared to hammer milling. Mixolab dough evaluation showed that a 15 % BSG inclusion with wheat flour significantly increased dough development time and flour water absorption; therefore, levels up to 20 % BSG were studied. Fermentation of BSG was carried out using a 'sponge and dough' method which pre- fermented all of the BSG in the formulation with a third of the wheat flour. A short (3 h) 'sponge and dough' process improved gas-holding properties of the composite doughs and gave higher loaf volume, more open and softer crumb as opposed to the straight dough method. This is probably primarily due to the more conditioned fibre component causing less mechanical disruption to the gluten network and dough expansion. At 20 % BSG inclusion, the composite wheat bread had 71.4 % more dietary fibre and substantially higher zinc and iron contents, among other minerals, when compared to commercial brown wheat bread.

4.1.2 Introduction

The rapidly increasing wheat consumption, adverse conditions for wheat cultivation and high importation prices in the developing countries of sub-Saharan Africa (SSA) pose a major economic problem (Mason *et al*., 2015). Whilst SSA wheat imports were at 23 metric tonnes (US $7.5 billion) in 2013, a 38 % growth was estimated within the next 10 years (Macauley, 2015). Food price increases are most detrimental to the poor populations (Wodon and Zaman, 2008), not only pushing them further below poverty lines but also compromising dietary quality and ultimately, child growth and development (Bibi *et al*., 2009; Meerman and Aphane, 2012). In order to reduce wheat importation and promote local grown underutilized crops, the use of composite flours has been encouraged in developing countries (Noorfarahzilah *et al*., 2014).

Barley brewer's spent grain (BSG), which represents 85% of total brewing by-products, is relatively inexpensive and available at large quantities irrespective of season (Mussatto *et al*., 2014). BSG represents a low cost cereal ingredient that has the potential to improve the nutritional value of bread by increasing both the protein and dietary fibre content (Ozturk *et al*., 2002), addressing some of the nutrition problems in those developing countries that have a high prevalence of malnutrition. However, achieving acceptable quality characteristics, such as loaf volume and shelf life, of high-fibre breads is a challenge. Inclusion of dietary fibre rich components weakens the gluten structure and overall baking quality of wheat dough, hence the decreased loaf volume and crumb elasticity (Katina, 2005). Therefore, the incorporation of BSG in bread formulations requires much effort in modification of its physicochemical properties through the use of various technologies.

Spent grain particle size reduction prior to incorporation in baked products has been widely practiced. For example, the particle size of BSG flour has been found to affect the quality of wheat biscuits (Guo *et al*., 2014), whereas bran particle size has been shown to affect loaf volume and texture (Zhang and Moore, 1999). Another well-known practice is sourdough fermentation in bread making. The use of bran sourdough has been found to compensate for the negative effects of added fibre on loaf volume and crumb texture. However, it has been suggested that improved quality using sourdough fermentation can only be obtained under its optimized conditions (Clarke, 2003). Although other studies have looked at spent grain inclusion in bread, there has been little published research concerning using various pre-

treatment technologies in combination, and the impact thereof on bread quality and nutritional properties. Therefore, this work will focus on examining pre-treatment technologies, i.e. particle size reduction in combination with sourdough fermentation, in the improvement of wheat-BSG composite dough with the aim of producing a low cost nutrient-rich bread from underutilized materials.

4.1.3 Materials and methods

4.1.3.1 Materials

Dried barley brewers spent grain (BSG) (7.7 g/100 g moisture as is basis; 21.1 % protein as is basis) was kindly provided by ABInBev (South Africa). The BSG was hammer milled with a Falling Hammer Mill 3100 (Falling Number, Huddinge, Sweden) to obtain a flour using a 500 μm screen.

BSG fractionation was achieved by using a double break roller Mill (Maximill, Kroonstad, South Africa). Four fractions were obtained from roller milling, namely: fine, medium-fine, medium-coarse and coarse. To obtain three final BSG fractions for analyses, the fine and medium-fine fractions were combined.

Particle size determination of the BSG fractions was done through sieve separation. Six sieves of different sizes were stacked on top of each other on a mechanical sieve shaker in ascending order (i.e. 180, 250, 500, 710 and 2000 μm screen opening size).

White wheat bread flour (14.1 g/100 g moisture as is basis) (Snowflake, Premier Foods, Isando, South Africa) was obtained from a local store.

4.1.3.2 Methods

BSG Sourdough Production

Pre-fermentation of BSG was performed as part of a 'sponge and dough' process of bread dough preparation, adapted and modified from a method developed by Artisans at Home (2012). 'Sponge' dough was prepared by mixing 132 g wheat flour (30 % w/w of total flour) with all of the BSG flour and yeast into a dough with 200 ml warm water (~ 50 °C). The 'sponge' was left to ferment for 3 h at 40 °C in a 'short sourdough' process until a pH of 4.5 was reached, or for 15 h in a 'long sourdough' process to reach a pH of 4.2.

Production of BSG-Wheat bread

BSG-wheat composite bread doughs were made using the 'straight dough' and the 'sponge and dough' methods. The fermented BSG (i.e. sponge) was prepared as described in *4.1.3.2* above, then gradually mixed using an electric mixer with other ingredients (as described below) to form a complete bread dough in the mixer. In the straight dough method, white

wheat bread flour (440 g as is basis), mixed with BSG flour where applicable, was measured into a mixing bowl. Other dry ingredients were added to the flour, i.e. instant dried yeast (4 % flour basis), premix (4 % flour basis), salt (2 % flour basis), sugar (4 % flour basis). The entire mixture was transferred into an artisan-type electric stand mixer with a dough hook attached. Once the mixer was powered on at a mixing speed of 2, warm water (70 % on an as is flour basis) at 50 oC was slowly added to the mixture. Once the dough had formed, after approximately 7 min mixing time, softened margarine (at ~ 25 oC) was added to the dough, which was thereafter mixed for another 2 min. The dough was placed on a table surface sprinkled with wheat bread flour and kneaded into a ball. The dough ball was placed in a greased stainless steel bowl and thereafter the bowl was inserted into a tightly sealed polyethylene bag. Proofing was done in an oven at 45 oC for 1 h until the dough had doubled in size. The dough was taken out and knocked back into a flat pancake, rolled into a cylinder shape and placed into a loaf tin (265 x 100 x 118 mm) with the crease at the bottom. The dough in the loaf tin was proofed once more for 1 h at 45 min. Baking was carried out at 200 oC for ~ 30 mins in a commercial rack oven. The bread was carefully removed from the loaf tin and allowed to cool on a cooling rack. The loaf height was measured, then the bread sliced and slices photographed.

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┌─────────────────────────┐
│  Mixing of white wheat bread │
│  flour, sugar, premix, salt and │
│  warm water (~ 50 °C).  │
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            │
            ▼
┌─────────────────────────┐        ┌─────────────────────────┐
│ Mixing in dough mixer (7 mins) │◄──│  Addition of fermented  │
└─────────────────────────┘        │  BSG 'sponge'           │
            │                       └─────────────────────────┘
            ▼
┌─────────────────────────┐
│ Addition of softened margarine │
│ (at ~ 25 °C)            │
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│ Mixing in dough mixer (2 mins) │
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│ Kneading of dough into a ball │
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│ Proofing at 45 °C for 1h │
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│ Knocking back of dough  │
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            ▼
┌─────────────────────────┐        BSG-wheat composite
│ Baking at 200 °C for ~30min │──►         bread
└─────────────────────────┘
```
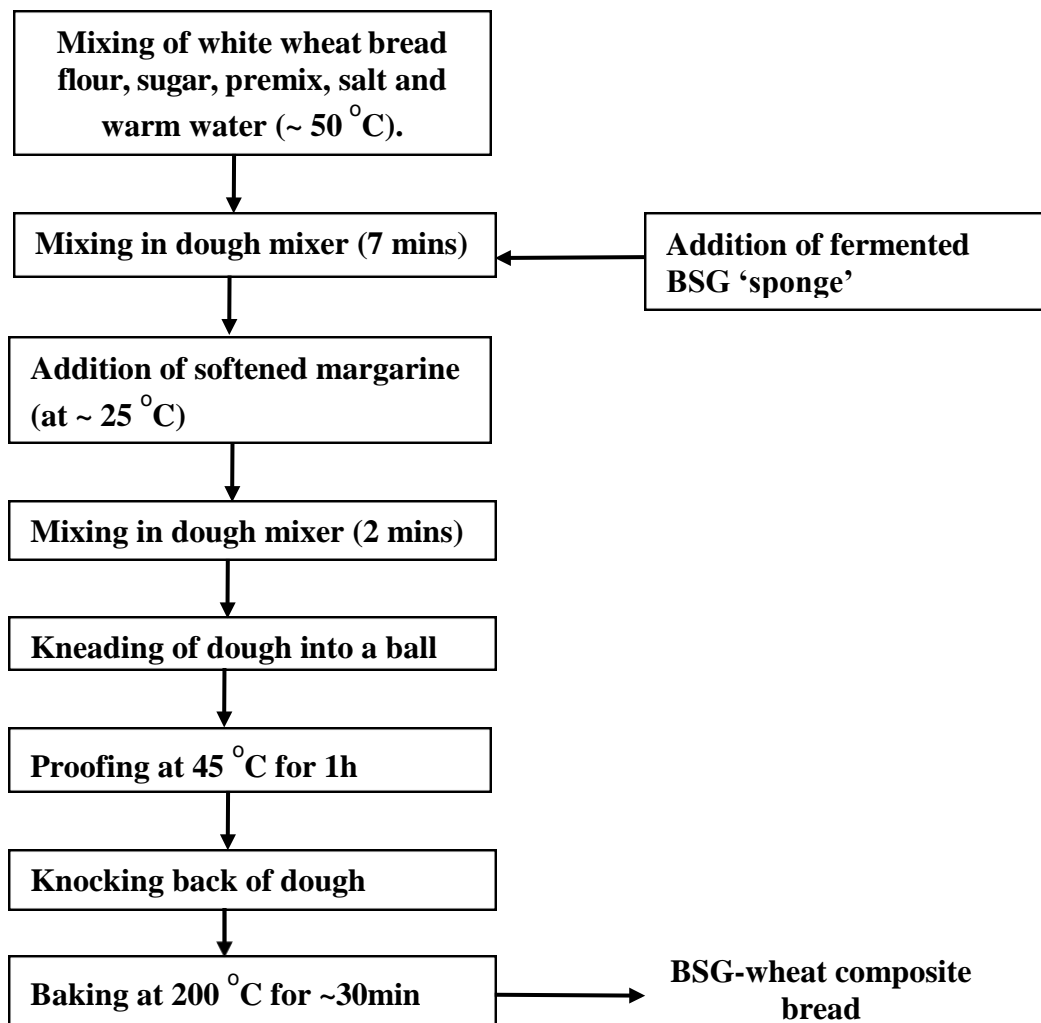
Figure 4.1.1: Procedure of making BSG-wheat bread composite bread using the 'sponge and dough' method adapted from the method of Artisans at Home (2012).

Proximate Analyses

Moisture and protein contents of the sorghum, BSG and wheat flours and breads were determined essentially according to the Approved Methods: 44-15A and 46-19, respectively, of the American Association of Cereal Chemists International (AACCI, 2000). Moisture content was determined by loss of weight of the samples after drying at 103 °C for 3 h. Crude protein was determined by a Dumas Combustion procedure (AACCI Approved Method: 44-15A). The nitrogen conversion factor used was 6.25, 5.7 and 5.38 for sorghum, wheat and barley products, respectively.

Wheat bread, BSG flour and BSG-wheat composite breads were also analysed for their mineral contents (Cu, Fe, K, Mg, Mn, P and Al). For the determination of minerals, approx. 1 g of each of the ground samples was digested with $HClO_4$ and HNO_3 Which lasted for 2 h. After cooling, the digested sample was transferred into a 250 ml flask and were make up with distilled water. The samples were then analysed by an atomic absorption spectrometry (model 210 VGP) (Buck Scientific, Norwalk, USA).

Dietary fibre and crude fat analysis were performed by the Southern Africa Grain Laboratory (SAGL), Pretoria, South Africa. Crude fat analysis was carried out using petroleum ether extraction and dietary fibre determined using 'In-House Method 012'.

Alveography

Alveography (Chopin NG Consistograph, Paris, France) was used to determine the rheological properties of dough according to AACCI approved method: 54-30A (AACCI, 2000) and in combination with the Alveograph NG Consistograph instructional manual (Chopin, 2010). Alveogram values: tenacity or resistance to extension (P, mm H_2O), extensibility (L, mm), deformation energy (W, J x 10^{-4}), and curve configuration ratio (P/L) of the dough were obtained.

Mixolab testing

Mixing and pasting behaviour of wheat flour and BSG composite doughs were studied using Mixolab Chopin$^+$ (Chopin, Tripette et Renaud, Paris, France), which measures the rheological properties of doughs by subjecting them to the stresses of mixing and temperature changes that occur during bread making. It measures the torque (in Nm) produced by the dough between two mixing blades, thus allowing the study of its rheological behaviour. For the test, the amount of flour and water needed was determined by the sample moisture and water absorption level, which was pre-determined using a simulation (Chopin S) under constant hydration. The settings used in the test were as detailed in the Mixolab Applications Handbook. The parameters obtained from the recorded graph provide information about the wheat protein stability when subjected to mechanical and thermal constraints, and both the gelatinization and gelling of starch (Huang *et al*., 2010). The parameters measured included:

initial maximum consistency (Nm) (C1), minimum torque (Nm) produced by dough passage subjected to mechanical and thermal constraints (C2), maximum torque produced during the heating stage (C3), minimum torque during the heating period (nm) (C4), and the torque (Nm) obtained after cooling at 50 °C (C5). The different curve slopes obtained were related to the flour different properties: speed of the protein network weakening due to heating (α); gelatinization rate (β) and cooking stability rate (γ).

Staling (measured using a texture analyser)

Bread loaves were stored in sealed clear plastic freezer bags, at ~28 °C for 3 days to mimic storage by the consumer. The firmness of the wheat and BSG-wheat composite sliced breads was evaluated daily according to the 74-10A compression test AACCI (1999). The measured firmness is an indication of freshness versus staling and is based on the theory that crumb peak force increases as the bread ages. Textural differences arising from difference in the formulations was also measured. For the tests, two bread slices of 12 mm thickness were placed on top of one another and positioned underneath a 25 mm diameter cylindrical probe, with the probe at the centre of the slices. The slices were compressed to a 3 mm distance and peak force was measured.

Crumb and Crust Colour

The colour of bread crumb and crust was quantified using a Minolta CR-400 colorimeter (Konica Minolta Sensing, Osaka, Japan), and results were presented in accordance with the Hunter Lab colour space. Parameters determined were L ($L = 0$ [black] and $L = 100$ [white]), a ($-a$ = greenness and $+a$ = redness), b ($-b$ = blueness and $+b$ = yellowness. All measurements done at least three times.

Stereomicroscopy

The microstructure of fresh broken bread crumbs was analyzed using a stereomicroscope (Zeiss Discovery V20, Jena, Germany) with a field of view of 3.5 mm, 1.8 µm resolution, and 64 µm depth of field.

Scanning Electron Microscopy (SEM)

Small pieced of crumb (~2 mm) were broken from the centre of fresh bread slices. These were thereafter frozen at -20 ^{o}C and then freeze-dried. Small pieces (< 0.5 mm) of freeze dried crumb were sectioned with a sharp razor blade and mounted on specimen stubs with double-sided carbon tape, the crumb sections were placed in such a way to ensure that the original surface of the crumb after freeze-drying was exposed for examination. The crumbs were sputter coated with carbon using an Emitech K950X carbon coater (Ashford, England) and viewed with a Zeiss 540 Crossbeam SEM (Zeiss, Oberkochen, Germany) operating at an accelerating voltage of 3 kV.

Statistical Analyses

All experiments were repeated at least twice. One-way analysis of variance (ANOVA) was performed. Means were compared at p = 0.05 using the Tukey Honestly Significant Test (HSD).

4.1.4 Results and discussion

4.1.4.1 BSG Protein, Moisture and Particle size

Due to the dried BSG being too granular, it was subjected to physical modification through particle size reduction by milling. Particle size analysis of the different milling fractions *(Table 4.1.1)* compared the efficiency of size reduction of BSG between roller milling (which yielded the three fractions: fine, medium and coarse) and hammer milling. The greatest degree of size reduction was achieved in the roller milling fine fraction, followed by the hammer-milled fraction. However, the low extraction yield of roller milling (i.e. 47.0 %) suggested it was a far less economically viable operation.

The moisture content of the whole unmilled BSG was significantly higher than that of the different milled fractions ($p < 0.05$) *(Table 4.1.1)*, except in the case of the coarse fraction, which had similar moisture content to the whole BSG. The moisture contents were in the range of 3.5-7.7 %, which is in agreement with BSG moisture content reported by Ktenioudaki *et al.* (2015). Hammer milled BSG had the lowest moisture content; after hammer milling, the flour was slightly warmer, and this can be implicated as causing moisture to evaporate. Because the larger and coarser fractions were mainly composed of husk material (*Figure 4.1.2*), the higher moisture content of these fractions (i.e. 5.8 and 6.1 %) can be attributed to the high water absorption capacity of the barley husk layers.

The protein contents of BSG fractions were inversely related to the degree of size reduction. The fine fraction had the highest protein content (28.4 %), whereas the coarse fraction had the lowest (11.0 %). Interestingly, whole unmilled BSG had 21.1 % protein, thus showing that particle size reduction by roller milling caused a fractionation effect on the different components found in the BSG. The finer fractions were enriched in protein, probably due to a greater content of aleurone cells, whereas the coarse fractions were mainly fibre-rich husks. However, considering that protein enrichment was only marginal, this method of particle size reduction did not represent an economically viable process due to the low extraction yield (47.0 %) as compared to hammer milling (100 %).

Table 4.1.1: Particle size distribution of hammer milled flour and roller milled flour fractions from dried barley malt spent grain

Milled Fraction	>2000 μm	<2000 μm - >710 μm	<710 μm - > 500 μm	<500 μm - >250 μm	<250 μm – >212 μm	<212 μm - >180 μm	<180 μm	Moisture (g/ 100 g)	Protein (g/ 100 g)
Whole BSG								$7.7^{e} \pm 0.0$	$21.1^{c} \pm 0.2$
Hammer Milled (100 % total BSG)	$0.0^{a} \pm 0.0^{1}$	$0.8^{a} \pm 0.1$	$1.3^{a} \pm 0.1$	$25.7^{b} \pm 3.8$	$33.2^{c} \pm 0.8$	$25.0^{c} \pm 2.0$	$14.1^{b} \pm 1.0$	$3.5^{a} \pm 0.0$	$22.8^{d} \pm 0.1$
Roller Milled									
Fine Fraction (47.0 % of total BSG flour)	$0.0^{a} \pm 0.0^{2}$	$0.6^{a} \pm 0.2$	$8.6^{b} \pm 0.6$	$56.0^{c} \pm 3.7$	$11.8^{b} \pm 0.9$	$7.1^{b} \pm 0.5$	$16.1^{b} \pm 3.0$	$5.0^{b} \pm 0.1$	$28.4^{e} \pm 0.1$
Medium Fraction (22.8 % of total BSG flour)	$0.0^{a} \pm 0.0$	$78.2^{b} \pm 0.5$	$13.1^{c} \pm 1.4$	$7.7^{a} \pm 0.4$	$0.6^{a} \pm 0.1$	$0.7^{a} \pm 0.1$	$0.1^{a} \pm 0.0$	$5.8^{c} \pm 0.0$	$19.2^{b} \pm 0.3$
Coarse Fraction (29.7 % of total BSG flour)	$3.7^{b} \pm 0.1$	$90.9^{c} \pm 0.3$	$2.1^{a} \pm 0.4$	$1.9^{a} \pm 0.2$	$0.5^{a} \pm 0.1$	$0.8^{a} \pm 0.1$	$0.1^{a} \pm 0.0$	$6.1^{e} \pm 0.0$	$11.0^{a} \pm 0.1$

[1] Particle size values presented as mean values of two milling trials (n=2) ± standard deviation; protein and moisture values presented as mean values of three repetitions (n= 3) ± standard deviation

[2] Values in the same column with different superscript letters ([abc]) differ significantly *(p<0.05)*

4.1.4.2 Effect of particle size reduction on the microstructure of BSG flour

The microstructure of the different BSG fractions after milling were compared with the unmilled BSG using stereomicroscopy *(Figure 4.1.2)*. Whole, unmilled BSG had a combination of both very small and very large (> 5 mm) particles. The barley husks had sharp edges and a rough appearance with remains of pericarp and aleurone material and possibly endosperm. This is in agreement with Forssell *et al*. (2008), who has described BSG structure as extremely heterogeneous and Ktenioudaki *et al*. (2012), who reported the presence of husks, fibre filaments and starchy endosperm remains. Together with empty aleurone cells, endosperm remains are present in BSG, depending of the evenness of malting (Mussatto *et al*., 2006).

The roller milled BSG produced four fractions that were separated based on particle size into three fractions; fine, medium and coarse. The coarse fraction constituted of mainly barley husks *(Figure 4.1.2),* which could not be successfully reduced further down to size. These husks had been scraped clean of most of their interior scraped off from most of their pericarp and endosperm remains. The medium fraction (~ 3 mm particle size), was essentially a combination of smaller and larger broken husks. The fine fraction was composed of flour with no visible husks nor pericarp remains. Hammer milling using a 500 µm opening screen produced a powdery BSG flour with the husk layers barely identifiable. On the contrary, broken husks were visible even in the finest roller milled fraction. This was probably an indication of incompatibility between the roller milling process and the BSG type of material.

Figure 4.1.2: Appearance of the different milled BSG fractions. Fine, medium and coarse fractions are products of roller milling process.

4.1.4.3 Composite wheat-BSG dough characteristics

Mixolab performance

The Mixolab parameters *(Table 4.1.2)* provide information concerning mechanical and thermal protein weakening and starch gelatinization (Marco and Rosell, 2008). Mixolab curves of white wheat bread flour and BSG flour obtained by hammer milling are shown in *Figure 4.1.3*. Flour water absorption of wheat flour blends increased with increasing BSG inclusion from 65.9 % (15 % BSG) to 67.9 % (20 % BSG), with both blends having significantly higher water absorption compared to the wheat flour alone (62.2 %) *(Figure 4.1.3)*. This confirms the findings of other studies which have shown the inclusion of fibre in the form of wheat bran (Xhabiri *et al*., 2013), barley β-glucan concentrate (Ahmed, 2015) and BSG (Stojceska and Ainsworth, 2008; Aprodu *et al*., 2016) to be directly related to flour water absorption. Dough development time (DDT) also increased greatly (p< 0.05), from 1.28 min (wheat control) to 8.19 min (15 % BSG).

As previously stated, BSG is essentially a lignocellulosic material with the main constituents being cellulose and non-cellulosic polysaccharides (mainly arabinoxylans), lignin and protein (Xiros and Christakopoulos, 2012) and some β-glucans (Gupta *et al*., 2010). Both soluble and insoluble fibres, particularly the β-glucans, have been implicated in tightly binding high amounts of water in dough, thus reducing the availability of water for development of the gluten network (Gill *et al*., 2002). The greater number of hydroxyl groups from the fibre probably enabled for more water interactions through hydrogen bonding (Rosell *et al*., 2001)

The maximum torque at C1, which is a measure of wheat dough stability, decreased slightly with increasing BSG inclusion. This showed that the inclusion of spent grain fibre had a weakening effect on the wheat dough. In contrast, Stojceska and Ainsworth (2008) found increased dough stability in BSG-wheat composite doughs at 10- 30 % BSG addition. Given that the BSG composition data was similar to that obtained in this study, the differences in dough behaviour could possibly be on account of differences in the physical properties of the dry milled BSG.

Both C3 and C4 increased with increasing BSG inclusion. C3 is an indication of starch gelatinization whilst C4 measures the amylase activity causing a reduction in viscosity due to physical breakdown of the starch granules. It was expected that gelatinisation would be impeded by the reduced starch content in the wheat-fibre blends (Collar *et al*., 2006) as well as the greater competition for water amongst the starch granules amidst the introduced fibre (Rosell *et al*., 2010). The magnitude of effects on dough behaviour during the high temperature stages depended on the BSG inclusion rate and possibly the nature of the added fibre.

Starch retrogradation (C5), like other Mixolab parameters, increased with the BSG level of inclusion. The high water absorption attribute of spent grain fibre in dough reduces water availability and consequently increases the rate of starch retrogradation (Stojceska and Ainsworth, 2008). From the physicochemical behaviour of the doughs measured by the Mixolab, it is clear that a substitution of more than 15 % of wheat flour with BSG weakens the dough and hampers viscoelastic behaviour. It seemed that increasing the BSG incorporation above the 20 % level could further deteriorate dough making quality. The question that arose was whether additional modification of BSG prior to incorporation as a bread ingredient would allow for BSG inclusion greater than 15 % by reducing the drastic effects thereof on final product quality. This was investigated through employing a sourdough fermentation process.
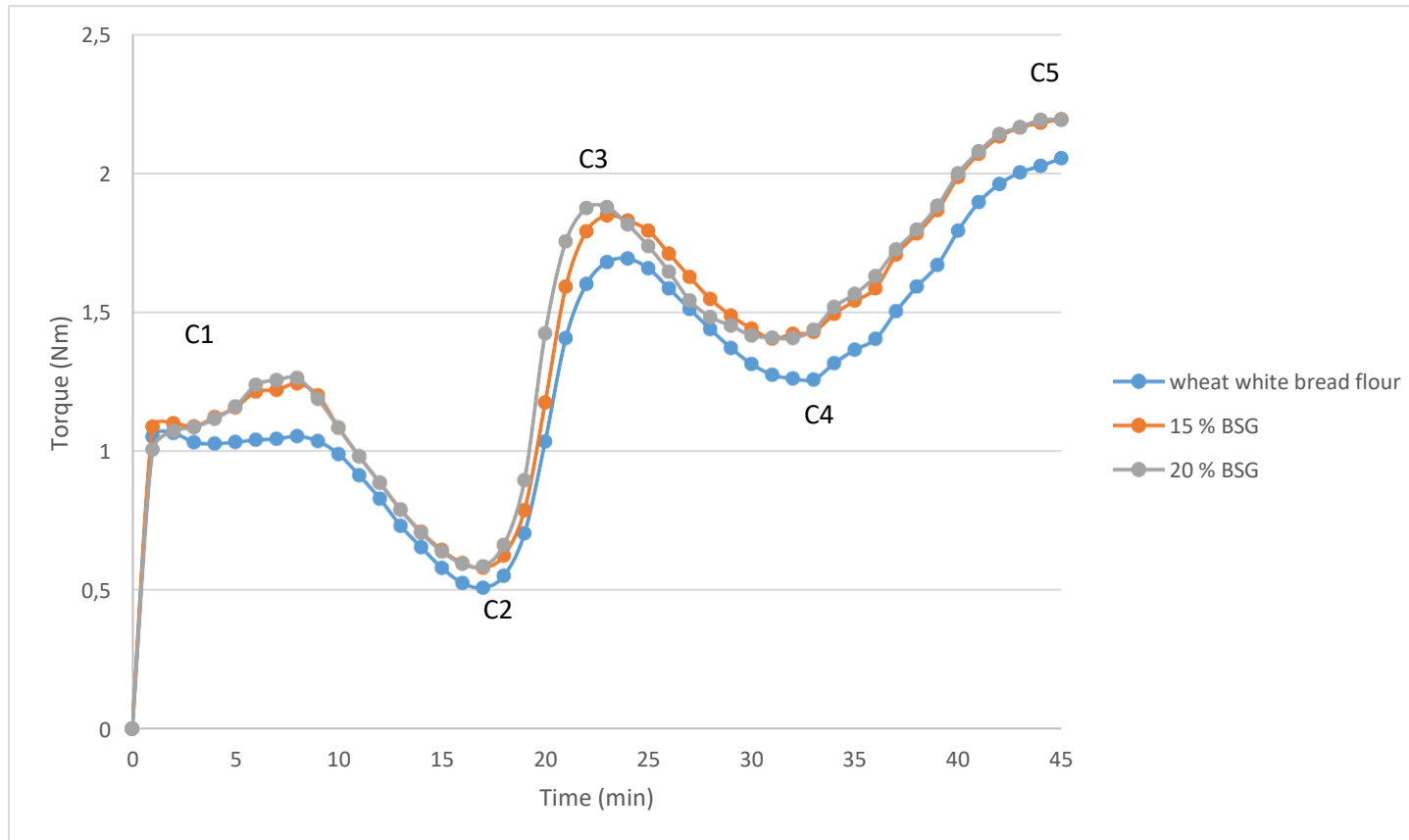
Figure 4.1.3: The effect of brewer's spent grain (BSG) inclusion on the Mixolab performance of wheat white bread flour

Table 4.1.2: Effect of BSG inclusion on the wheat-BSG on Mixolab dough mixing and thermo-mechanical parameters

Dough	Water Absorption	Development Time	Dough Stability at C1	C2	C3	C4	C5
	(%)	(min)	(min)	(Nm)	(Nm)	(Nm)	(Nm)
100 % Wheat	62.2[a] ±1.1	1.28[a] ±0.11	9.65[a] ±0.25	0.50[a] ±0.01	1.70[a] ±0.00	1.25[a] ±0.00	2.05[a] ±0.01
85 % wheat + 15% BSG	65.9[b] ±0.1	8.19[b] ±0.13	8.94[a] ±0.19	0.58[ab] ±0.03	1.85[b] ±0.02	1.40[b] ±0.03	2.19[a] ±0.05
80 % wheat + 20% BSG	67.9[b] ±0.1	7.54[b] ±0.48	7.89[a] ±0.87	0.59[b] ±0.02	1.90[b] ±0.01	1.45[b] ±0.07	2.45[a] ±0.37

[1] Values presented as mean values ± standard deviation of two experiments

[a] Numbers in columns with different superscript letters differ significantly ($p < 0.05$)

Alveograph characteristics

The alveograph characteristics of BSG-wheat composite doughs with and without pre-fermentation are shown in *Table 4.1.3* and *Figure 4.1.4*. As expected, 100 % wheat dough *(Figure 4.1.4 A)* was able to inflate and hold more air when inflated by alveography as compared to composite doughs of wheat and BSG flour. The gas-holding ability of wheat dough was due to the formation of a strong viscoelastic gluten network when wheat flour is hydrated (Gallagher *et al*., 2004). This was drastically impaired as BSG was included to the dough formulation due to its high insoluble fibre content.

The dough stability (P) of the composite doughs with 15 % (88.3) and 20 % BSG inclusion was significantly higher than that of 100 % white wheat bread flour (66.0). The extensibility (L) of wheat dough (38.0 mm) was reduced to 37.7 and 29.3 mm at 15 and 20 % BSG inclusion, respectively. It was thus clear that with inclusion of BSG, the viscoelastic behaviour of wheat dough was reduced. The energy required to inflate the dough (W) was increased with BSG inclusion compared to wheat dough on its own. Also, both the dough stability and deformation energy were reduced with increasing BSG inclusion rate from 15 to 20 %. Increasing the spent grain inclusion also reduced the dough inflation size (*Figure 4.1.4*), irrespective of the method of dough preparation (i.e. sponge and dough versus straight-dough method). This shows that the higher content of fibre material in the dough induced more disruptions to the gluten network and thus weakened the dough.

Doughs prepared with pre-fermentation of BSG still had higher stability and reduced extensibility (*Table 4.1.3*). However, the deformation energy of composite dough at 20 % BSG inclusion was higher with the pre-fermentation (102.0 J) compared to the straight dough (82.7 J), the former is more similar to that of wheat dough (99.3 J). The use of fermented BSG therefore counteracted the weakening effect of BSG inclusion on the dough viscoelastic properties by restoring some of the dough strength.

A major difference was observed between composite doughs with 15 % BSG inclusion, with the different methods of dough preparation (*Figure 4.1.4 B and D*). It appeared that the sponge and dough method (D) gave dough of improved extensibility and gas-holding ability. Preparation of a 'sponge' allows the BSG flour to pre-ferment and mimics a softening process of rough fibre particles. It was previously noted that sourdough fermentation of bran not only improves the solubility of cell wall components but also the gluten network, and hence gas retention (Katina *et al*., 2006). According to Corsetti *et al*., (1998), the improved protein network with

sourdough fermentation is possibly a result of proteolytic activity, which modifies the physical properties of gluten. The pre-conditioning step reduced the adverse effects of BSG inclusion on the gas-holding properties of the dough and was therefore expected to yield better bread quality compared to utilizing a conventional straight dough method.

Table 4.1.3: Effect of BSG inclusion and method of bread dough preparation- straight dough or 'sponge and dough' method, on the dough extensibility.

Type of Dough	Figure 4.3 codes	Stability	Extensibility	Curve configuration ratio	Deformation energy
		(P)	(L, mm)	(P/L)	(W, J x 10^{-4})
100 %Wheat	(A)	$66.0^{a} \pm 2.6^{1}$	$38.0^{c} \pm 1.7$	$1.76^{a} \pm 0.1$	$99.3^{ab} \pm 4.6$
15 % BSG	(B)	$88.3^{b} \pm 4.2$	$37.7^{c} \pm 0.6$	$2.34^{ab} \pm 0.1$	$127.0^{c} \pm 3.6$
20 % BSG	(C)	$86.3^{b} \pm 4.0$	$29.3^{b} \pm 1.2$	$2.75^{b} \pm 0.6$	$82.7^{a} \pm 5.1$
15 % BSG S&D	(D)	$156.3^{d} \pm 10.0$	$37.7^{c} \pm 1.5$	$4.15^{c} \pm 1.9$	$160.0^{d} \pm 11.3$
20 % BSG S&D	(E)	$125.7^{c} \pm 3.5$	$16.7^{a} \pm 0.6$	$7.55^{d} \pm 0.4$	$102.0^{b} \pm 5.3$

[1]Means ± Standard deviation (n=2)

[a]Values in columns with different superscript letters differ significantly (p<0.05)

S&D= sponge and dough method
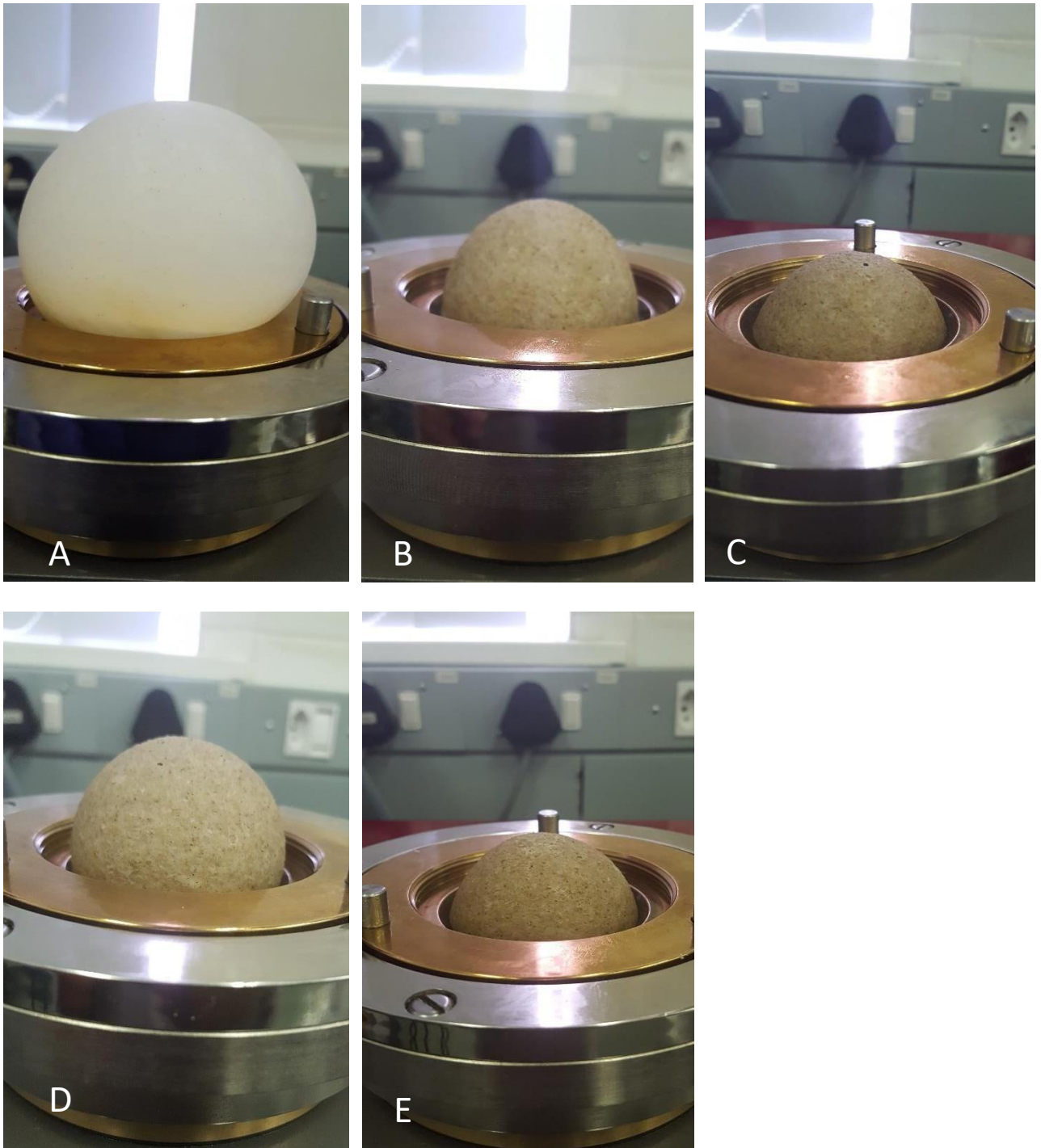
Figure 4.1.4: Alveography showing the effect of BSG inclusion and method of bread dough preparation, straight dough or 'sponge and dough' method, on dough gas-holding. **A.** Wheat. **B.** 15 % BSG and wheat composite. **C.** 20 % BSG and wheat composite. **D.** 15 % BSG and wheat composite with 'sponge and dough' method. **E.** 20 % BSG and wheat composite with 'sponge and dough' method.

4.1.4.4 Effects of particle size reduction on bread quality characteristics

Loaf height and weight

The type of milled BSG fraction used in the wheat flour blends affected both the loaf height and loaf weight *(Table 4.1.4)*. The loaf height was significantly lowered (p <0.05) by the inclusion of all the different spent grain fractions. The negative effect on loaf height was compounded with increasing BSG inclusion rates. Composite bread with 20 % of the low and high protein BSG fractions had similar loaf heights, i.e. 95.5 mm, which was nearly twice as high as that of bread with 40 % BSG levels.

A negative correlation between loaf volume and the amount of spent grain inclusion has been previously reported (Waters *et al*., 2012; Stojceska and Ainsworth, 2008). The decrease in volume of spent grain-containing breads is due to a gluten dilution effect and mechanical disruption of the gluten network formation by the addition on fibre (Sullivan *et al*., 2011). The presence of fibre in wheat dough reduces gluten hydration (Lai *et al*., 1998), interferes the viscoelastic network, ultimately yielding poor gas-holding dough (Gan *et al*., 1995). The gluten protein network in the dough plays a major role in retention of the carbon dioxide produced during fermentation and initial stages of baking (Goesaert *et al*., 2005).

The loaf weight, which gives an indication of the denseness or airiness of bread, was directly proportional to the level of BSG incorporation *(Table 4.1.4)*. Nonetheless, there were differences between the effect of the type of BSG fraction on the loaf density and height. This is probably due to the differences in protein and fibre composition as well as particle size distribution in the BSG fractions as previously seen in *Table 4.1.1*. The protein contents of the low and high protein BSG flours were 22.8 and 28.4 %, respectively. The high protein content in the BSG flour would be implicated in introducing more starch-protein and protein-water interactions that would impede viscoelastic network development and gelling properties, thus affecting the loaf density. According to Singh *et al*. (2011), the presence of proteins can impede absorption of water and swelling of starch granules. The high water-binding capacity of fibre may also result in increased water retention during baking, reducing the loaf volume and increasing loaf density (Sudha *et al*., 2007). Due to the less deleterious effects of the lower protein BSG, it can be concluded that the presence of barley protein in BSG flour plays a critical role in the loaf height and density and should not be overlooked.

Fractionation of different BSG components by sieving and milling has been employed before (Kissell *et al*., 1979). The authors found that as total lipids, residual starch and β-glucan became more concentrated in the finer BSG fractions and the occurrence of embryo and aleurone cell-protein layers increased. Barley β-glucan, when added to wheat flour during baking, was shown to bind to high amounts of water (Gill *et al*., 2002). This explains the poorer bread making performance of the high protein BSG fraction. Fractionation of BSG may have been a successful tool to enrich the protein of BSG flour, but it did not produce a flour with compositional properties that are suitable for compositing into wheat bread.

Table 4.1.4: Effect of inclusion of different milled BSG fractions in wheat bread on the loaf height and weight

Bread formulation	Loaf height (mm)	Loaf weight (g)
Wheat standard	$113.5^d \pm 2.1$	$646.3^a \pm 15.7$
20 % Low protein BSG	$95.5^c \pm 2.1$	$683.8^b \pm 6.7$
20 % High protein BSG	$95.5^c \pm 0.7$	$690.0^b \pm 4.4$
40 % Low protein BSG	$48.0^b \pm 1.4$	$714.8^c \pm 10.6$
40 % High protein BSG	$40.5^a \pm 2.1$	$716.8^c \pm 7.3$

[1] Values presented as mean values ± standard deviation of four repetitions (n=4)

[a] Numbers in columns with different superscript letters differ significantly (p<0.05)

Crumb structure and appearance

The visual quality of baked products makes up part of the physical attributes that consumers use in the perception of quality and acceptability. It is therefore crucial to evaluate the modifications of the composite breads imposed by the incorporation of BSG. The reduction in loaf volume and crumb porosity as a result of increasing BSG inclusion was observed in the cross-sectional views of these breads (*Figure 4.1.5)*. There was negative correlation between the addition of the high protein BSG fraction with crumb porosity; at 40 % spent grain level, these composite breads had a denser visual crumb appearance and gas cells that

were much smaller and fewer. It is clear that the fibre caused a reduction in the dough's ability to withstand pressure from carbon dioxide production within gas cells in the dough. The darker brown colour of the crumb at the highest level of inclusion was indicative of the appreciable moistness of the crumb even after baking. Spent grain fibre particles were visible in the crumb of all breads, and more so in 40 % High Protein BSG bread, which evidently gave a much coarser looking crumb. Some of the composite breads, i.e. 20 % low protein BSG and 40 % low protein BSG, had a 'flying top' (a top crust that is unattached from the crumb). This defect occurs when the top crust bursts open under the pressure of the expanding gas, instead of rising gradually with the bread crumb. The likely causes have been reported to be inadequately conditioned gluten or insufficient proofing in the pan (NIHM Kolkata, n.d). Since the proofing conditions were controlled, the former cause could be the cause.

| Wheat
Standard | 20 % Low
Protein
BSG | 20 % High
Protein
BSG | 40 % Low
Protein
BSG | 40 % High
Protein
BSG |
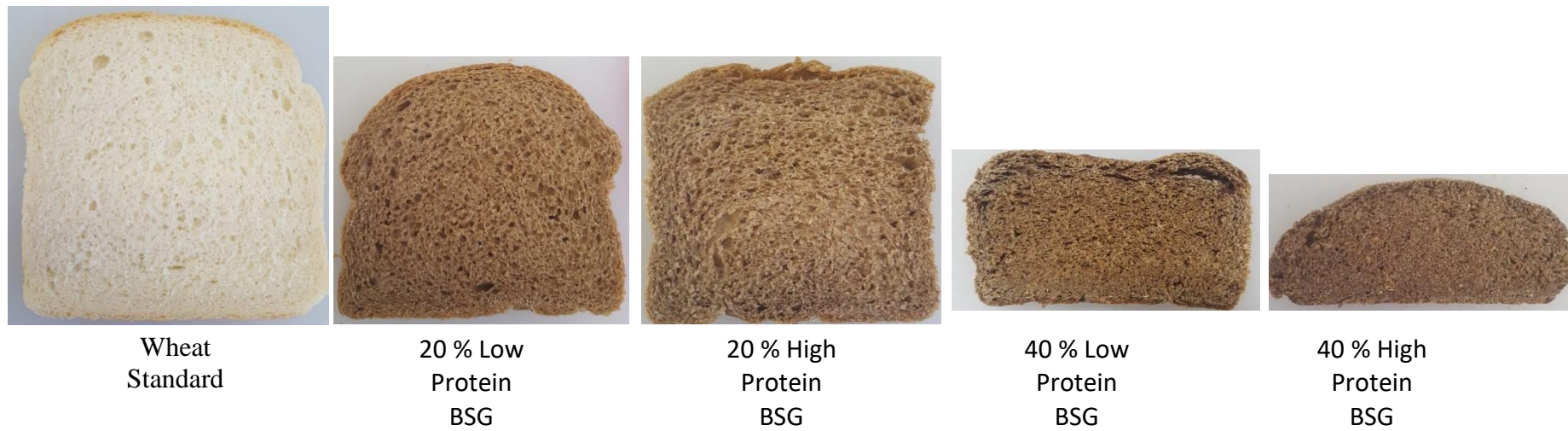
Figure 4.1.5: Effects of BSG inclusion and fractionation of BSG flour on the bread crumb visual quality

4.1.4.5 The effects of pre-fermenting BSG flour on bread quality characteristics

Loaf height and weight

As expected, even with the different dough preparation methods used, wheat substitution with BSG impacted the loaf height negatively (*Table 4.1.5*). Similar results were obtained by Waters *et al*. (2012) whereby increasing the inclusion levels of BSG and BSG sourdough into wheat dough resulted in lower loaf specific volume compared to wheat bread.

In this work, at the same inclusion levels, the sponge and dough method brought about a slight increase in loaf height (*Table 4.1.5*). This effect was only significant ($p < 0.05$) at 20 % spent grain addition. When BSG sourdough in wheat dough was added at levels higher than 10 %, a slight increase in the specific volume in the final breads compared to the composite breads with non-fermented BSG was observed (Waters *et al.,* 2012). Previous studies showed fermentation of wheat bran in high-fibre wheat bread development resulted in an increase in loaf volume (Katina *et al*., 2006).

Pre-fermentation of the BSG through the 'sponge and dough' method is likely to cause proteolytic modification of the protein component, subsequently improving the gluten network. Conditioning (softening) of the fibre components also occurred, as was noted by the softer textural feel of the fermented BSG 'sponge' (no data). These physicochemical changes on the BSG, prior to being added in bread dough, would impart a less deleterious impact on dough expansion and therefore, loaf volume. The sponge and dough procedure was also studied by Pagani *et al*. (2006), which gave improvements in loaf volume against a conventional straight dough procedure.

It was also crucial to determine the sourdough fermentation conditions for BSG and the effects thereof on final bread quality. This was done by varying the fermentation time. The 'Long sponge-dough' implies that pre-fermentation of BSG was carried out for a 15 h time period as compared to short fermentation of 3 h. Lengthening the fermentation time diminished the loaf height significantly ($p <0.05$). In a previous study, the volume/weight ratio of durum wheat breads made using a sourdough method was significantly lower compared to those made from a straight dough method (Rinaldi *et al*., 2015). This was attributed to the high level of sourdough addition (15 % v/w of dough) and the level of acidification. Intense acidification has been reported to decrease bread volume (Barber *et al*.,

1992; Katina *et al*., 2009). In this study, the pH of the dough was 4.5 after a 3 h fermentation (not tabulated) and dropped further to 4.2 after 15 h.

Table 4.1.5: Effects of dough preparation method on the loaf weight and height of BSG-Wheat composite bread

Bread	Loaf Weight (g)	Loaf Height (mm)
Wheat Standard	653.3[a] ± 5.4[1]	103.0[c] ± 4.2
15% BSG	694.9[b] ± 0.1	86.5[ab] ± 0.7
20% BSG	723.2[bc] ± 3.3	78.0[a] ± 1.4
15% BSG S&D	706.9[bc] ± 15.8	95.5[bc] ± 2.1
20% BSG S&D	732.3[bc] ± 0.2	94.5[bc] ± 0.7
20% BSG Long S&D	723.8[d] ± 7.0	82.5[a] ± 4.9

[1]Values presented as mean values ± standard deviation; (n=2)

[a] Numbers in columns with different superscript letters differ significantly ($p < 0.05$)

S&D= 'sponge and dough' method.

Crumb structure and appearance

In line with the improvements in loaf volume due to the sponge and dough method, the crumb structure and porosity of BSG composite breads was notably improved *(Figure 4.1.6)*. At all levels of BSG inclusion, the crumb structure of these breads was characterised by the presence large gas cells located at crumb centre supported by numerous smaller gas cells located at the periphery, similar to the wheat control crumb characteristics. The occurrence of larger and more heterogeneous gas cells is as a result of a more complex fermentation process that is associated with sourdough bread-making (Corsetti, 2013). However, the bread crumb porosity decreased with increasing BSG inclusion, as anticipated. Increasing the BSG inclusion from 15 to 20 % gave the loaves a flat top, which is a bread making defect that occurs due to poor gas retention and gas cells having collapsed or pierced during dough expansion (Hemdane *et al*., 2016). However, the use of sponge-dough method helped retain the rounded loaf top, which is a characteristic feature of wheat bread. It has been noted by Hammes and Gänzle (1998) that if baker's yeast is added with sourdough during dough preparation, the contribution of gas production by sourdough microflora is of little significance, as is the case in this study where 4 % (w/w total flour basis) instant dried yeast

was used. This supports the notion that it is gas retention and not gas production that is affected by sourdough addition (Clarke *et al.,* 2002), hence the more risen loaf top obtained with the 'sponge and dough' process.

| White wheat bread | 15 % BSG | 20 % BSG | 15 % BSG S&D | 20 % BSG S&D | 20 % BSG Long S&D |
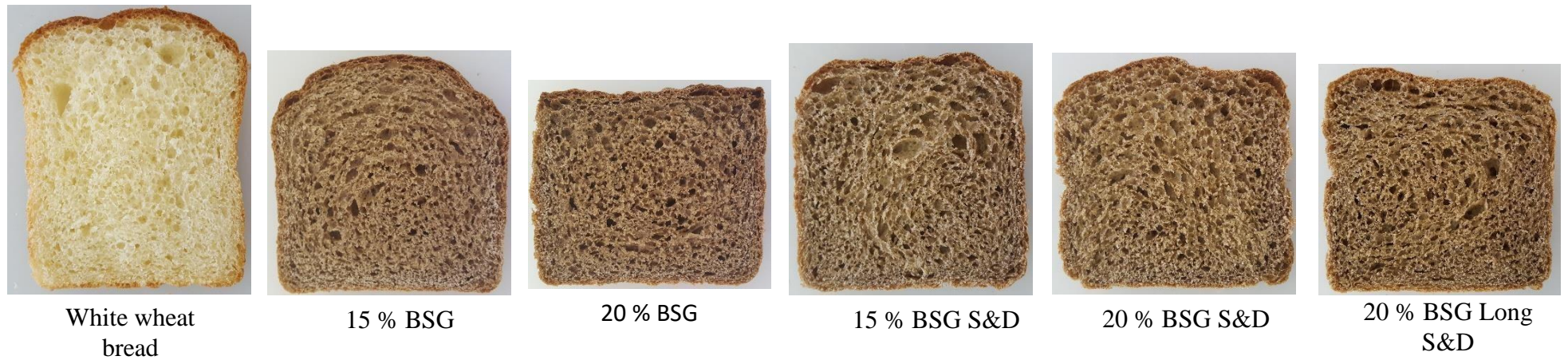
Figure 4.1.6: Effect of BSG inclusion and method of bread dough preparation on the bread crumb visual quality of wheat breads. S&D = sponge and dough method

Crumb microstructure by SEM and stereomicroscopy

From the microstructure of the different breads *(Figures 4.1.7 and 4.1.8)*, observations about the gas cells characteristics can be made. In the control wheat white bread, the microscopy confirmed the presence of both small and large gas cells in the crumb, as also seen in the cross-sectional images in *Figure 4.1.6*. The same observations were made in the wheat brown bread crumb, which served as a standard. However, the control (wheat white) bread had more distinct of gas cells. This is probably due to the fact that wheat brown bread is characterised by the presence of wheat bran in the formulation, which has been seen to restrict and force gas cells to expand in a particular dimension (Gan *et al*., 1992). Similar to wheat breads, sponge and dough breads had a more heterogeneous crumb morphology, characterised by large areas with very little aeration and other areas with large cavities.

Bread crumb of 20 % BSG bread (straight dough method) had less cavities in the crumb compared to the sponge and dough bread. The gas cells were not easily visible. This is highly due to the presence of large husk material from the BSG (indicated by white arrows) that caused mechanical disruption of the starch-gluten matrix.

Microscopy of the crumb with longer fermentation of BSG (i.e. 15 h), with visible barley husks *(Figure 4.1.7)*, showed that although pre-fermentation may have improved aeration, it did not alter the physical structure of the fibrous BSG material within the crumb. It can be proposed that the sponge-dough method conditioned but did not physically modify the integrity of the BSG material sufficiently to reduce its dire effects on the crumb aeration. Other authors have had to utilize the combination of sourdough and enzymes such xylanase to solubilise fibre for production of high-fibre breads of bran (Katina, 2005) and BSG (Ktenioudaki *et al.,* 2015). The enzyme activity that causes solubilisation and breakdown of cell wall components (Katina, 2005).

In this study, it can be concluded that the short sourdough fermentation process used in combination with fine particle size modified the BSG component sufficiently to reduce negative effects on dough aeration and crumb structure formation.
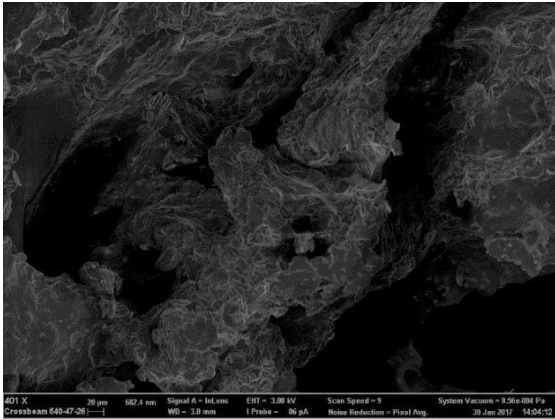
Wheat (white)
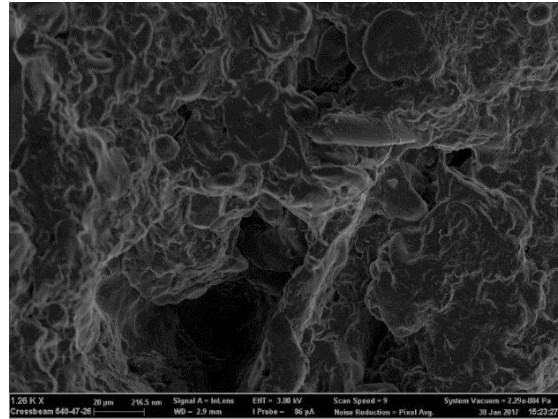
Wheat (Brown)
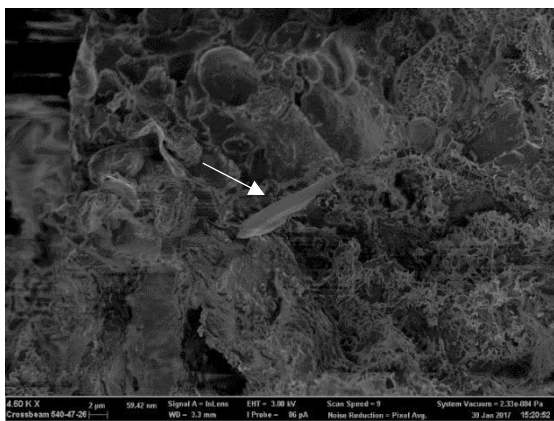
20 % BSG
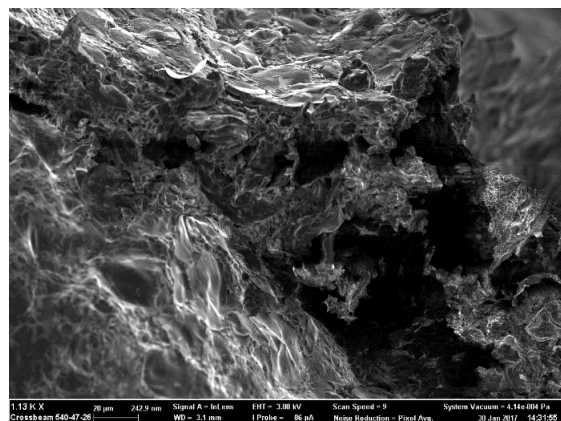
20 % BSG S&D
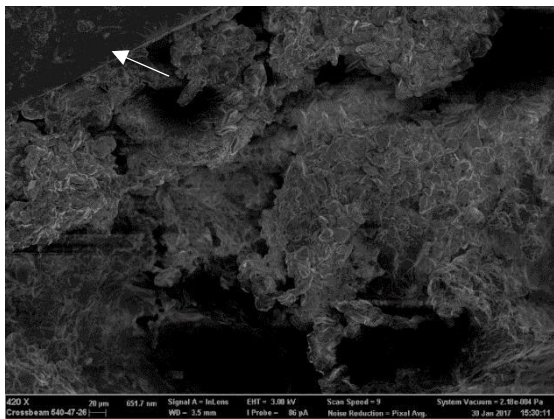
20 % BSG Long S&D

Figure 4.1.7: Stereomicroscopy showing the effects of BSG inclusion and the method of dough preparation on the bread crumb microstructure. White arrows show the fibrous BSG husk material within the crumb. S&D= 'Sponge and Dough' method.

White wheat	15 % BSG	20 % BSG
15 % BSG	20 % BSG S&D	20 % BSG Long S&D
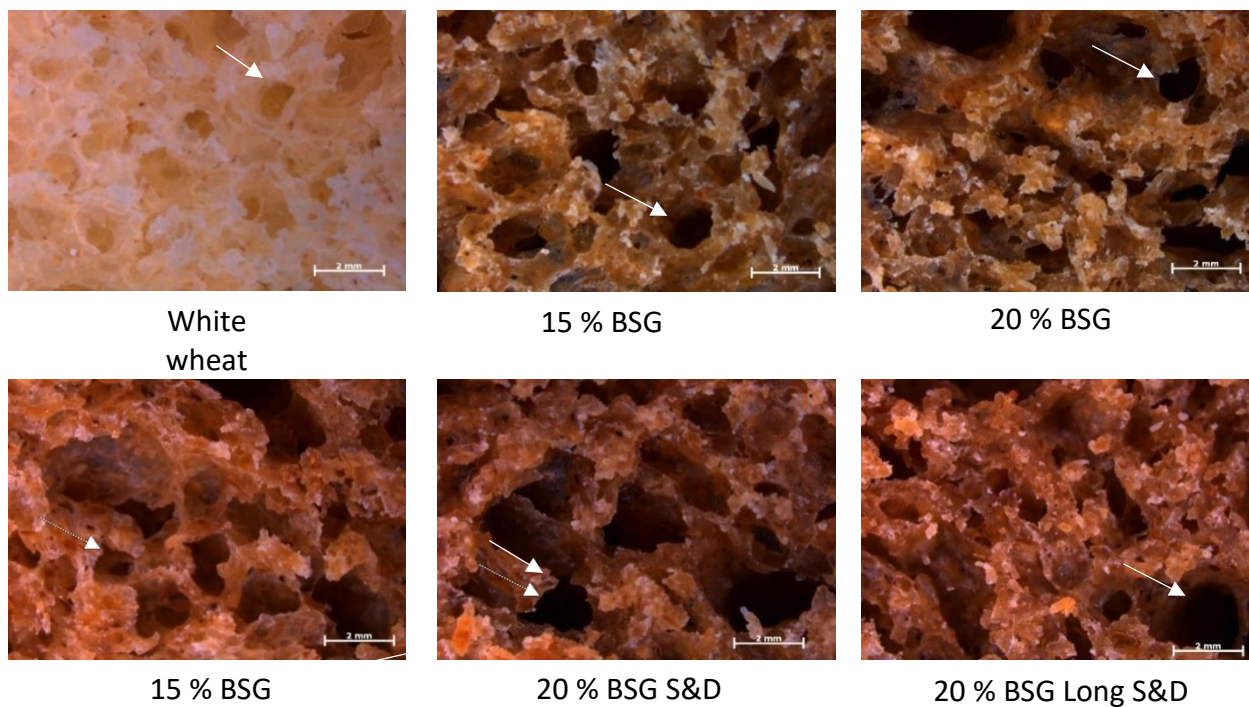
Figure 4.1.8: Scanning electron microscopy showing the effect of BSG inclusion and different methods of bread dough preparation on the crumb structure of wheat bread.

S&D= 'Sponge and Dough' method.

White arrows indicate gas cells.

4.1.4.6 Effect of BSG inclusion on the crust and crumb colour

Colour forms part of the visual quality and therefore plays a role in the consumer's perception of quality. The crumb colour of breads containing spent grain was significantly darker; the L values (an indication of the lightness of the colour) of the crumb decreased significantly ($p < 0.05$) with increasing spent grain incorporation from 15 to 20 % *(Table 4.1.6)*. The higher a* and lower b* values indicated that BSG composite breads were more red than green and more blue than yellow compared to the control wheat bread, respectively. Crust colour was not greatly affected by the spent grain addition, as the colour parameters did not follow a notable trend. This is likely as a result of the outer colour of loaves being more controlled by external factors such as temperature and caramelisation discoloration.

The characteristic dark colour on BSG-containing bread was as a result of the dark colour of spent dried grain material. Similar findings were obtained in the manufacture of spent grain containing breadsticks (Ktenioudaki *et al*., 2012) and in BSG enriched breads (Stojceska and Ainsworth 2008; Waters *et al*., 2012). BSG has a brownish colour when moist and is more suitable for incorporation in off-white products such as baked products prepared from wholemeal flour (Hassona *et al*., 1993; Miranda *et al*., 1994). Dark particles with roasted colours were also present in the dried BSG used in this study, probably because of the high temperatures of the drying process.

Interestingly, the crumb L* values of all composite breads increased slightly with utilization of the 'sponge and dough' method *(Table 4.1.6)*. A lightening trend of crust colour was reported to be due to the addition of fermented BSG. This effect is attributed to a reduction of polyphenols and fatty acids (Corsetti and Settanni, 2007) and exhaustion of free sugars in the dough by fermentative activity (Waters *et al*., 2012). Consequently, there would be less polymerisation of endogenous phenolic compounds as well as less enzymatic and Maillard associated browning seen in the final product.

Table 4.1.6: Effect of spent barley flour inclusion on the tristimulus colour values of the crust and crumb of composite wheat bread

Bread	Crust			Crumb		
	L	a	b	L	a	b
Wheat Standard	$48.9^a \pm 1.3^1$	$9.3^c \pm 0.5$	$4.8^{cd} \pm 0.8$	$67.5^c \pm 0.7$	$0.4^a \pm 0.1$	$10.1^d \pm 0.5$
15% BSG	$47.8^b \pm 1.3$	$8.1^b \pm 0.5$	$5.7^d \pm 1.1$	$52.0^b \pm 0.7$	$4.0^{bc} \pm 0.2$	$6.4^c \pm 0.4$
20% BSG	$44.6^a \pm 0.9$	$6.8^a \pm 0.4$	$2.6^{ab} \pm 0.8$	$48.9^a \pm 0.6$	$4.4^d \pm 0.1$	$5.2^b \pm 0.4$
15% BSG S&D	$47.6^b \pm 0.9$	$7.5^{ab} \pm 0.7$	$3.6^{bc} \pm 0.9$	$52.3^b \pm 0.9$	$3.9^b \pm 0.2$	$4.8^b \pm 0.6$
20% BSG S&D	$48.0^b \pm 0.5$	$6.4^a \pm 0.5$	$2.7^{ab} \pm 0.4$	$49.5^a \pm 0.8$	$4.4^{cd} \pm 0.2$	$3.4^a \pm 0.7$
20% BSG Long S&D	$44.6^a \pm 1.0$	$7.4^{ab} \pm 0.4$	$1.0^a \pm 0.6$	$50.3^a \pm 0.3$	$4.6^d \pm 0.1$	$4.3^{ab} \pm 0.5$

[1]Values presented as mean values ± standard deviation of four measurements taken from two loaves for each bread type

[a] Numbers in columns with different superscript letters differ significantly (p<0.05)

colour parameters measured using a colour meter, calibrated with a standard white tile (L=97.3, a= -1.2 , b=2.9)

L (100=white; 0=black), a (+ =red; - = green), b (+ = yellow; - = blue).

S&D= 'Sponge and dough' method.

4.1.4.7 Effect of BSG inclusion on bread textural and staling properties

All breads were stored for 3 days at ~28 °C to mimic typical day-to-day storage of bread by consumers. *Table 4.1.7* shows the changes in bread firmness of the breads at different spent grain inclusion rates as well as different dough preparation methods (i.e. straight dough versus sponge-dough). At day 1, the significant increase (p<0.05) in bread crumb firmness with increasing BSG inclusion was indicative of the negative effect on the initial bread texture of the final product. This finding is important as texture is a significant part of the sensory attributes used as a guideline of freshness and overall bread quality and is in agreement with other studies (Stojceska and Ainsworth, 2008; Ktenioudaki *et al.,* 2015).

The highest firmness on day 3 was observed for the 20 % BSG bread sample, with no sourdough (2.14 N). This is due to the arabinoxylan, glucan and xylooligosaccharides in the formulation, hampering with the gluten network, which is important in the crumb morphology and ultimately crumb texture (Katina, 2005). In this study, the best results for crumb softness was with the pre-fermented BSG composite breads, which had lower crumb firmness initially and throughout all the days of storage. Therefore, with the sponge-dough procedure, staling was notably retarded. The improvement in shelf life is probably due to amylolytic starch modifications which cause slower retrogradation (Waters *et al*., 2012). Sourdough has also been reported to positively affect moisture redistribution in the crumb, hence causing delayed crumb moisture loss over the storage period (Rinaldi *et al*., 2015).

Table 4.1.7: Effect of BSG flour inclusion and storage on the crumb firmness (N) of BSG-Wheat composite breads

Bread	Days of storage		
	1	**2**	**3**
Wheat white bread	$0.47^a \pm 0.06^1$	$0.57^a \pm 0.01$	$0.67^a \pm 0.03$
15% BSG	$0.72^{ab} \pm 0.06$	$1.13^{bc} \pm 0.18$	$1.79^d \pm 0.06$
20% BSG	$1.57^d \pm 0.21$	$1.92^d \pm 0.03$	$2.14^e \pm 0.06$
15% BSG S&D	$0.68^{ab} \pm 0.04$	$0.84^{ab} \pm 0.00$	$1.09^b \pm 0.14$
20% BSG S&D	$1.18^{cd} \pm 0.03$	$1.29^c \pm 0.11$	$1.46^c \pm 0.01$
20% BSG Long S&D	$0.88^{bc} \pm 0.03$	$1.06^{bc} \pm 0.05$	$1.38^{bc} \pm 0.11$

[1]Values presented as mean values force values (N) ± standard deviation of two readings for each bread; n=2

[a] Numbers in columns with different superscript letters differ significantly (p<0.05). S&D= 'Sponge and dough' method.

4.1.4.8 Effect of BSG inclusion on the bread nutritional properties

The nutrient composition of BSG flour and BSG-wheat composite breads versus standard wheat brown bread is shown in *Table 4.18*. BSG flour had a high dietary fibre content (51.4 % db), which is similar to that found by other researchers (Farcas *et al*., 2014; Stojceska and Ainsworth, 2008; Waters *et al*., 2012). The differences in dietary fibre content between the wheat brown bread and 20 % BSG bread were significant (p<0.05), with the latter containing of 71.4 % more dietary fibre. Protein content was also higher in BSG-containing bread compared to the wheat brown bread. The same trends were observed in the protein and dietary fibre of BSG-wheat composite bread-sticks (Ktenioudaki *et al*., 2012). The implications in human nutrition are attractive. Dietary fibre has been shown to help prevent cardiovascular diseases, irritable colon, colon cancer and constipation, among others (Rodriguez et al., 2006). Dietary fibre has prebiotic effects, as ingestion of BSG selectively stimulates the growth and or activity of bacteria in the colon, thus providing digestive and health benefits (Gibson and Roberfroid, 1995). With regard to protein, Waters *et al*. (2012) showed that essential amino acids make up 30 % of the total BSG protein, thus making it suitable for fortification of wheat based products.

The fat content of BSG bread in thus study was also within the range reported in literature *(Table 4.1.8)*, i.e. between 3.4 and 4.4 % (Stojceska and Ainsworth, 2008). According to Waters *et al.* (2014), wheat white flour contains some fat (1.81% w/w). BSG total fat content is substantially higher (i.e. 7.12 % w/w), with essential fatty acids accounting for over half of total BSG lipids.

The mineral composition of the breads showed that BSG incorporation increased the copper, iron, zinc, aluminium by appreciable levels *(Table 4.1.8)*. This is due to the high contents of these minerals present in the BSG flour, which is higher than those in wheat (Ktenioudaki *et al.,* 2015). Conversely, other minerals such as magnesium and phosphorus were present in lower quantities in the composite bread compared to the wheat bread.
Due to micronutrient deficiencies amongst children, food regulations in South Africa have made it mandatory for wheat flour to be fortified with vitamins and minerals; zinc and iron being paramount (Department of Health, 2003). At 20 % wheat replacement with BSG, the iron and zinc contents were 65.7 and 29.4 mg/kg, respectively. These values are well above the fortification standards provided in legislation; i.e. 34.69 mg/kg iron and 20.07 mg/kg zinc.

Table 4.1.8: Nutrient composition of BSG flour, wheat and BSG-wheat composite breads (g or mg/kg; dry basis)

Nutrient	Brown wheat bread	BSG flour	20 % BSG bread
Total Dietary Fibre (g/100g)	9.1 *	51.4*	15.6*
Crude Fat (g/100g)	1.0*	8.7*	3.9*
Protein (g/100g)	$16.0^a \pm 0.0$	$23.6^c \pm 0.1$	$17.4^b \pm 0.1$
Copper (Cu) (mg/1000g)	$0.8^a \pm 0.0$	$7.8^c \pm 0.6$	$1.9^b \pm 0.2$
Iron (Fe) (mg/1000g)	$35.3^a \pm 1.5$	$234.2^b \pm 41.0$	$65.7^a \pm 3.2$
Potassium (K) (mg/1000g)	$2804.0^c \pm 70.1$	$274.2^a \pm 2.2$	$1296.2^b \pm 57.9$
Magnesium (Mg) (mg/1000g)	$869.2^b \pm 18.2$	$2005.5^c \pm 82.5$	$606.5^a \pm 17.3$
Phosphorus (P) (mg/1000g)	$2333.9^b \pm 18.1$	$4702.2^c \pm 43.7$	$1979.9^a \pm 117.7$
Manganese (Mn) (mg/1000g)	$10.2^b \pm 0.0$	$35.1^c \pm 1.4$	$6.5^a \pm 0.5$
Aluminium (Al) (mg/1000g)	$11.4^a \pm 1.0$	$632.5^b \pm 78.6$	$25.3^a \pm 3.2$
Zinc (Zn) (mg/1000g)	$15.1^a \pm 4.9$	$91.2^b \pm 7.1$	$29.4^a \pm 7.7$

Values presented as mean values of 2 replications (n=2) ±standard deviation

[abc] Values in the same column with different superscript letters differ significantly ($p < 0.05$)

* Analysed by the Southern African Grain Laboratory (SAGL).

4.1.5 Conclusions

A short sourdough fermentation of BSG through a 'sponge and dough' method in combination with particle size reduction produces bread of greater volume, reduced crumb hardness, improved crumb structure and nutritional properties. It appears that physicochemical modification of BSG prior to its use as a functional ingredient is the key to good quality, high fibre wheat-BSG composite breads. The technologies used in this study are common and easily accessible and with their aid, BSG inclusion rate in wheat bread can successfully be pushed to 20 % (flour basis) without drastically affecting quality characteristics. This is higher than what is suggested in literature (i.e. 10 % and less). Investing in the production of good quality bread from BSG represents one of the viable solutions needed for replacement of high wheat importation and combatting mineral deficiencies in African countries.

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4.2 RESEARCH CHAPTER 2: CHEMICAL AND PHYSICAL TREATMENTS OF DOUGHS OF NORMAL AND HIGH PROTEIN DIGESTIBILITY SORGHUM FLOURS WITH MODIFIED KAFIRIN EXPRESSION FOR IMPROVED DOUGH FUNCTIONALITY

4.2.1 Abstract

Sorghum is a popular grain-crop in Africa, where it is well suited for cultivation and could potentially replace wheat in breads, which is largely imported. However, sorghum prolamin proteins (kafirins) are naturally confined in rigid structures called protein bodies and lack the desirable visco-elastic, gas-holding properties of wheat gluten. Hence, the gluten-free breads made from sorghum exhibit poor quality. Also, sorghum possesses poor protein digestibility and inadequate amounts of the essential amino acids, lysine and tryptophan. Biofortified transgenic sorghum lines with high lysine and high protein digestibility (TG-HD) have improved dough functionality.

The functionality of sorghum doughs from these lines was investigated with the aim of improving kafirin functionality in the dough system. This was done by employing sourdough fermentation, manual dough sheeting and chemical treatment with glacial acetic acid. Upon baking with normal sorghum flour, the combination of sheeting (15 passes) and sourdough inclusion (50 % w/w of total flour) produced bread with a more aerated crumb and greater volume compared to the untreated control. TG-HD lines and their null controls were treated with glacial acetic acid, followed by water addition. The TG-HD sorghum doughs had 38 and 42 % higher extensibility compared to their null controls. This was attributed to the greater accessibility of α-kafirins in the invaginated protein bodies of HD sorghum. Elevated temperature (>50 ºC), glacial acetic acid treatment and combination thereof, reduced dough extensibility. Transmission electron microscopy of treated doughs revealed disrupted protein bodies by treatment with glacial acetic acid and in combination with sheeting. Scanning electron microscopy showed that the starch granules were intact, suggesting the changes in dough properties were due to protein modification. This work showed that TG-HD sorghums have better dough tensile properties compared to normal sorghums, and potentially have better bread making properties. However, beyond releasing kafirins from their confinement, mobilising the inert kafirins as functional components for a viscoelastic dough development is complex.

4.2.2 Introduction

Sorghum is a popular grain-crop in Africa, particularly in West Africa, where 25% of the world's sorghum is produced (ICRISAT, n.d). Its reliability is due to ability to grow in hot, arid environments. Therefore, it has the potential to replace wheat in breads, thus reducing high costs due to wheat importation. However, the sorghum prolamin proteins, kafirins, are encapsulated in endosperm protein bodies (Taylor *et al*., 1984) and lack the desirable viscoelastic gas-holding properties of wheat proteins (Goodall *et al*., 2012). Hence, wheat-free breads made from sorghum have poor quality (Schober *et al*., 2007). The doughs have much lower elasticity and cohesiveness, resulting in lower loaf volume, poor texture and crumb characteristics (Houben *et al*., 2012).

Adding to these problems is sorghum's poor protein quality in terms of lack of the essential amino acid lysine and poor digestibility (Henley *et al.,* 2010). A possible solution could be utilizing protein-biofortified sorghum varieties, which have high protein digestibility and high lysine (HDHL) as vehicles for protein-energy malnutrition alleviation and delivery of bread of better quality. Transgenic sorghums have been developed by the Africa Biofortified Sorghum project (Biosorghum, 2010) by using RNA interference technology for suppression of the synthesis of specific kafirin subclasses. There is evidence from Elhassan *et al*. (2017) of possible improved dough functionality of HDHL sorghum lines. Not only do these biofortified sorghums hold promise towards producing good quality breads, they also have over 60 % higher lysine content (Henley *et al*., 2010) than normal sorghums.

Cohesive maize doughs of improved extensibility can be obtained through sheeting. However, starch pre-gelatinization is a prerequisite (Khuzwayo, 2016). Pre-gelatinized starch can act as a binder in gluten-free systems (Sozer, 2009). Sheeting involves passing dough through a set of rolls (Patel and Chakrabarti-Bell, 2013) and applies shear forces to the dough, which could potentially free kafirins from the protein bodies (Hamaker and Bugusu, 2003). Glacial acetic acid as an effective solvent, followed by addition of water to precipitate the kafirins out of solution, was used for the first time in kafirin microparticle preparation (Taylor, 2008; Taylor *et al.*, 2009).

Therefore, this chapter aimed to investigate physical and chemical treatments of protein biofortied transgenic sorghums and their null controls to release kafirins from their protein body confinements to improve their participation in sorghum flour dough functionality.

4.2.3 Materials and Methods

4.2.3.1 Materials

Decorticated (90 % decortication rate), finely milled red sorghum meal (fine Mabele) (Nola, Randfontein, South Africa) was reduced to size to obtain 250 μm flour particle size by using a 2-stage process. First, size reduction was done using a 500 μm screen in a hammer Laboratory Mill 3100 (Falling Number, Huddinge, Sweden), then the flour was further separated through a 250 μm mesh sieve to obtain the desired particle size.

Crushed whole grains of two non-tannin, white tan-plant, transgenic high protein digestibility (TG-HD) sorghum lines (TG-HD-1 and TG-HD-2) and their normal protein digestibility controls (NC1 and NC2) were obtained from DuPont Pioneer, Johnston, Iowa, produced in an approved controlled field trial. The TG-HD sorghum lines were produced through recombinant DNA technology (genetic engineering) and had suppressed expression of α-A, B1, B2 and γ-kafirin 1 and 2 kafirin subclasses technology, as described by Da Silva *et al.* (2011). To obtain a flour, the grains were milled using a hammer Laboratory Mill 3100 (Falling Number, Huddinge, Sweden) fitted with a 500 μm screen.

Isolated protein bodies from conventionally bred (non-transgenic), high protein digestibility sorghum (P850029) and normal protein digestibility sorghum NK283 were obtained in dry form (freeze-dried and milled), as described in work by Duodu (2000).

4.2.3.2 Methods

Sorghum dough preparation

Sorghum flour (100 g) was mixed with water (80 ml) at ~ 25 °C and formed into a dough. The dough was prepared with and without pre-gelatinized flour. In the latter case, a third of the flour (33.3 g) was pre-gelatinized using the custard method; by heating a slurry of the flour with all the water (60 ml) in a microwave oven at 800 W for 2 min. The mixture was allowed to cool to ~ 25 °C before mixing with the rest of the flour (66.7 g). Water lost during pre-gelatinization was determined and added back into the pre-gelatinized flour.

Sheeting of dough

Sorghum dough sheeting was done according to the method of Khuzwayo (2016) for maize dough. A pasta dough sheeter (Ibili Menaje, Bergara, Spain) with approximately 3 mm gap was used. A piece of sorghum dough was passed through the sheeter while turning the handle in a clockwise direction (away from the operator), folding it to double the thickness, turning the sheet by 90° after each sheeting pass. This operation was repeated to a total number of 15 passes. Sheeting was performed at ambient temperature (22 °C).

Sorghum sourdough preparation

Traditional sourdough was prepared by mixing finely milled sorghum flour (100 g) with distilled water (110 g). The mixture was mixed to a thick paste, covered and left to spontaneously ferment at 30 °C for 72 h until the sourdough reached a pH between 3.3 and 3.7. A portion (10 g) of the fermented sorghum flour was then used as a starter (backslopping) for a fresh mixture of sorghum flour and water. The mixture was fermented at 30 °C to a pH of 3.4-3.7 (approx. 48 h). This sourdough was then used for dough preparation.

Glacial acetic acid dough preparation with sorghum flours

Transgenic high protein digestibility sorghum flours with modified kafirin expression and normal protein digestibility sorghum flours were treated with glacial acetic acid followed by treatment with water, as described by Taylor (2008). Glacial acetic acid (16 ml) was pippeted into a 100 ml glass beaker containing a magnetic stirrer bar. Sorghum flour (20 g) was added to the beaker with constant stirring. The mixture was heated slowly with continuous stirring to ~ 50 °C (within 5 min) in a closed fume hood. The beaker was covered with parafilm during heating. The magnetic stirrer bar was removed and the mixture was left covered overnight. The dough mixture was then removed from the glass beaker, placed on a stainless steel tray and formed into a dough ball. A small well was created at the center of the dough and distilled water at ~ 25 °C was pippeted (2- 12 ml) into the well and the dough was reformed/kneaded as the water was being taken up. The amount of water added was recorded.

Glacial acetic acid dough preparation with isolated protein bodies

Protein body doughs were prepared as described for kafirin microparticle preparation by Taylor (2008), Taylor *et al.* (2009) and kafirin dough preparation with glacial acetic acid by Elhassan (2016). Glacial acetic acid (5 ml) was pippeted into in a glass beaker containing 1 g of isolated protein body flour (freeze-dried and milled). The mixture was covered with parafilm and continuously stirred with a magnetic stirrer whilst heating to ~ 50 $^{\circ}$C in a closed fume hood. The magnetic stirrer bar was removed and the mixture was left to air-dry on a stainless steel tray overnight.

Production of sorghum bread

This was performed as described by Khuzwayo (2016), with some modifications. The additional ingredients to sorghum flour were per 100 g flour: sugar (6 g), instant dried yeast (4 g) and water (80 ml) at ~ 25 °C. Pre-gelatinized flour (prepared as previously described above) was added together with the rest of the ingredients, including the sourdough (80% w/w of flour), where applicable. The dough was formed into a ball and sheeted for up to 15 sheeting passes, 74 mm length, 55 mm width, 66 mm depth) coated with a baking pan release spray. Proofing in a water bath was done at 40 °C for 45 minutes. Baking was at 200 °C for ~ 25 minutes. The loaves were carefully removed from the bread tin and allowed to cool on a cooling rack. Loaf height was determined. Cut bread slices were photographed.

4.2.3.2 Analyses

Tensile Properties (Kieffer rig)

The tensile properties of sorghum doughs from normal and transgenic sorghum flours were evaluated using a Kieffer rig mounted on an EZ Test (Shimadzu, Tokyo, Japan) texture analyser. The prepared sorghum dough was flattened out evenly using a ruler to cut out uniform dough strips (70 mm length, 20 mm width, 5 mm thick). The dough strips were placed over the vertical struts (30 mm apart) of the Kieffer rig and firmly held at both ends with the operator's thumb and index finger. The hook that was centred between the two vertical struts extended the doughs at a constant rate of 1 mm/s over a distance of 150 mm (maximum displacement of the texture analyser). The test was performed at either ambient temperature (23-25 $^{\circ}$C) or above the T_g of kafirin (>50 $^{\circ}$C), within ±5 min to prevent the doughs from

drying out and cooling below the T_g of kafirin. The temperature of 50 ºC was maintained by placing dough strips in zip-lock bags inside a sealed Styrofoam cooler with beakers of warm water placed inside. A force x distance graph was obtained with corresponding values for the peak force (N) and extensibility until rupture (mm). At least two independent experiments were conducted per dough treatment, with two repetitions in each.

Derivation of extensibility and rheological parameters

Extensibility parameters were calculated using formulae from Abang Zaidel *et al.* (2008). *Equation 1* was used to calculate the length of the dough (l_0) in its original position (y_0) before being extended. The distance (d) between the two vertical struts on either side of the hook was 30 mm. *Equation 2* used the hook displacement (yt) (extensibility until rupture) to calculate the final length of the doughs at break (lt). *Equation 3* was used to determine the actual force (Fa). This equation took into consideration the measured force (Fm) which was assumed to be divided equally over both sides of the hook (Figure 5.1). *Equation 4* shows the expression of the angle of deformation (α) in terms of the measured force (Fm) and actual force (Fa) since multiple forces were acting on the doughs it caused the actual force (Fa) to act at an angle.

Equation 1: lo= 2√[(d/2)2+ (yo)2]

Equation 2: lt = 2√[(d/2)2+ (yt)2]

Equation 3: Fa =Fm.lt/4(yt+ yo)

Equation 4: sin α = (Fm/2)/Fa = (yt+ yo)/ (lt/2)

The calculated extensibility parameters were used to determine the rheological properties of the doughs. Hencky strain (εH) was calculated using *Equation 5*. *Equation 6* gave strain rate which was derived by dividing Hencky strain by time. The hook speed (v) was 3.3 mm/s. As is shown in *Equation* 7, the final cross-sectional area (At) of the dough was 7.065 mm^2, assuming it to be the same as the original cross-sectional area of dough (A0). The stress (δ) that acted on the dough was calculated by dividing the actual force (Fa) by the final cross-sectional area of the dough cylinder (At), as in *Equation 8*. *Equation 9* was then used to calculate the extensional viscosity (ηE) of the dough by dividing the strain rate with stress (δ).

Equation 5: εH = ln (lt/lo) = [2√[(d/2)2+ (yt)2]]/ [2√[(d/2)2+ (yo)2]]

Equation 6: $\dot{ε}$ = 4v(yt+ yo)/ lt2

Equation 7: At = Ao

Equation 8: σ = Fa/At

Equation 9: ηE = $\acute{\varepsilon}$ / σ

The following parameters were calculated by using the above formulae: The true/ peak force (N), extensibility (mm), peak stress (kPa), extensional viscosity (kPa.s), strain at maximum hook displacement (%), Hencky strain (εH), Young's modulus (kPa) and extensional viscosity (ηE).

y_0= dough original position (mm)
y_t = final hook displacement (mm)
α = angle of deformation (º)
d = distance between the two struts)(mm
F_m = measured force (N)
F_a = actual force (N)
l_o = original length (mm) of dough
l_t = final length at fracture (mm) of dough
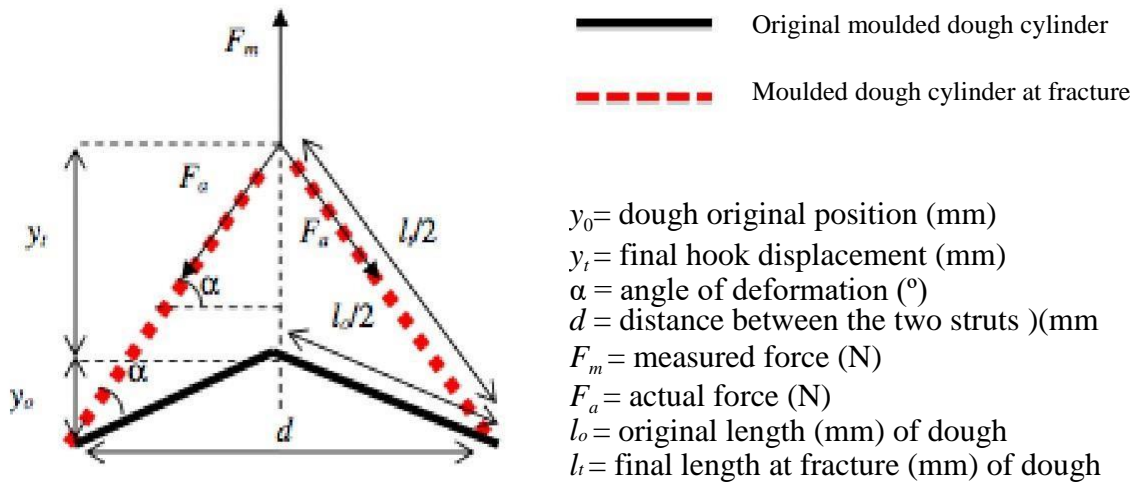
Figure 4.2.1: Diagram illustrating the forces acting on a dough strip and its changes in length when stretched by a hook on a Kieffer rig. Adapted from Abang Zaidel *et al.* (2008).

Scanning Electron Microscopy (SEM)

Small pieces (<2 mm diam.) of air-dried doughs from normal (conventionally bred) sorghum flours, prepared by sheeting and glacial acetic treatment, were mounted on specimen stubs with double-sided carbon tape. The crumbs were sputter coated with carbon and viewed with a Zeiss 540 Crossbeam SEM (Zeiss, Oberkochen, Germany) operating at an accelerating voltage of 3 kV.

Transmission Electron Microscopy (TEM)

Glacial acetic prepared sorghum doughs, sheeted doughs and normal doughs, as well as isolated protein body doughs were studied by TEM. Air-dried lumps of dough were prepared for TEM by fixing small pieces (<2 mm diam.) in 2.5% glutaraldehyde in 0.075 M phosphate buffer overnight at ~ 5 °C. Samples were then rinsed with the phosphate buffer 3 times at 10

min intervals each and then post-fixed with aqueous osmium tetroxide under a fume hood for 1 h before rinsing again 3x with the phosphate buffer. The pieces were then dehydrated in an ethanol series (30, 50, 70, 90 and 100 % ethanol) for 15 min each. Some of the sample pieces were transferred into plastic tubes and infiltrated with 33 and 66 % ethanol and LR-White resin mixture for 1 h each before infiltration with 100 % LR-White resin overnight at ~ 5 °C. Samples were finally infiltrated with fresh 100 % LR-White resin before polymerizing in the oven at 50 °C for 3 days. Thin slices of 100 nm were obtained from polymerized samples using a diamond microtom knife and were stained in uranyl acetate for 15 min before rinsing with triple distilled water and gently dried with a filter paper. Samples were examined with a Philips CM10 TEM (Eindhoven, The Netherlands) operated at 80kV.

Statistical Analyses

All experiments were repeated at least twice. One-way analysis of variance (ANOVA) was performed. Means were compared at $p = 0.05$ using the Tukey's Honest Significant Difference Test (HSD).

4.2.4 Results and discussion

4.2.4.1 Dough treatment by sheeting in combination with flour pre-gelatinisation and sourdough fermentation of normal sorghum flours

Effect of inclusion of pre-gelatinized sorghum flour on dough handling properties

Sheeting was used in this study to apply mechanical energy through shearing in attempt to release the kafirin proteins from protein bodies and hence to understand their role in dough functionality. Sheeting was also applied to attain uniform distribution of the pre-gelatinized flour throughout the dough.

Producing cohesive sheets from sorghum bread dough without pre-gelatinizing a portion of the flour prior to sheeting was not possible. Sorghum dough without flour pre-gelatinization had a crumbly texture and poor handling properties; both before and after the sheeting process. This is because sorghum flour lacks the unique viscoelastic properties of wheat flour due to its lack of gluten, and exhibits poor rheological properties in terms of pliability, extensibility, and rollability (Bansal *et al*., 2012). It was apparent that pre-gelatinization of part of the sorghum flour had a positive effect on the cohesiveness of bread dough *(Figure 4.2.2).* As sorghum flour seemed to have a high water uptake but poor water holding ability, the addition of pre-gelatinized starch played a major role in improving water retention in the dough.

At a 10 % level of flour pre-gelatinization, the dough became increasingly sticky and soft and had resultant holes and tears throughout the sheets after undergoing 15 sheeting passes *(Figure 1 b)*. Although also sticky, tears were not observed at 20% flour pre-gelatinization. Dough with 30% flour pre-gelatinization had superior texture and cohesiveness. Dough stickiness resulting from sorghum flour prepared with hot or boiling water is a function of starch gelatinization (Kulamarva *et al*., 2009).

Pre-gelatinized starch has been found to play the role of a binder, thus improving non-wheat dough functional properties (Sozer, 2009). Improved dough rheological properties in rice pasta production (Brites *et al*., 2010) as well as in maize bread dough (Khuzwayo, 2016) have been reported. The combination of dough sheeting and starch pre-gelatinization has not been previously reported for sorghum flours, but has been found to improve the handling properties of maize dough (Khuzwayo, 2016).
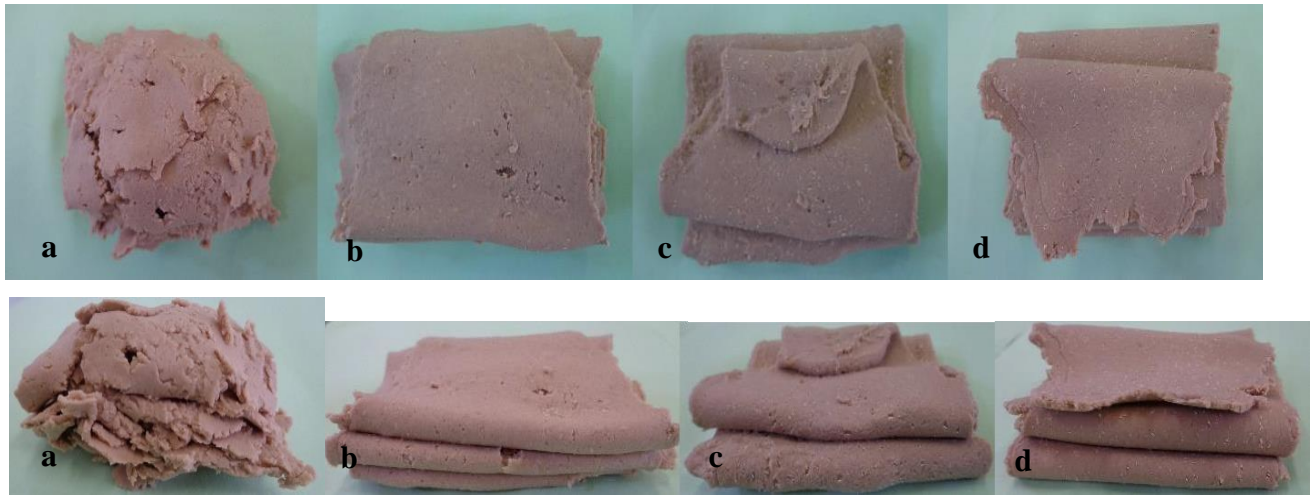
Figure 4.2.2: Images illustrating the effect of different levels of pre-gelatinized sorghum flour inclusion on the sheetability and dough handling properties of sorghum bread dough after 15 sheeting passes. **(a)** Control (No pre-gelatinized flour), **(b)** 10% pre-gelatinized flour, **(c)** 20% pre-gelatinized flour, **(d)** 30% pre-gelatinized flour.

Effect of sourdough addition dough handling properties

Sorghum sourdough, prepared by traditional fermentation, was incorporated in sorghum bread dough along with pre-gelatinized flour to determine its effect on the dough properties. These doughs were sheeted for uniform distribution of all components and analysed for sheetability and thereafter appearance and texture.

The addition of sourdough to the sorghum dough had dramatic effects on the texture of the dough, especially in terms of the softness and stickiness. Inclusion of sourdough in the dough made the dough notably softer. At 75 % sourdough inclusion, sheeted dough had visible tearing *(Figure 4.2.3)* and was characterised by a non-uniform surface with holes. The dough appearance was not smooth due to the difficulty of removing the dough sheets from the sheeting rollers caused by the increased stickiness of the dough. The best surface appearance and texture was obtained at 25 % sourdough inclusion. This dough was softer than dough without sourdough inclusion but still maintained its cohesiveness throughout the sheeting process.

Rheological analyses of maize dough by Falade *et al.* (2014) showed that the addition of maize sourdough resulted in a lower modulus of elasticity, indicating a softer and less elastic dough.

The impact on dough textural characteristics was attributed to starch granule modifications. Proteolytic activity also features in sourdough fermentation and plays a crucial role in the overall quality of sourdough bread (Gänzle *et al.*, 2008). Reduced resistance to deformation and increased liquid-like behaviour of brown rice batters made by treatment with proteases was previously reported by Renzetti and Arendt (2009), this was as a result of lower water holding capacity of the hydrolysed proteins rather than weakened protein structures.
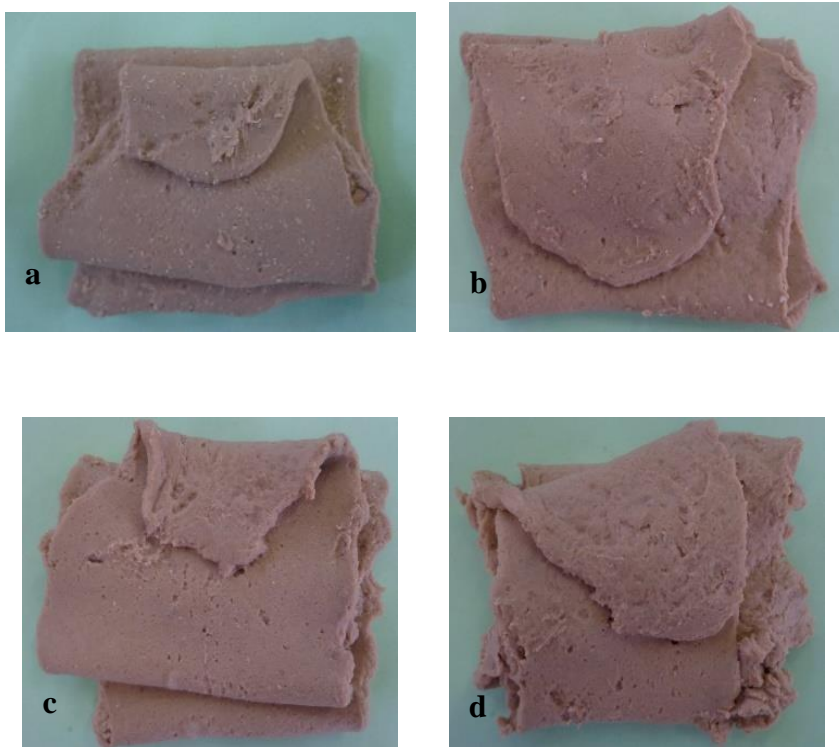
Figure 4.2.3: Images illustrating the effect of different levels of sorghum sourdough inclusion on the sheetability and dough handling properties of sorghum bread dough after 15 sheeting passes, prepared with 20 % pre-gelatinized flour.

(a) control (no sourdough), **(b)** 25 % sourdough, **(c)** 50 % sourdough, **(d)** 75 % sourdough.

Effect of inclusion of pre-gelatinized sorghum flour on bread quality

Following the improvements obtained in visual dough properties, the effect of pre-gelatinized flour on the bread making quality of sorghum flour was investigated in order to determine the optimum level of pre-gelatinized flour. All the doughs were of similar height before proofing

(Table 4.2.1). After baking of the sorghum doughs, the loaf height varied significantly with the level of flour pre-gelatinization.

Using 20 % pre-gelatinized flour gave the best results, yielding the highest loaf height after baking. However, the surprisingly small differences between loaf heights with the different levels of pre-gelatinized flour despite the differences in their dough handling properties is indicative of the complexity of bread making with sorghum flours. Above the optimum level of pre-gelatinized flour addition of 20 %, the rounded top of sorghum bread was not present (*Figure 4.2.4*). The dough fell flat during baking, thus affecting the final loaf volume. Also, the top/crust surface of bread with 30 % pre-gelatinized flour had the most visible cracks and a darker colour *(Figure 4.2.4 Aa)*. This can be attributed to more gas and moisture being lost through the top surface during baking.

Crumb appearance *(Figure 4.2.4 B)* of the breads provided information on the porosity and hence has-holding capacity conferred by different formulations. At 10 and 20 % pre-gelatinized flour inclusion, the crumb structure was the most porous, whilst at 30 % compact crumb structure was observed. A compact crumb shows that the dough did not trap carbon dioxide well and expand during fermentation and baking. As stated, the dough softness had been reduced as the amount of pre-gelatinized flour increased. It therefore seemed that a softer and less rigid dough was more preferable in obtaining a more open bread crumb.

Compared to the wheat bread, the crumb of all breads with pre-gelatinized flour was still moist in the centre of the loaf. This was due to the crumb not baking evenly through and the sorghum dough retaining too much moisture. Without pre-gelatinization, a dry floury portion in the centre of the crumb was observed *(Figure 4.2.4 Bb)*. This further shows that pre-gelatinization of part of the flour not only acted as a binder in the dough but also a moisture retainer in the crumb of the sorghum bread.

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Table 4.2.1: Effect of different levels of pre-gelatinized flour inclusion on the loaf height of sorghum bread prepared with dough sheeting (15 passes)

	Dough height before proofing (mm)	Loaf height (bread) [mm]
Wheat standard	22.5 ± 0.7^1	49.5 ± 3.5
Control (No pre-gelatinized flour)	$22.3^a \pm 0.3$	$28.5^{ab} \pm 0.7$
10 % pre-gelatinized flour	$22.3^a \pm 0.4$	$27.8^a \pm 0.4$
20 % pre-gelatinized flour	$22.3^a \pm 0.4$	$30.5^b \pm 0.7$
30 % pre-gelatinized flour	$21.8^a \pm 0.4$	$27.0^a \pm 0.0$

[1] Values presented as means ±standard deviation, n=2. Mean values in the same column but with different superscripts differ significantly ($p < 0.05$)

Figure 4.2.4: Effect of inclusion of pre-gelatinized flour on **(A)** crust and **(B)** crumb appearance of sorghum bread prepared with dough sheeting at 15 passes.

(a) Wheat standard, **(b)** sorghum control (no pre-gelatinized flour), **(c)** 10 % pre-gelatinized flour, **(d)** 20 % pre-gelatinized flour, **(e)** 30 % pre-gelatinized flour.

Effect of sorghum sourdough addition on sorghum bread quality

Sourdough fermentation has been shown to have beneficial effects in various gluten-free dough-based systems and carries an 'additive-free' image (Chavan and Chavan, 2011). For these reasons, it was used in this study together with flour pre-gelatinization, as a technological aid to the bread making performance of sorghum flour.

The levels of sourdough addition used were 25, 50 and 75 % (w/w of total flour). The loaf height of breads increased slightly with increasing sourdough addition *(Table 4.2.2)*. The highest loaf height was obtained with 50 % sourdough inclusion, followed by 75 %. It also appeared that at the highest addition of sorghum sourdough, the loaf had a more flattened top compared to all the other treatments. Sourdough sorghum breads had cracks on the top crust surface, through which gas was presumably lost. These are the same observations reported by Khuzwayo (2016) with 60- 80 % maize sourdough breads. The crumb structure of all loaves showed visible gas cells, however, this was most pronounced in sorghum bread with 50% sourdough added. At this level of sourdough addition, the aerated crumb was characterised by large gas cells in the centre and smaller ones towards the periphery. Clarke *et al.* (2002) reported that in contrast with chemical dough acidification, sourdough addition increased the loaf specific volume. The findings were related to the reduced dough elasticity and firmness observed in rheological tests. Structural changes in the protein network was suggested to enable better expansion of the dough. In this study, it can be highlighted that although textural dough modifications by sourdough addition are desirable, a maximum threshold exists, resulting in a soft dough that collapses easily during carbon dioxide production to give poor loaf volume.

In agreement with findings with maize sourdough bread making (Falade *et al.,* 2014), sourdough leads to improvement of sorghum gluten-free bread quality characteristics.

Determining the optimum level of sourdough addition is crucial. In this study, 50% of sourdough inclusion gave the best bread-making quality in terms of loaf volume and crumb structure appearance.

Table 4.2.2: Effect of sourdough inclusion on the loaf height of normal sorghum bread prepared with dough sheeting at 15 passes and 20 % (w /w of total flour) pre-gelatinized flour

	Loaf height before (dough) [mm]	Loaf height after (bread) [mm]
Control (0 pts SD: 20 pts pre-gel: 80 pts sorghum flour)	$22.0^a \pm 0.0^1$	$30.5^a \pm 0.4$
25 pts SD: 20 pts pre-gel: 55 pts sorghum flour	$22.8^a \pm 0.4$	$31.3^{ab} \pm 0.4$
50 pts SD: 20 pts pre-gel: 30 pts sorghum flour	$22.3^a \pm 0.4$	$34.5^c \pm 0.7$
75 pts SD: 20 pts pre-gel: 5 pts sorghum flour	$22.4^a \pm 0.4$	$32.3^b \pm 0.4$

[1]Means ±standard deviation, n=2. Mean values In the same column but with different superscripts are significantly different ($p < 0.05$)

pts= parts, SD= sorghum sourdough, pre-gel= pre-gelatinized sorghum flour,
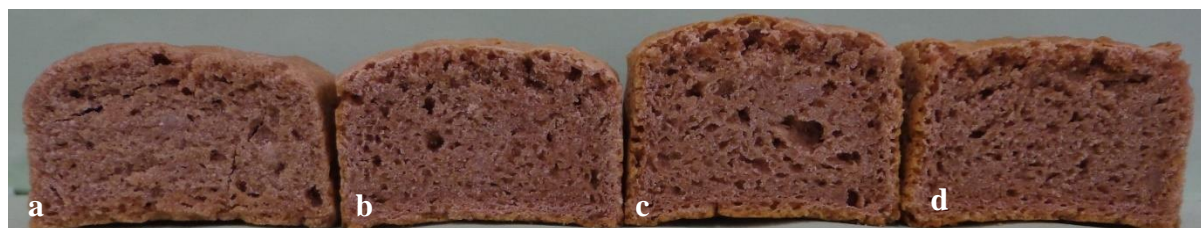
Figure 4.2.5: Effect of different levels of sorghum sourdough inclusion on crumb and crust appearance of sorghum bread prepared with 20% pre-gelatinized flour in combination with dough sheeting (15 passes).

(a) control (no sourdough), **(b)** 25 % sourdough, **(c)** 50 % sourdough, **(d)** 75 % sourdough.

4.2.4.2 Glacial acetic acid treatment of doughs of normal sorghum and sorghum with modified kafirin expression

Effect of glacial acetic acid treatment in combination with sheeting on sorghum dough handling properties

Treatment of normal sorghum flours using glacial acid was carried out to investigate its effects on protein body disruption in order to release the encapsulated kafirins for improved dough functionality. This was applied in conjunction with physical treatment of the dough by sheeting. After glacial acetic acid treatment, all the dough pieces were crumbly both before and after being subjected to a single sheeting pass, as expected for gluten-free dough systems *(Figure 4.2.6)*. There was no obvious effect of raising the temperature of the doughs to 50 $^{\circ}$C *(Figure 4.2.6 B)*. Following sheeting by up to 5 passes, both the sorghum doughs with water and with glacial acetic acid had better ability to hold together.

Dough handling properties and ability to withstand mechanical forces of sheeting without crumbling were improved with glacial acetic acid treatment followed by treatment with water. Taylor *et al.* (2009) found that when water is slowly added to a solution of kafirin in glacial acetic acid, phase separation occurred and the kafirin precipitated out. This was because the solvent became more hydrophilic. The authors further reported the formation of kafirin aggregates that were similar to zein microspheres with this treatment. The addition of water to the glacial acetic acid-formed sorghum doughs could have had a similar effect on the kafirin in the dough, thus releasing it into solution to participate in dough functionality.
Sorghum doughs prepared with glacial acetic acid treatment followed by treatment with water were more cohesive upon sheeting compared to glacial acetic acid treatment on its own *(Figure 4.2.7 B)*. This was more so for sorghum doughs held overnight; where there was extended solvent and flour interaction time.

(A)
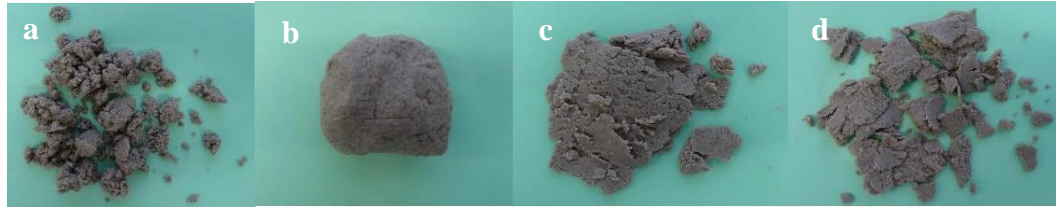
CONTROL (WATER SOLVENT)
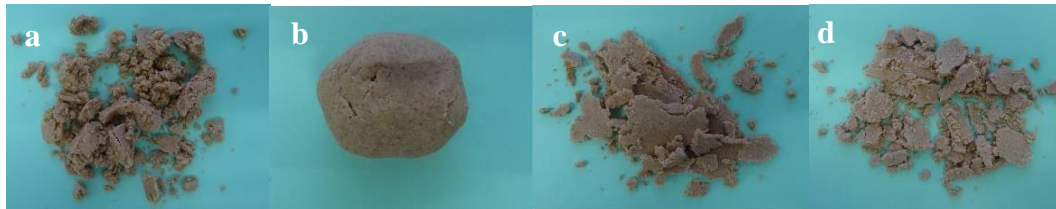
GLACIAL ACETIC ACID SOLVENT

(B)
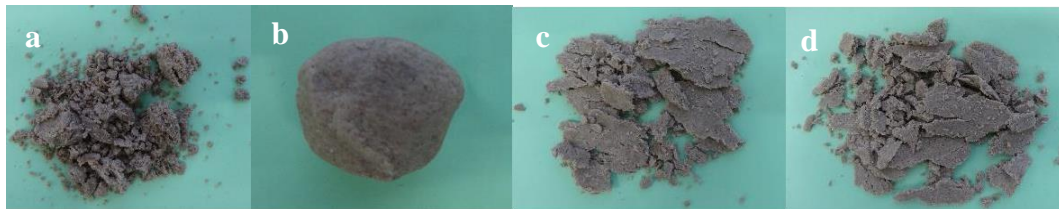
CONTROL (WATER SOLVENT)

GLACIAL ACETIC ACID SOLVENT

Figure 4.2.6: Sorghum flour doughs formed with glacial acetic acid (80 % by flour weight), followed by treatment with water. **(A)** Dough held at ambient temperature (± 18.3 °C) overnight; **(B)** dough heated to 50 °C and held overnight at ambient temperature; **(a)** Dough pieces, **(b)** dough rolled into a ball, **(c)** after one sheeting pass, **(d)** after five sheeting passes.

(A)
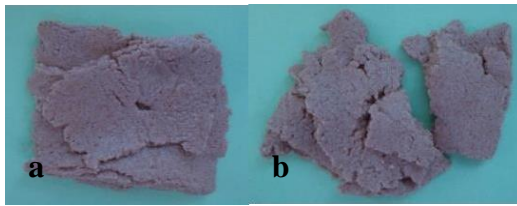
CONTROL [80 % (w/w flour) water]
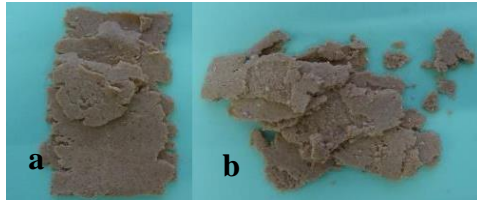
GLACIAL ACETIC ACID [80 % w/w flour]

(B)
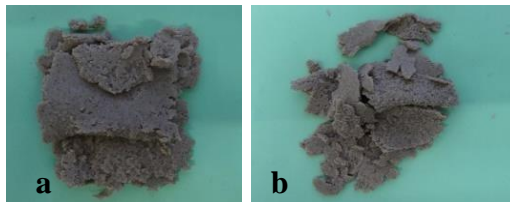
CONTROL [80 % (w/w flour) water] + 2 ML WATER
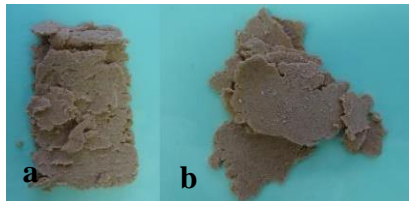
GLACIAL ACETIC ACID [80 % w/w flour]+ 2 ML WATER

Figure 4.2.7: Effect of glacial acetic acid treatment on the sheeting properties of sorghum flour dough.

(A) Without water addition (precipitation step), **(B)** with 2 ml water addition.

(a) After one sheeting pass, **(b)** after five sheeting passes.

Sorghum doughs treated with glacial acetic acid as solvent also had greater extensibility upon manipulation/ stretching by hand *(Figure 4.2.8)*. Kafirin doughs prepared using the same technique as this study showed the presence of fibrils both on the exterior and within the kafirin dough as a result of dough preparation with glacial acetic acid (Elhassan *et al*., 2018).

Tensile tests were performed on the dough at the maximum level of water addition/uptake before the dough turned into a slurry. To maintain the required dough temperature (i.e. either ~ 18 or 50 ºC), tensile tests were performed within ~ 3 min of dough cutting. It was observed that glacial acetic acid treatment in combination with sheeting of sorghum doughs caused a

1.5x increase in solvent (water) uptake compared to the control dough. The same observations were made by Sly *et al.* (2014) when dilute organic acids were used in zein dough systems. Studies on wheat dough showed that water absorption was increased with the addition of a mixture of organic acids (isolated from fully proofed San Francisco sourdough) in the absence of salt (Maher Galal *et al*., 1978).

As acetic acid is a proton-rich solvent, protonation of proteins such as zein occurs (Li *et al.*, 2012). This prevents molecular aggregation and causes partial unfolding of zein. The similarities between zein and kafirin with respect to amino acid composition, solubility, structure and molecular weight (Shull *et al.*, 1991) suggest similar mechanism of dissolution for the kafirin proteins, allowing kafirin the ability to participate in dough formation and thus impart improved handling properties on the dough.

The visual and sheeting properties of sorghum doughs only served as preliminary indications of the effects of glacial acetic acid on sorghum dough handling properties. The protein body microstructure and associations with other components in the dough were examined to confirm the effectiveness of these treatments in improving dough functionality of the kafirins. It was hypothesized that the treatments could potentially have more pronounced effects in disrupting kafirin confinement in the transgenic high protein digestibility sorghum lines that already possess a modified, less rigid protein body morphology.
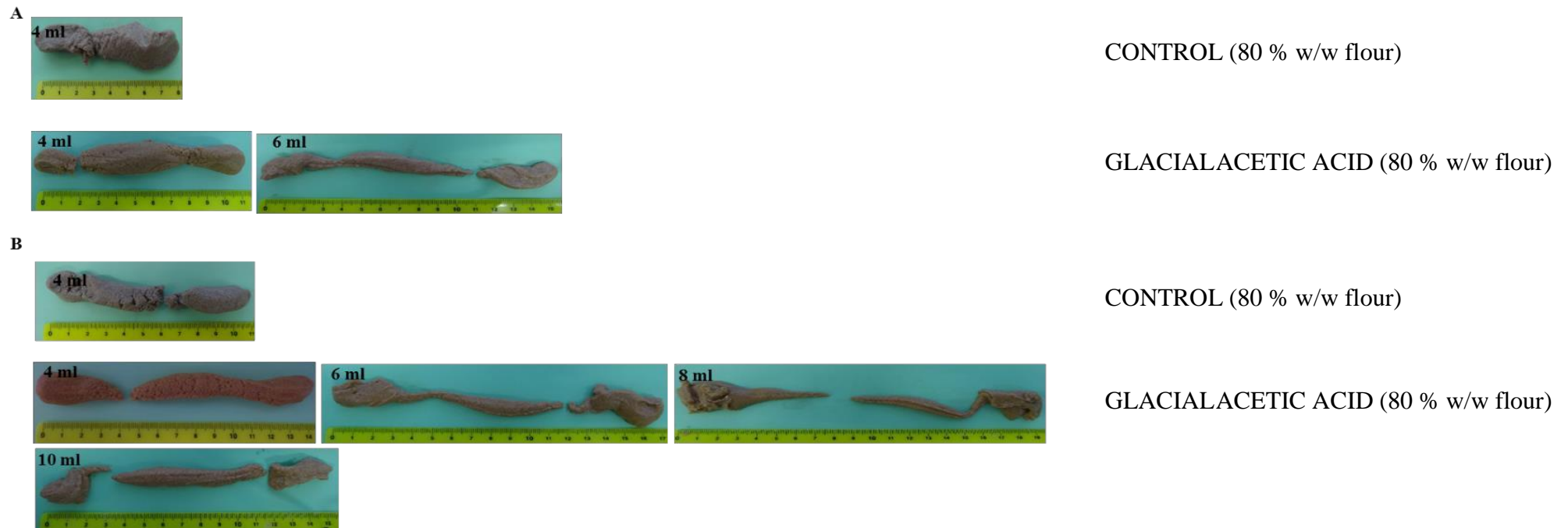
Figure 4.2.8: Effect of treatment with water on the extensibility of glacial acetic acid prepared sorghum. Total amount of water added for precipitation is labelled on the image. **(A)** Below 50 ºC ((± 18 ºC), **(B)** above 50 ºC (± 55 ºC)

Effect of glacial acetic acid treatment on the dough tensile properties of transgenic high protein digestibility sorghum flours

Tensile properties were initially analysed on normal and transgenic high protein digestibility sorghum flour doughs prepared with water or glacial acetic acid. However, since the doughs were too brittle *(Figure 4.2.9)*, pre-gelatinised flour was required to increase the firmness and cohesiveness of the dough. Flour pre-gelatinization was therefore a prerequisite in all the sorghum doughs to produce extensible doughs that could be analysed with the kieffer rig. It was observed that sorghum dough, without the pre-gelatinized flour (*Figure 4.2.9 A)*, was unable to hold on to moisture and was very fragile upon handling.
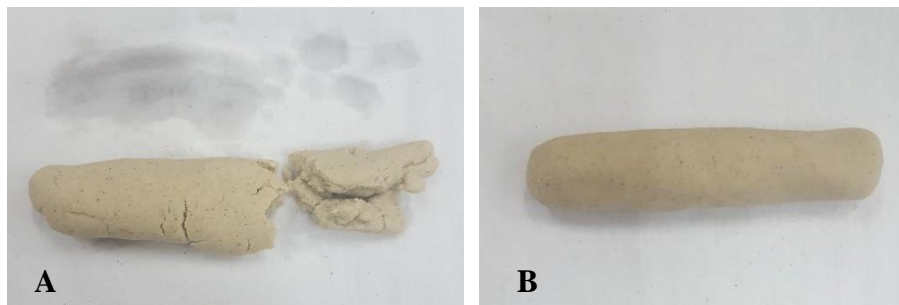
Figure 4.2.9: Hand rolled dough pieces of transgenic sorghum (TG-HD1) with high protein digestibility and modified kafirin expression. **(A)** Without pre-gelatinized flour, **(B)** with pre-gelatinized flour

Tables 4.2.3 and *4.2.4* show ANOVA results for peak stress and % strain, respectively. The sorghum line (high protein digestibility versus normal), dough treatment and their interaction had a significant effect ($p < 0.05$) on the peak stress and % strain, respectively, of sorghum doughs, except in the interaction of sorghum variety and temperature. Peak stress, an indication of dough softness, of all the doughs ranged widely from 10.7 kPa to 45.9 kPa *(Table 4.2.5)*. A significant difference in peak stress between TGHD and NC doughs was only evident at elevated temperatures (i.e ±50 °C) with a glacial acetic acid solvent; doughs of TGHD sorghum flours were significantly softer ($p < 0.05$). Temperature increase clearly induced dough softness in all the sorghum line doughs as the % strain of all the sorghum line doughs, prepared with water as a solvent was significantly reduced ($p < 0.05$). Furthermore, a comparison between the two solvents showed that glacial acetic acid treatment gave lower % strain of all doughs compared to water. Sly (2013) found that preparation of zein-maize starch

doughs with dilute organic acid produced softer, more fibrous doughs but these became increasingly brittle as the temperature was reduced below zein's T_g. It therefore appeared that the use of glacial acetic acid as a solvent, followed by treatment with water, of doughs prepared from TGHD and NC sorghum lines yielded doughs that behaved similar to zein-maize starch doughs prepared with dilute organic acids.

The dough extensibility measurements (*Table 4.2.5)* also showed that the TGHD and NC doughs with water as a solvent at ambient temperature were the most extensible compared to those prepared with glacial acetic acid and at elevated temperatures. The extensibility of *TG-HD1* and *TG-HD2* doughs was 25 and 42 % higher respectively, compared to the *NC* doughs. Since no other variable existed except the sorghum line, it can be concluded that the effect was likely due to the difference in kafirin expression on flour properties. Elhassan (2016) reported that TG-HD sorghum flours produced stronger and more elastic doughs than their null controls. The improved dough properties were attributed to reduced endosperm compactness due to suppression of expression of certain kafirin subclasses (spceifically the γ-kafirins). The reduction in the level of these cysteine-rich kafirins, and therefore reduction in hydrophobicity probably allow for more protein-starch and protein-water interactions through hydrogen bonding. Protein bodies in TG-HD sorghum lines are altered to have a folded/invaginated morphology instead of the normal spherical shape (Da Silva *et al*., 2011). This suggests that there is possibly greater accessibility of the kafirins in the irregularly shaped protein bodies of these TG-HD sorghums to be mobilised for dough viscoelasticity. Also, the TG-HD lines have reduced amounts of γ-kafirin and are therefore able to bind more water due to their reduced hydrophobicity (Elhassan, 2016).

Dough extensibility was significantly reduced ($p < 0.05$) at higher temperature (i.e. ± 55 $^{\circ}$C). This could be due to the rapid softening and therefore reduction in dough elastic behaviour, as shown by reduction of the Young's modulus as well. This is contrary to Goodall *et al*. (2012) who obtained more viscoelastic doughs with conventionally bred HD sorghum varieties above glass transition temperature. However, Elhassan *et al*. (2017) reported a reduction in the G' (storage modulus) of TG-HD sorghum doughs at high temperatures (above 90 $^{\circ}$C). Although the principle holds, the differences in dough preparation with this author (90 % solvent, flour basis, compared to the 80 % used in this study), as well as use of pre-gelatinized flour in the doughs makes the temperature effects on dough rheological properties not directly comparable.

The increased force required to extend doughs of all sorghum doughs with glacial acetic acid indicated that the doughs were too stiff, which was probably evidence of the inadequate plasticisation. These doughs fractured easily upon handling. All the doughs were prepared with the same amount of solvent addition. With wheat dough, addition of organic acids was found to increase water absorption but reduced dough stability and mixing tolerance (Maher Galal, 1978).

Table 4.2.3: ANOVA table for peak stress

Source of Variation	Mean Square	DF	F	P
Sorghum line	281.26	3	78.90	0.000
Solvent	1431.85	1	401.68	0.000
Temperature	0.19	1	0.052	0.820
Line*Solvent	422.97	3	118.66	0.000
Line*Temperature	184.26	3	51.69	0.000
Solvent*Temperature	50.02	1	14.03	0.001
Line*Solvent*Temperature	128.96	3	36.18	0.000
Residual	3.56	48		

DF= degrees of freedom, F= F-test, P= significance level.

Table 4.2.4: ANOVA table for % strain

Source of Variation	Mean Square	DF	F	P
Sorghum line	646.2	3	46.26	0.000
Solvent	10563.9	1	759.17	0.000
Temperature	1976.4	1	141.48	0.000
Line*Solvent	938.1	3	22.38	0.000
Line*Temperature	88.5	3	2.11	0.111
Solvent*Temperature	2666.6	1	190.88	0.000
Line*Solvent*Temperature	676.3	3	16.07	0.000
Residual	670.6	48		

DF= degrees of freedom, F= F-test, P= significance level.

Table 4.2.5: Effect of sorghum line, dough temperature and solvent type on the tensile properties of sorghum doughs with high protein digestibility and their null controls**.**

Sorghum line	Approx. temp at dough measurement	Solvent	Peak force	Extensibility	Peak stress	Strain at maximum displacement		Extensional viscosity from peak stress	Young's modulus from peak stress2
	(ºC)		(N)	(mm)	(kPa)	%	(εH)	(ηE, kPa.s)	(E, kPa)
NC1	18	Water	$0.260^{bc} \pm 0.007^{1}$	$16.0^{e} \pm 1.4$	$13.2^{ab} \pm 0.4$	$130^{e} \pm 3$	$0.269^{e} \pm 0.021$	$403^{a} \pm 14$	$3.56^{e} \pm 0.21$
NC2	18	Water	$0.226^{ab} \pm 0.010$	$15.8^{e} \pm 0.8$	$11.5^{a} \pm 0.5$	$132^{e} \pm 4$	$0.275^{e} \pm 0.027$	$350^{a} \pm 18$	$3.16^{e} \pm 0.24$
TG-HD1	18	Water	$0.319^{cde} \pm 0.012$	$21.3^{f} \pm 1.8$	$16.3^{bc} \pm 0.6$	$153^{f} \pm 6$	$0.425^{f} \pm 0.037$	$493^{a} \pm 14$	$6.89^{g} \pm 0.39$
TG-HD2	18	Water	$0.264^{bc} \pm 0.018$	$27.2^{f} \pm 3.2$	$13.5^{ab} \pm 0.9$	$155^{f} \pm 4$	$0.464^{f} \pm 0.052$	$414^{a} \pm 22$	$6.22^{f} \pm 0.33$
NC1	50	Water	$0.215^{ab} \pm 0.011$	$11.2^{bc} \pm 0.5$	$11.0^{a} \pm 0.6$	$117^{cd} \pm 2$	$0.152^{cd} \pm 0.018$	$375^{a} \pm 32$	$1.66^{cd} \pm 0.14$
NC2	50	Water	$0.223^{ab} \pm 0.024$	$10.9^{bc} \pm 0.6$	$11.3^{a} \pm 1.2$	$114^{c} \pm 1$	$0.132^{c} \pm 0.012$	$404^{a} \pm 54$	$1.49^{bcd} \pm 0.10$
TG-HD1	50	Water	$0.274^{bcd} \pm 0.025$	$15.7^{e} \pm 1.9$	$14.0^{abc} \pm 1.3$	$129^{e} \pm 3$	$0.254^{e} \pm 0.028$	$428^{a} \pm 38$	$3.56^{e} \pm 0.53$
TG-HD2	50	Water	$0.210^{ab} \pm 0.014$	$13.9^{de} \pm 1.0$	$10.7^{a} \pm 0.7$	$121^{d} \pm 2$	$0.192^{d} \pm 0.019$	$345^{a} \pm 22$	$2.05^{d} \pm 0.27$

Table 4.2.5: Continued

NC1	18	GAA	$0.353^{def} \pm 0.016$	$7.2^a \pm 1.6$	$18.0^{cd} \pm 0.8$	$108^b \pm 2$	$0.078^b \pm 0.008$	$833^f \pm 61$	$1.41^{bc} \pm 0.07$
NC2	18	GAA	$0.524^i \pm 0.067$	$5.6^a \pm 1.1$	$22.4^f \pm 2.3$	$104^{ab} \pm 1$	$0.035^{ab} \pm 0.006$	$1760^b \pm 220$	$0.94^{ab} \pm 0.03$
TG-HD1	18	GAA	$0.358^{ef} \pm 0.068$	$6.6^a \pm 0.4$	$19.7^{de} \pm 2.3$	$106^{ab} \pm 1$	$0.055^{ab} \pm 0.002$	$791^d \pm 180$	$1.06^{bc} \pm 0.03$
TG-HD2	18	GAA	$0.438^h \pm 0.041$	$5.9^a \pm 0.7$	$21.4^e \pm 1.1$	$105^{ab} \pm 1$	$0.048^{ab} \pm 0.005$	$1070^c \pm 85$	$1.10^{bc} \pm 0.08$
NC1	50	GAA	$0.410^{fg} \pm 0.068$	$9.6^b \pm 0.8$	$19.2^{de} \pm 0.5$	$107^e \pm 1$	$0.071^b \pm 0.007$	$853^b \pm 50$	$1.35^{bc} \pm 0.13$
NC2	50	GAA	-	-	-	-	-	-	-
TG-HD1	50	GAA	$0.285^{bcde} \pm 0.036$	$10.7^{bc} \pm 1.1$	$15.0^{abc} \pm 1.8$	$108^a \pm 0$	$0.076^b \pm 0.003$	$596^{ab} \pm 50$	$0.92^{abc} \pm 0.10$
TG-HD2	50	GAA	$0.165^a \pm 0.017$	$12.6^{cd} \pm 0.7$	$12.1^a \pm 1.1$	$118^{cd} \pm 4$	$0.167^{cd} \pm 0.030$	$502^a \pm 177$	$1.86^{bcd} \pm 0.39$

[1] Means ± Standard Deviation of two independent experiments, with two replications for each dough (n= 4)

[2] GAA= Glacial Acetic Acid

TG- HD= Transgenic High Digestibility sorghum

NC= Null Control/ normal digestibility sorghum

ε_H = Hencky strain/true strain

- Data lost

Values amongst all dough treatments ([abcdefg]) in columns with different superscript letters differ significantly (p< 0.05)

Effect of glacial acetic treatment on the microstructure of isolated protein bodies and TG-HD sorghum doughs

The protein body structure of the normal and high protein digestibility sorghums with modified kafirin expression protein was studied to test the hypothesis that increased availability of kafirins would positively influence sorghum dough properties. TEM was used to examine the effect of glacial acetic acid treatment and precipitation with water, prepared at $\pm$ 55 $^{\circ}$C, on releasing proteins from isolated sorghum protein bodies *(Figure 4.2.10),* and in TG-HD sorghum flours, non-transgenic sorghum flours and their null controls *(Figure 4.2.11)*. The isolated protein bodies from high protein digestibility sorghum were densely packed and $\sim$ 2 μm in diameter. As expected, their structure had a folded/ invaginated morphology *(Figure, 4.2.10a*, black arrows) as described by Oria *et al.* (2000) and Da Silva *et al.* (2011). This is different from the normal protein bodies, which are characterised by being mainly spherical with a presence of an outer dark-stained rings *(Figure 4.2.10b)*. Upon glacial acetic treatment, the high digestibility isolated protein bodies appeared to be dispersing and somewhat elongated *(Figure 4.2.11c)*. The normal protein bodies had a less visible outer membrane that seemed to have been dissolved by the glacial acetic acid treatment. Some merging of neighbouring proteins was also seen, probably as a result of the absence of a protective membrane barrier.

The glacial acetic acid treated TG-HD sorghum flours *(Figure 4.2.11)*, like the glacial acetic acid treated isolated protein bodies, showed some modifications in protein body structure. Both transgenic lines, TG-HD1 and TG-HD2, had some presence of small sized protein bodies ($\pm$ 500 nm) budding from larger protein bodies ($\pm$ 2 μm) *(Figure ,4.2.11)*, which is suggestive of release of proteins from their encapsulation in the rigid structures. This was not observed in their respective null control doughs. It can be concluded that the effect of treatment with glacial acetic acid at elevated temperatures ($\pm$ 55 $^{\circ}$C) of sorghum doughs is more effective in disrupting the abnormally shaped PBs, than the normal shaped/ unmodified protein bodies, due to an increased accessibility to the kafirins because of their greater surface area.

The glacial acetic acid treatment of the doughs in this study followed the method of kafirin microparticle formation as described by Taylor (2008). The author initially dissolved kafirin in glacial acetic acid to form a viscous solution, followed by addition of water to form microparticles. It was proposed that α-kafirin would precipitate out first, followed by β-kafirin

which would stabilise the freed α-kafirin. Lastly, the hexapeptide γ-kafirin repeat (PPPBVHL) would stabilise the microparticle surface by using a self-assembly mechanism.

Upon release from their confinement, protein body-free kafirins probably followed a similar mechanism of microparticle formation, to form protein structures of smaller size. Kafirin microparticles are reported to have a spherical structure, similar to protein bodies, but slightly larger in size than (Taylor *et al.*, 2009). However, smaller protein bodies were seen to have formed from larger ones in this study, thus suggesting that aggregation of protein structures into larger microparticle did not occur. This was possibly due to the difference in the systems being studies. Taylor (2008) made use of isolated kafirin whereas the doughs in this study comprised of whole sorghum flour.

The nature of the transgenic sorghum varieties (TG-HD1 and TG-HD2) entails supressed γ-kafirin synthesis, which results in absence of or reduced expression of the γ-kafirins (Da Silva *et al.*, 2011). This would make it easier for the glacial acetic acid treatment to have an effect in solvating the kafirins in the less stable protein bodies. The proposed effects of glacial acetic treatment and kafirin precipitation by water treatment were thus more pronounced with the TG-HD sorghums compared to normal sorghum lines.
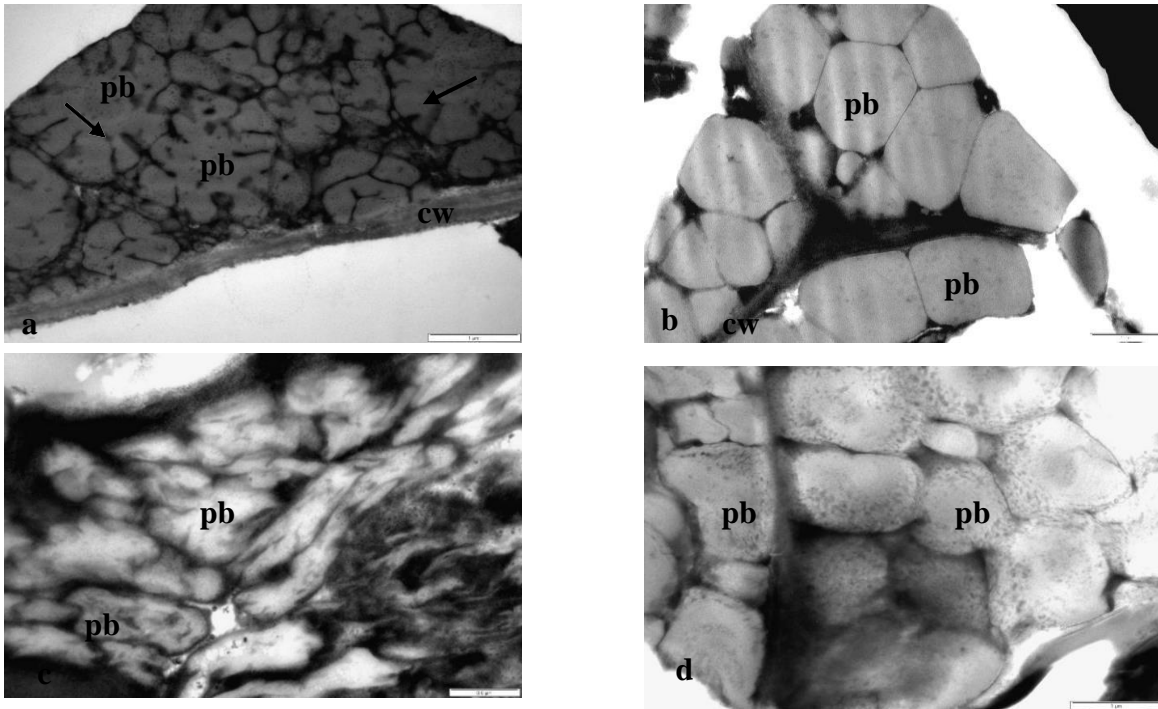
Figure 4.2.10: Effect of glacial acetic acid treatment followed by treatment with water, at ±55 °C, on the structure of isolated protein bodies from conventionally bred sorghum.

(a) high protein digestibility protein bodies (P850029), **(b)** normal digestibility sorghum pbs (NK283) as is , **(c)** high protein digestibility protein bodies (P850029), glacial acetic acid, ±55 °C, **(d)** normal digestibility sorghum protein bodies (NK283), glacial acetic acid, ±55 °C. Where **pb**= protein body; **cw**= cell wall, arrows show invaginations.
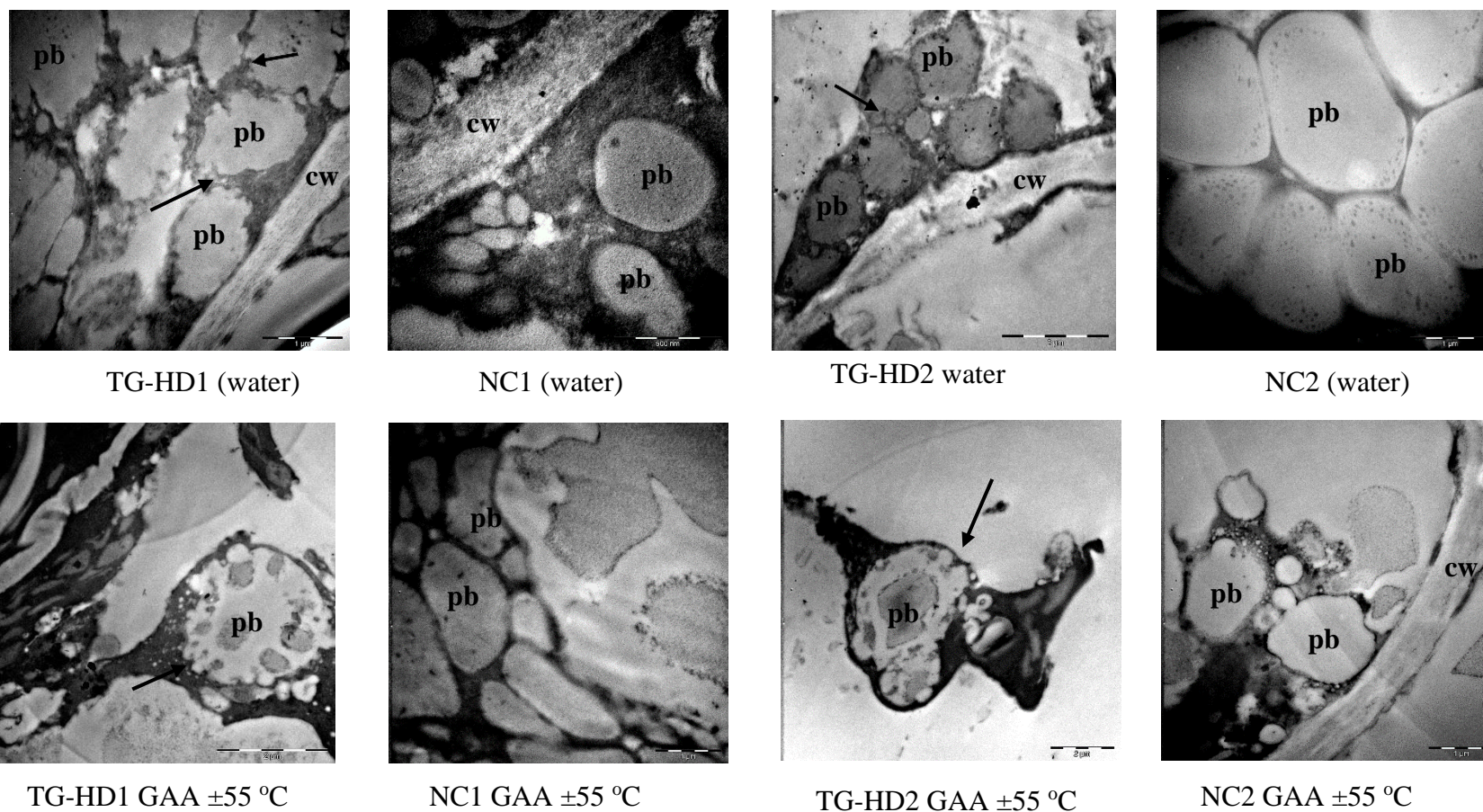
Figure 4.2.11: Effect of glacial acetic acid treatment followed by treatment with water, at ±55 °C, on the protein body microstructure of transgenic sorghum flour doughs (TG-HD1 and TG-HD2) and their null controls (NC1 and NC2). **TG**= Transgenic; **HD**= High protein Digestibility; **NC**= Null Control, **pb**= protein body; **cw**= cell wall, arrows show invaginations

Effect of sheeting on the microstructure of normal protein digestibility sorghum doughs

Protein bodies of normal protein digestibility sorghum doughs had a distinct spherical morphology *(Figure 4.2.12)* and did not show significant deformation following sheeting treatment on its own. Only upon the combination of sheeting and glacial acetic acid treatment of sorghum dough was the protein body morphology clearly affected. Protein bodies with this combination treatment appeared irregular and elongated.

A study by Hamaker and Bugusu (2003) showed that application of a high shear force (measured as specific mechanical energy) is required to disrupt the structure of maize protein bodies. The authors observed dispersal of zein with no noticeable protein body structures upon application of medium-high shear forces of extrusion processing. During sheeting, a range of stresses and strains are applied, causing the shape of the dough to change (Dobraszczyk and Morgenstren, 2003). In wheat dough, the mechanical stress was proposed to cause changes in the protein network in the dough (Feillet *et al*., 1977), associated with biochemical and rheological changes properties of the gluten proteins (Kim et al., 2008).

The protein microstructure changes that occur during sheeting of gluten-free dough have not been reported. It was highly likely that the shear forces applied in this study were not sufficient to affect proteins at the microstructural level. It can be concluded that dough sheeting represents a mechanical process that has potential to release kafirin from the confinement within their protein bodies, but at this point, significant effects can only be obtained when coupled with a chemical treatment with an organic acid. This mechanical disruption causes formation of smaller protein structures in the glacial acetic acid treated doughs *(Figure 4.2.12)*. This probably increases the surface area available for kafirins to precipitate out upon water addition and possibly form matrixes with other components in the dough.
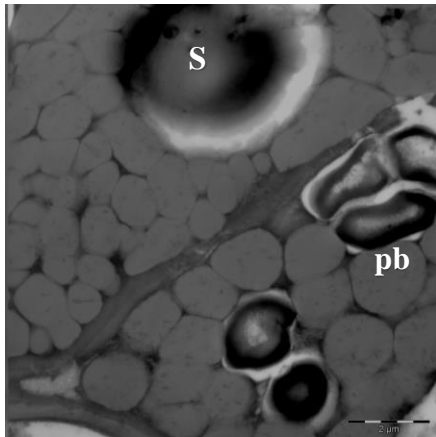
Sorghum dough control
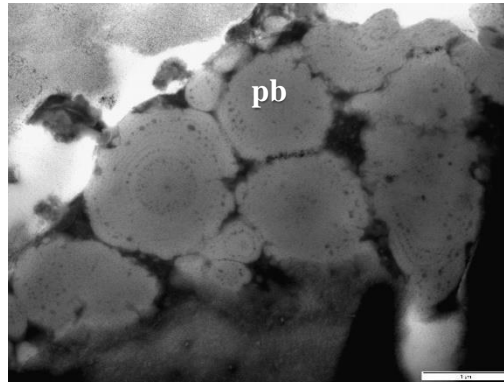
Sheeted sorghum dough
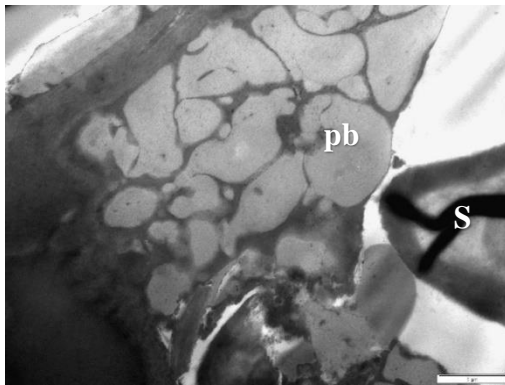
Sheeted GAA treated
sorghum dough

Figure 4.2.12: Effect of physical treatment through sheeting of doughs of normal sorghum flour flours on the protein body morphology (obtained through TEM). pb= protein body; S= starch granule.

SEM of sorghum doughs

SEM showed that in all sorghum doughs, the starch granules remained intact and not disrupted by the shear force of sheeting, elevated temperatures above T_g of hydrated kafirin (i.e. ±55 °C), nor the chemical treatment with glacial acetic acid as a solvent *(Figure 4.2.13)*. The temperatures used in this study were much lower than the starch gelatinisation temperature ranges (i.e. 67- 73 °C) reported for sorghum grown in southern Africa (Beta and Corke, 2001). The apparent intact microstructure of starch granules of the treated doughs suggested that it was unlikely the the starch component contributed to the changes in dough tensile properties. Hence, the modifications in the protein component of the sorghum flour, as shown in TEM

micrographs *(Figure 4.2.13)*, indicate that the effect on dough properties were more protein-related.
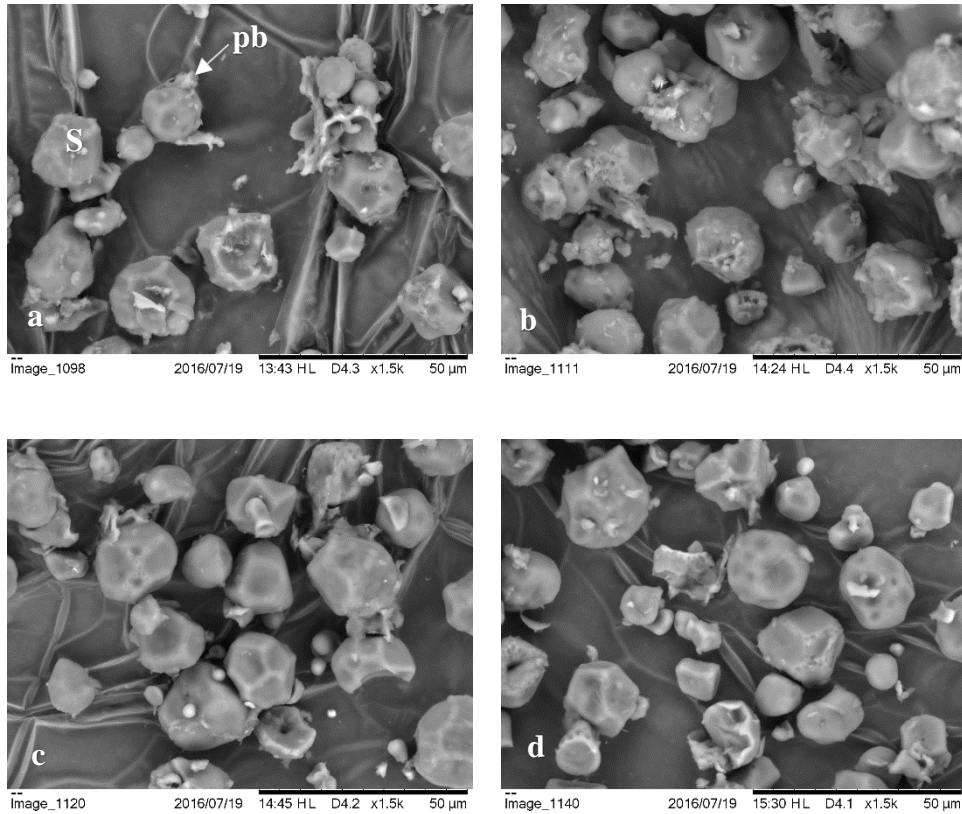
Figure 4.2.13: Effect of sheeting, elevated temperature (±55 ⁰C) and glacial acetic acid treatment on the microstructural properties of doughs from normal sorghum flours (obtained through SEM).

(a) Control sorghum dough (water, no sheeting), **(b)** sorghum dough ±55 ⁰C + sheeting, **(c)**sorghum dough + glacial acetic acid+ sheeting, **(d)** sorghum dough ±55 ⁰C + glacial acetic acid + sheeting.

S= intact starch granule, pb= protein body.

4.2.5 Conclusions

Sorghum dough handling properties can be improved by utilizing a combination of physical and chemical treatments, namely: flour pre-gelatinization, dough sheeting, sourdough inclusion and treatment with glacial acetic acid solvent. Dough sheeting technology represents a potentially viable method for the physical manipulation of sorghum doughs to improve handling properties. Release of kafirin from protein bodies by chemical treatment of sorghum doughs with glacial acetic acid and combination with a sheeting process is observed. However, these doughs have reduced extensibility and elastic behaviour. It therefore seems that mobilising the inert kafirins as functional components for a viscoelastic dough development in a dough system which comprises of other flour components (mainly starch) is a complex task. This study shows that TGHD sorghums with modified kafirin expression exhibit better dough properties, prior to these treatments, compared to normal sorghums. With technologies targeted at further mobilising the 'freed' kafirins, they might have better bread-making properties.

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5 GENERAL DISCUSSION

This general discussion will firstly critically review the major experimental methodologies applied in this study. Secondly, significant findings with respect to the technologies that could be used to improve the handling and rheological properties wheat-free dough systems with sorghum and barley brewer's spent grain will be explained. Lastly, the application of this work and possible future research directions will be discussed.

5.1 Critical review of methodology

Stable viscoelastic masses exhibiting similar viscoelastic properties to wheat gluten can be formed by dissolving kafirin in glacial acetic acid and precipitating the protein with rapid coacervation with cool water (Elhassan *et al*., 2018). King (2015) also formed viscoelastic zein dough at elevated temperatures (above hydrated zein's T_g). With hydrated zein, the temperature, at which cohesive and extensible doughs are formed was found to be ~ 28 ℃ (Lawton, 1992). Water hydrated kafirin, on the one hand, has a T_g of around 29 ℃ (Schober *et al.*, 2008). Therefore, maintaining or exceeding the T_g of kafirin was believed to be important in this study. Although careful measures were taken to monitor the dough temperature as well as keeping the apparatus and mixing tools used (spatulas, trays) at elevated temperature, maintaining the dough above 50 ℃ at the point of tensile property testing was a challenge. Sorghum doughs were kept in zip-lock type bags in a water bath above 50 ℃ in attempt to maintain the dough temperature. However, due to the ambient considerably lower temperature (~22 ℃) in the Kieffer rig testing environment, loss of heat from the doughs was inevitable. An ideal testing environment would be one that allows testing of tensile properties in a temperature controlled environment above 50 ℃.

Processing technologies that apply strong shear forces such as extrusion cooking have been suggested to disrupt protein bodies in order to release kafirins to realize their role as functional proteins in a gluten-free dough system (Hamaker and Bugusu, 2003). The authors stated that a specific mechanical energy of about 100 kJ/kg (with extrusion cooking) was required to begin to disfigure maize protein bodies. The sheeting procedure used in this study was performed manually, and therefore it was not practical to quantify the forces applied to the sorghum doughs. Seeing that sheeting was not found to cause significant changes in sorghum protein body morphology in this work *(Table 5.1)*, obtaining positive effects would be easier if all parameters were known. For the purpose of quantification of the forces

required to manipulate kafirins in the dough system and replication in a large-scale baking system, in an ideal situation, the required sheeting forces should be measured and controlled.

Sourdough fermentation is potentially an inexpensive, additive-free technology that is simple to apply across both the modern and poorer communities of sub-Saharan Africa. In this study, short and long (3 h versus 15 h) BSG fermentation processes were with the aim of trying to reduce the time and facilities required for producing BSG-wheat composite bread compared to conventional wheat bread. Apart from its convenience, it was further found that the short sourdough fermentation process gave better loaf volume *(Table 5.2)*, which is an added advantage. The reduced time would consequently reduce costs of production, especially on a large scale, as well as increase the chances of this bread making method being adopted. However, it would have been beneficial to characterize the microflora involved in the BSG sourdough.

Barley brewer's spent grain is well-known to contain substantial amounts of silica, mainly located in the husk (Macleod, 1979). Silicates have long been thought to be inert compounds that are not absorbed in the gastrointestinal tract (Dobbie and Smith, 1982). However, due to the main route of silica excretion being through the kidneys into urine, it is now thought that long-term excessive silicon intake may cause formation of renal stones (Jugdaohsingh, 2007). For these reasons, the direct and indirect inclusion of silicates in food needs to be controlled. However, the determination of silica content was out of the University of Pretoria's capacity and that of the independent laboratories that were approached.

5.2 Important research findings

Table 5.1: Summary of the effects of particle size reduction in combination with BSG sourdough fermentation on the dough and bread quality characteristics

	Fractionation (by roller milling)	Particle size reduction in combination with BSG pre-fermentation
BSG flour quality	Disintegration of large, sharp barley husk layers Increased protein content	Not applicable
Dough extensibility (alveography)	Not applicable	Improved extensibility (and gas-holding ability)
Loaf height	Decreased	Increased and decreased with increasing BSG inclusion and fermentation time
Loaf weight	Increased	Increased
Crumb structure	Dense, moist crumb with little aeration	Rounded loaf top. Increased porosity (presence of large gas and smaller gas cells).
Crumb microstructure (SEM)	Not applicable	Physical structure of the fibrous BSG husk in crumb still intact
Staling	Not applicable	Lower crumb firmness initially and throughout all the days of storage
Colour	Darker crumb appearance	Lighter crust colour

Table 5.2: Summary of the effects of different technologies in improving dough and bread making with normal and high protein digestibility sorghum.

	Flour pre-gelatinization	Sheeting	Sheeting plus flour pre-gelatinization	Sourdough inclusion plus flour pre-gelatinization	Glacial acetic acid treatment	Glacial acetic acid treatment ±55 ºC	Glacial acetic acid treatment ±55 ºC plus sheeting
Normal sorghum lines							
Dough handling properties	Cohesive	Soft, crumbly	Cohesive, uniform	Soft, cohesive	Crumbly	Crumbly	Cohesive
Dough extensibility	Extensible	Not extensible	Extensible	Not applicable	Increased	Increased	Not applicable
Loaf height	Not applicable	Not applicable	Increased with increasing proportion of pre-gel flour and decreased	Increased with increasing proportion of sourdough and decreased	Not applicable	Not applicable	Not applicable
Crumb structure	Not applicable	Not applicable	Increased porosity with increasing proportion of pre-	Increased porosity and uniformity	Not applicable	Not applicable	Not applicable

			gel flour and decreased				
Protein body microstructure	Not applicable	Mostly intact	Not applicable	Not applicable	Not applicable	Disrupted	Disrupted
Transgenic, high protein digestibility sorghum lines							
Dough extensibility	Not applicable	Not applicable	Not applicable	Not applicable	Reduced	Reduced	Not applicable
Protein body microstructure	Not applicable	Disrupted	Not applicable	Not applicable	Not applicable	Disrupted	Not applicable

BSG fractionation

Table 5.1 summarizes some of the effects of fractionation on BSG flour and bread quality characteristics. In this study, fractionation of whole BSG was achieved through a roller milling process. Fractions of different particle sizes and compositions (particularly in protein) were obtained. A major finding was that a protein-enriched BSG material could be obtained, which also has reduced particle size due to absence of large husk material. However, this fraction is not suitable for bread making as it affects the loaf volume negatively and imparts a gritty texture and mouthfeel (as observed during an informal sensory evaluation), probably due to a high ratio of the finer aleurone cells amongst the coarse husks. This enrichment technique can possibly be valuable for other application purposes.

BSG particle size reduction in combination with sourdough fermentation

It seems reducing the pH through a lactic acid fermentation will be beneficial in improving dough and bread making properties with both wheat-free cereal ingredients- BSG and sorghum. The BSG was subjected to a sourdough process to overcome difficulties with the coarse texture (sharpness and grittiness) of the material. Dough extensibility and ultimately the loaf volume and crumb structure were significantly improved with sourdough fermentation. This can be attributed to the sourdough process acting as a pre-conditioning step for the fibrous material. The activity of endogenous extracellular cellulolytic enzymes in the sourdough probably played a role in the partial depolymerisation of cellulose. In fact, the solubility of cell wall components as well as the gluten network and gas retention can be improved by sourdough fermentation (Katina *et al*., 2006). Use of reduced particle size BSG not only increased the available surface area for sourdough modifications but also assisted in better physical incorporation of BSG into the dough. The BSG used, in its unmilled form, had a range of particle sizes, up to larger than 5 mm. This in agreement with Forssell *et al*. (2008) who described BSG as extremely heterogeneous. It is thus beneficial to modify the large, sharp husks of BSG prior to developing a bread dough, as BSG is known to not only cause a gluten dilution effect but also induce mechanical disruption to the gluten network (Sullivan *et al*., 2011).

Sorghum flour pre-gelatinization

Sorghum flours do not form a cohesive dough, regardless of their nature, either normal or high protein digestibility type. Sorghum doughs are too soft and crumbly upon handling. Further, exerting mechanical force to the already weak doughs had deleterious effects. Pre-gelatinizing a portion of the sorghum flour was hence investigated to act as a binder in the dough system. The pre-gelatinized flour improved the cohesiveness, sheetability, water holding ability and the overall handling properties of the dough *(Table 5.2)*. This huge improvement allowed sheeting of the sorghum dough. In wheat-free flours, pre-gelatinization can enable the development of a viscoelastic batter that has the ability to trap and retain gas during proofing (Onyango *et al.*, 2009).

Sheeting in combination with flour pre-gelatinization and sourdough fermentation

Sheeting of the sorghum doughs seemed to cause even distribution of major dough components (i.e. starch and protein) to produce uniform doughs. Furthermore, the doughs became smoother and more cohesive with increasing sheeting passes. A total 15 sheeting passes was used in this study, according to findings from Khuzwayo (2016), which gave significant improvements in maize dough texture. The author reported that with sheeting, maize doughs were stronger and more extensible. The combination of sheeting, flour pre-gelatinization and sourdough was also previously reported by Khuzwayo (2016). However, these technologies has not been explored with sorghum dough flours. The resultant doughs had improved texture; the dough softness and smoothness increased with increasing sourdough inclusion.

Due to increasing dough stickiness, which was an obstacle to the sheeting process, the optimum sourdough inclusion rate was found to be 50 % (w/w of total flour). Above this level, dough properties not only deteriorated but the loaf volume was reduced *(Table 5.2)*. The improvements of wheat-free bread quality with sourdough inclusion are in agreement with Falade *et al.* (2014). The effects are associated with metabolic activities of the naturally occurring microorganisms, such as lactic acid fermentation, proteolysis and exopolysaccharide production (Moroni *et al.*, 2009). However, intense acidification has been reported to diminish bread volume (Barber *et al.*, 1992; Katina *et al.*, 2009).

Glacial acetic acid treatment of sorghum doughs

The high protein digestibility sorghum lines showed better extensibility on their own compared their null controls and the treatment with glacial acetic acid *(Table 5.2)*. This

finding could possibly due to insufficient plasticisation of these doughs as they are different to the normal digestibility sorghums. These transgenic lines have a suppressed expression of specific kafirin subclasses and possess protein bodies with an irregular, invaginated morphology (Da Silva *et al.*, 2011). This morphology is believed to allow greater accessibility of the α-kafirins for participation in dough functionality (Goodall *et al*., 2012). Also, these sorghum lines are assumed to have bound more water compared to normal sorghum flours due to the reduced amount of γ-kafirin and hence reduced hydrophobicity (Elhassan, 2017).

Protein body modification by sheeting and glacial acetic treatment

The disruption of the protein bodies, through the combination of sheeting and glacial acetic acid treatment is a novel finding that represents a step closer to mobilisation of the kafirins. This is because the kafirins are not regarded as functional proteins, with one of the major drawbacks being their encapsulation in rigid protein bodies (Hamaker and Bugusu, 2003; Taylor *et al*., 2016).

The mechanism of protein body modification by glacial acetic acid can be explained using a hypothetical model adapted from Taylor (2008) with respect to kafirin microparticle preparation with glacial acetic acid and subsequent coacervation with water *(Figure 5.1)*. The glacial acetic acid served to solvate the kafirin, followed by addition of water to form microparticles as the kafirins precipitate out. Glacial acetic, having a low dielectric constant (Merck Chemical Data Base, 2005), is considered a hydrophobic solvent, hence was found to be suitable to dissolve the hydrophobic kafirin (Taylor, 2008). Precipitation of the kafirins occurs as water is added to the solution, probably causing increased interactions of the amide groups of the kafirin residues with the water and the acetic acid interacting through hydrogen bonding with the water (Taylor, 2008). As more water is added, a point where the kafirin can no longer be soluble is reached and it precipitates out. It was also suggested by Taylor (2008) that increased mobility of the kafirin molecules may be expected as water is added which may enable increased number of protein/protein interactions to occur resulting in aggregation into larger molecules. It was proposed that α-kafirin would precipitate out first, followed by β-kafirin which would stabilise the freed α-kafirin. Upon release from their confinement, protein body-free kafirins probably followed a similar mechanism, to form protein structures of smaller size. With the flours investigated in this study, aggregation of the kafirin proteins may have not occurred in a similar order or to the same extent because unlike working with

isolated kafirin alone, the dough system in this study consisted of all flour components and the kafirins were initially encapsulated in protein bodies *(Figure 5.1)*.
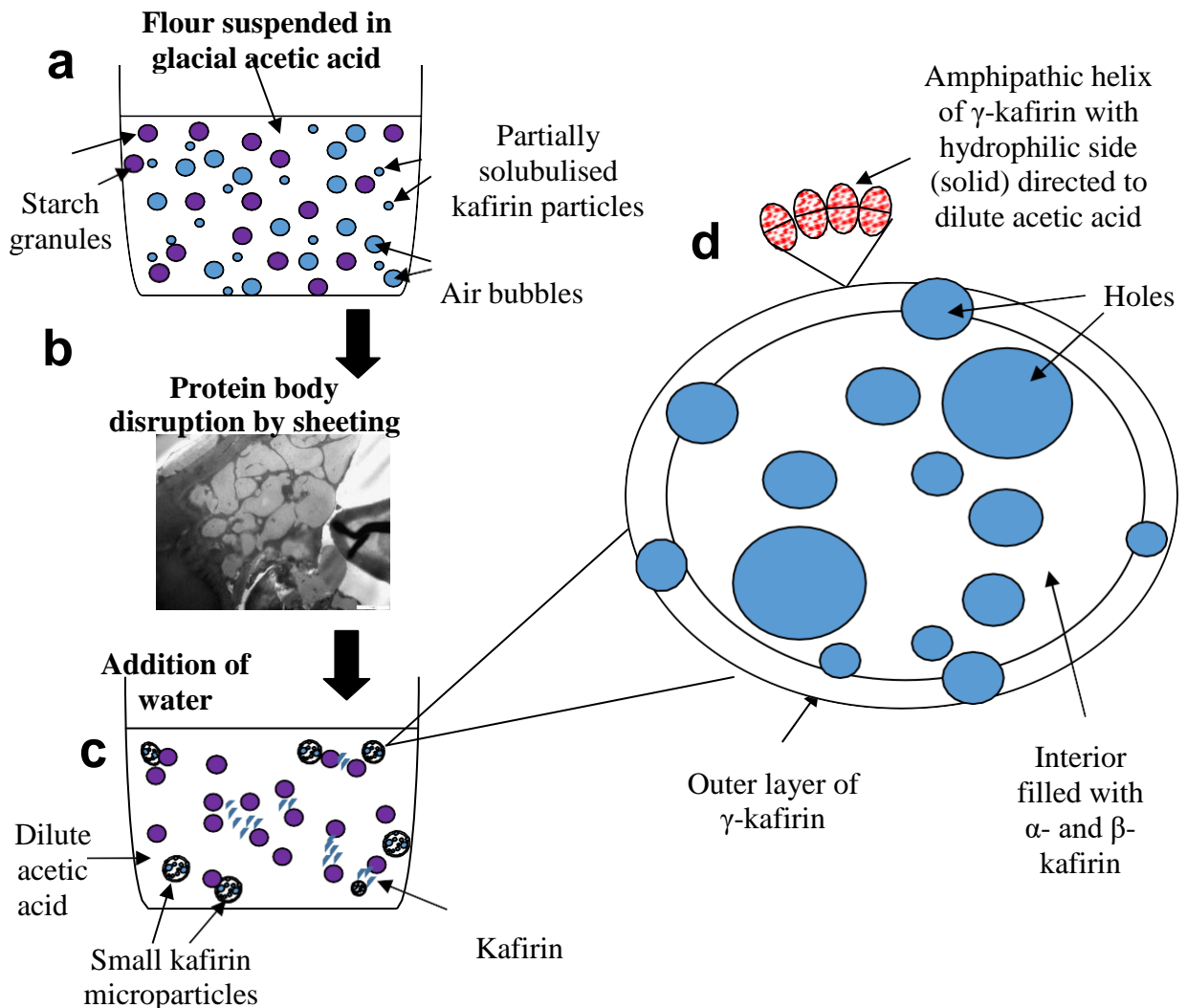
Figure 5.1: Hypothetical model to describe the formation of some kafirin microparticles and protein matrixes amongst the starch granules with glacial acetic acid treatment of the sorghum flour, followed by water addition. **a**: Suspension of sorghum flour in glacial acetic acid showing entrapped air bubbles and particles of partially solubulised protein bodies, **b**: protein body disruption by dough sheeting, **c**: On addition of water, few microparticles form as discrete particles whilst some kafirins are freed to interact with starch granules. **d**: Expanded view of a single kafirin microparticle (not to scale) [adapted from Taylor (2008)].

5.3 Way Forward

The benefits of BSG particle size reduction in combination with sourdough pre-fermentation technology in reducing the adverse effects imparted on bread quality characteristics has been demonstrated in this study. Future work should therefore look at determining the effects on sensory attributes and how consumers would perceive these. This is because sourdough acidity in wheat breads, contrary to rye breads, is only deemed desirable in mild quantities (Katina, 2005). Longer fermentation times are associated with development of stronger flavours when compared to short time processes (Molard, 1994). It could be desirable to determine whether the short 'sponge and dough' fermentation applied in this study resulted in acceptable bread flavour. Also, it has been suggested that carefully optimised conditions such as fermentation time and temperature should enable full exploitation of the benefits of sourdough fermentation (Katina, 2005). The optimum conditions for BSG fermentation and the microflora involved in producing a nutritionally and technologically valuable ingredient for application in other foods should be determined.

The tensile tests performed on the sorghum doughs as well as determination of texture visually and by feel indicated that the doughs were too soft. Although some textural improvements with inclusion of pre-gelatinized starch were obtained, the dough softness hindered further rheological analyses using frequency sweep tests and alveography, thus suggesting that these dough improvements obtained may still not be significant enough to produce bread. Further dough improvements could possibly be investigated, such as inclusion of hydrocolloids and proteins in the form of emulsifiers. The latter have been found to increase the elastic recovery of doughs, reduce crumb firmness and staling rate in sorghum bread (Onyango *et al.*, 2009).

This research has highlighted the importance of cross-fertilisation of different technologies, ideas and methods as a tool that can broaden the possibilities of bread making with non-wheat materials. Applying sheeting, pre-gelatinization, sourdough fermentation and organic acid treatment in combination with other technologies such as enzyme treatment and hydrocolloid inclusion could be valuable for other non-wheat cereal materials that are available in abundance in sub-Saharan African countries, such as maize and cassava.

6 CONCLUSIONS AND RECOMMENDATIONS

With regard to improving the functionality of BSG-wheat composite doughs, a major hurdle is the sharp and large fibrous husk particles. This study reveals the important factors and pre-treatment technologies that allow value-addition to BSG as a bread ingredient. Firstly, physically modifying the BSG material by particle size reduction is shown to be a method that can be used either as a BSG protein-enrichment method, or simply as a method of producing a uniform high-fibre flour for easier incorporation with wheat flour. The former, however, limits the amounts of BSG that can be composited with wheat flour, due to undesirable gritty texture of husk cells and drastic reductions in loaf volume. Protein-enriched BSG isolate can perhaps be utilized as an inexpensive nutritious food ingredient. Secondly, the use of a short and practical 'sponge and dough' sourdough method of producing the BSG-wheat composite breads versus the conventional straight-dough method produces more extensible doughs. The improved loaf volume and crumb structural properties are also in agreement with the well-known benefits of this 'sponge and dough' method, in particular, partially solubilizing the BSG components and hence reducing the mechanical disruption to the gluten network. The future focus here should be directed towards optimising the sourdough fermentation process and characterising the sourdough microflora for improved degradation of the cellulolytic components. It is also suggested that the application of exogenous enzymes such as cellulases and proteases be investigated.

By utilizing dough sheeting, flour pre-gelatinization and sourdough fermentation, sorghum dough and bread making quality is substantially improved. Pre-gelatinizing a portion of the flour presents a major breakthrough in producing cohesive and extensible non-wheat doughs. Inclusion of sourdough increases the dough softness, which seems favourable for dough expansion during carbon dioxide production. Dough sheeting technology plays a role in producing uniform doughs with enhanced cohesiveness, possibly as a result of even distribution of the dough components such as the starch and protein. The effects of the manual sheeting used in the study were only clearly observed when used on glacial acetic acid-treated doughs. These invasive treatments were successful in freeing the kafirins from their encapsulation. However, surprisingly these effects do not translate into increased dough extensibility when sorghum lines with modified kafirin expression are used. This can be attributed to the effects on kafirin functionality being masked by other components in the dough system, mainly starch, as well as inadequate use of plasticizer as these specific

sorghum lines are reported to have reduced hydrophobicity and therefore greater water binding. Nevertheless, it is recommended that these techniques of freeing of kafirins from protein bodies be further investigated with application of methodologies targeted at mobilising the 'freed' kafirins in order to realise their functionality in dough-based foods. In the meantime, the use of sheeting in combination with flour pre-gelatinisation of sorghum-wheat composite flours possess great potential for application into sorghum-wheat composite doughs.

The outcome of this research highlights the need and importance of investing in technologies for modifying non-wheat flours in order to improve their dough making quality. Further potential can be realised through the importation and integration of practices and ideas across bread making with non-wheat doughs.

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8 RESEARCH OUTPUT FROM THIS WORK

Magabane, I.E., Taylor, J. and Taylor, J.R.N. 2017. Chemical and physical treatments of doughs of normal and high protein digestibility sorghum flours with modified kafirin expression for improved dough functionality, Poster at the 22nd Biennial International SAAFoST Congress and Exhibition, Century City Convention centre, Cape Town.