Minimal processing of underutilised ingredients for food gel applications

by

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This thesis is dedicated to Christos P.

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List of Abbreviations

GWL	Green whole lentils
RSL	Red split lentils
RWL	Red whole lentils
YP	Yellow pea
PM	Pearl millet
M	Maize
W	Wheat
S-A	Potato starch
S-B	Maize starch
S-C	Pea starch
YPPI	Yellow pea protein isolate
BM2	2 hours of ball milling
BM4	4 hours of ball milling
RVA	Rapid visco analyser
PV	Peak viscosity
BD	Break down
TV	Trough viscosity
SB	Set back
FV	Final viscosity
Pt	Peak time
PT	Peak temperature
pI	Isoelectric point
pH	potential hydrogen
RCF	relative centrifugal force
RPM	revolutions per minute
λ_{em}	Emission light
λ_{ex}	Excitation light
T_o	Onset temperature
T_p	Peak temperature
T_c	End temperature
ΔE	enthalpy
G’	Storage modulus
G’’	Loss modulus
LVR	Linear viscoelastic region
γ	Shear strain
ω	Frequency
RVA	Rapid visco analyser
DSC	Differential scanning calorimeter
TPA	Texture profile analysis

List of Symbols

cPA	centi Pascal
min	minutes
sec	seconds
g	grams
mg	milligram
^{o}C	degrees Celcius
nm	nanometers
µm	micrometers
kDa	kilodalton
kcal	kilocalories
w/v	Weight per volume
w/w	Weight per weight
mm	millimetres
cm	centimetres
µl	microliters

List of Conference attendance

June 2020 - Postgraduate conference in Food Science - Oral presentation

‘Functionalising proteins with novel processing’

July 2022 - Total Food - Poster presentation

‘*The functionality of physically modified lentil flours’*

November 2022 - EFFOST - Poster presentation

‘Ball milling a tool for changing the mechanical properties and structure of lentil heat-induced gels’

Abstract

Finding an alternative to chemical ways to modify the functional properties of food materials has increased over the last few years. Ball milling has been used recently as a method of physical modification of isolated components (starch, protein) from various botanical sources using mechanical force (centrifugal, impact).

This research introduces ball milling as a method of physical modification of non-fractionated flours from lentils and pearl millet. The impact of the ball mill on the physicochemical and functional properties as well as the morphology of the legume and cereal flours was studied between the native flours and over 2 and 4 hours of ball milling.

Ball milling decreased the birefringence, crystallinity and onset starch gelatinisation temperature of the lentils suggesting the disruption of the starch molecules' intermolecular bonds and the loss of radial orientation. Lower solubility and hydrophobicity as well as the observation of protein aggregates indicated partial denaturation and aggregation of the lentils' proteins after ball milling. Protein aggregation was responsible for decreasing the viscosity of the lentils after ball milling by inhibiting starch hydration and swelling. However, such changes were not observed for the pearl millet indicating a more resistant structure to the ball milling forces.

Having identified the impact of ball milling on the pasting of lentils, the research continued with the application of the ball-milled material on gel formation. Starch was the major component in lentil flours and responsible for the gel network formation. However, protein aggregation induced by the ball milling forces impacted the formation of a strong starch-based gel in lentils by disrupting amylose retrogradation. Lentils formed softer gels showing either a starch-based or a protein-starch (interpenetrating) gel network after ball milling.

This research has highlighted the use of ball milling on non-isolated flour modification and the potential advantages of using these flours for food gel applications.

1 Chapter: Introduction and Literature review

1.1 Introduction to thesis

1.1.1 Overview

The production of plant-based products to replace animal products including yoghurt and cheese has gained attention in recent years (Dobson et al., 2022; Grasso et al., 2020; Min et al., 2022; Nagaprabha et al., 2018; Shand et al., 2007), as a result of growing concerns surrounding the impact of animal protein consumption on the environmental impact and human health (He et al., 2020). Over 70% of the energy used for traditional meat production is lost through wastewater. The use of land, water and fuel for meat production is a dozen times higher in comparison to the production of soybean-based foods. The CO_2 emissions are 250 times higher to produce beef and meat in comparison to legumes. A vegan diet could reduce land use by 50-60%, with even partial replacement of meat with plant-based foods reducing the use of land by 15%. Therefore, decreasing meat production would lead to more sustainable means of food production protecting the environment and natural resources (He et al., 2020).

Consumers have also shown an increased interest in healthier food, nutritionally rich and less processed products (Iriti & Varoni, 2017). However, the differences in the nutritional profile of meat and plant-based products can create a challenge in the decision to shift to a plant-based diet. For example, meat patties contain about 24 g of protein, but plant-based patties contain about 18 g of protein. Yoghurt from cow's

milk contains 3.3 g of protein and plant-based yoghurt 1.6 g of protein (Nolden & Forde, 2023). However, the average values of macronutrients in plant-based alternative products including meat and dairy analogues contain lower saturated fat (1.1%), higher dietary fibres (3.6%), higher carbohydrates (8.4 g) in comparison to animal-based (saturated fat; 6.4%, nil dietary fibres and carbohydrates 4.0%) (Tonheim et al., 2022).

Due to the changes in consumers' health habits and increase in demand for plant-based products a need to explore new materials was created (Du et al., 2014). Cereals and legumes are examples of plant-based materials providing a combination of macro and micro-nutrients to produce a novel food with high protein content and low-fat content (Joshi et al., 2017). Lentils and millet are used as staple foods in African, Indian, and Mediterranean countries to produce soups, porridge, and other traditional foods. These grains are also known for growing under harsh environmental conditions for example low rainfall and poor soil/low fertiliser (Iriti & Varoni, 2017; Srichuwong et al., 2017). Hulled and de-hulled types of lentils were used in this thesis from the legumes category and the pearl millet (PM) from the cereals category. Moreover, other legumes (yellow pea; YP) and cereals (maize; M, wheat; W) were used as comparators.

Research and commercial use of plant-based sources typically rely on the inclusion of a purified protein ingredient. The conventional wet extraction methods (alkali, acid, water) for plant-based proteins require a high amount of water, chemicals and energy and produce by-products. For example, 1 kg of legume protein isolate requires approximately 3.57

kg seed, 80 kg water, 22 kg hexane, 40 g NaOH, 40 g HCl and 30 MJ energy and 30 wt% of the initial material is converted to a by-product (Berghout et al., 2015; Schutyser et al., 2015).

Recently, significant research has been conducted into the use of technologies to reduce the amount of water, chemicals and energy required for protein extraction and functionalisation processes. The recent technologies are based on physical processes like pulsed electric field, ultrasound (Eze et al., 2022), dry fractionation (Saldanha do Carmo et al., 2020) and air classification (Reinkensmeier et al., 2015).

This thesis will evaluate another technology, the application of ball milling to legume and millet grains as a method of functionalising ingredients, without purification. Ball mills are already used on an industrial scale in the pharmaceuticals, chemistry, and material industries for size reduction (Piras et al., 2019) both in dry (<10% moisture) and wet conditions. It has been observed that milling promotes changes in the structure of the starch and protein isolates (Gonzalez-Jordan et al., 2017; Palavecino et al., 2019; Yang et al., 2019) impacting ingredient functionality. For example, enhancing foaming (Mei et al., 2019), cold-set gelation (Liu et al., 2017) and pasting properties (Loubes et al., 2018a). Published research on the impact of ball milling on flours has been limited to the physical, chemical, and functional properties of extracted or isolated material (starch, proteins) and there is only a little research on whole grain flours (Juarez-Arellano et al., 2021; Mei et al., 2019). Most research focuses on the milling of wheat and rice but there

is not enough evidence regarding the effect of milling alternative grains like lentils and millet (Thakur et al., 2019).

The second part of the thesis research will focus on using modified flours in gel production. The gel properties of plant-based ingredients are fundamental to the texture of cheese and yoghurts. An understanding of those properties would help to drive consumer acceptability of alternative and sustainable product formats (Shand et al., 2007). Gel microstructures can hold a vast amount of water and nutrients (Fernández et al., 2014) therefore producing food low in calories while also being soft, elastic, and easy to swallow by the elderly and people with dysphagia (Nazir et al., 2017). However, there is no information to the best of the author's knowledge on the production of gel-like products from ball-milled and unfractionated legumes and millet flours.

1.1.2 Objectives

The objectives of this thesis were:

- To investigate the effect of ball milling on the structural and functional properties of lentil and millet flour.
- To utilise the native and ball-milled flour samples to produce sustainable plant-based, minimally processed heat-set gels.

1.1.3 Structure of the thesis

This thesis consists of five chapters including two result chapters focusing on each objective, Chapters 3 and 4, respectively. Chapter 3 describes the work undertaken to investigate the changes to the starch and protein components due to the ball milling protocol. Chapter 4 details

the structural, rheological, and textural characterisation of the heat-set gels prepared using several types of native and ball-milled modified lentil flours. Materials and methods common to both result chapters are detailed in Chapter 2. Conclusions with recommendations for further research are made in Chapter 5.

A review of the literature providing justification for the selection of the materials including a discussion on the agricultural characteristics of the plants and the composition of the grains was conducted. A discussion on the morphological characteristics of the grains was also conducted. The structural modification and functionality of ball-milled ingredients follow in the rest of Chapter 1.

1.2 Agricultural information on the lentil and millet plants

1.2.1 Taxonomy and general information on lentil and millet production

The taxonomy of the legumes and cereals used in the experimental part of this thesis can be found in Table 1.1.

Table 1.1 Taxonomy of the legumes and cereals used in this thesis.

Kingdom	Legumes	Cereals
Clade	Angiosperms, Eudicots	Angiosperms, Monocots
Order	Fabale	Poales
Family	Fabaceae	Poaceae
Species	*Lens culinaris* (lentils) *Lathyrus aphaca* (yellow pea)	*Pennisetum glaucum* (pearl millet) *Zea mays* (maize) *Triticum durum* (wheat)

This thesis will study the pulse called lentil or *Lens culinaris* belonging to the *Leguminosae* family. The name lentil originates from the lens-like shape of the grains and varies in colour, from yellow, and green to red and black (Erskine et al., 2009; Samaranayaka, 2017). Lentils have various names around the world including dhal and masoor (Joshi et al., 2017). Lentils are a convenient food to prepare with the cooking time being shorter (25 min) than other legumes (>70 min) (Dhull et al., 2022).

Two of the most common types of lentil grains in the market are the green and red lentils named after the colour of their grain. Green lentils are usually sold as whole seeds and red as split (de-hulled) seeds because their hull is easier to remove (Kaale et al., 2022). The climate and soil of Canada create favourable conditions for lentil production,

especially for the green and red lentil types. Canada has been leading lentil production since the 1960s when lentils were used as alternative crops in lieu of fallow practices (Erskine et al., 2009).

Drought-resistant cereal crops are an important addition to the world's food basket and a solution to malnutrition in developing countries (Singh, 2011). One example is millet, a name used for various small seeds belonging to the grass family *Poaceae*. Millet flour is used in ready-to-eat products (porridge, infant foods), and gluten-free products (baked goods) however, is still considered an underutilised crop due to a lack of processing technologies and food preparation (Kaur et al., 2019; Wrigley et al., 2017).

There are three types of millets based on the size of the grain including major millets (sorghum, pearl, and finger millet), minor millets (foxtail kodo, barnyard, little, proso) and pseudo millets (amaranth, buckwheat) (Nikita et al., 2022). India is the leading producer contributing more than 90% of the pearl millet production, it is a very important food source for smallholder farmers and also provides them with financial security (Jukanti et al., 2016a).

This research will specifically investigate Pearl millet (*Pennisetum glaucum*) known as Bajra, belonging to the *Gramineae family*. Pearl millet is one of the most popular millet species due to its high yield potential (Duodu & Dowell, 2019).

1.2.2 Agricultural benefits of the lentil plants

The lentil plant can grow under various soil types from loamy to alluvial with a pH of 6.0-8.0. It is highly tolerant in drought conditions with average annual rainfall of 20-25 cm. The plant has shallow roots that easily manage water absorption. Lentils are nitrogen-fixing plants and often grow in rotation with other crops usually, cereals including wheat. Nitrogen fixation occurs due to the symbiotic relationship of the lentil plant with nitrogen-fixing bacteria called Rhizobium. Nitrogen enhancement increases soil fertility and reduces the use of fertilisers. Lentil plants do not host pathogens and thus there is a lower risk of cereal crop infestation. The plant germination starts after ten days of sowing and is ready to harvest after three to four months (Cokkizgin & Shtaya, 2013; Erskine et al., 2009; Joshi et al., 2017; Samaranayaka, 2017).

1.2.3 Agricultural benefits of the millet plant

Millet is a crop resistant to drought and elevated temperatures, growing under low annual rainfall (40-60 cm) (Wrigley et al., 2017) and other harsh environmental conditions including infertile soils with poor water-holding capacity and short growing seasons (Rani et al., 2018). Pearl millet is tolerant of pests and diseases and is cultivated in various forms of cropping including monocrop in sandy soils, intercropping with legumes and mixed cropping. It is also highly tolerant in acidic and saline soils and grows in undernourished soils (Jukanti et al., 2016b). The pearl millet has a higher photosynthesis and water-use efficiency than C_3 plants thus, is recognised as a C_4 plant and is compliant with climate-

smart agriculture (CSA). The plant has a strong and deep root system. Pearl millet cultivars are classified into two maturing groups: tall plants (3-6 m height) belonging to the late group (130-150 days) and short plants (1.50-3 m height) belonging to the early group (60-95 days). There is a large variability in plants' colour from pale yellow, brown, grey, or purple (Queroz, 1991). Yield of pearl millet relies on environmental and growing conditions (Duodu & Dowell, 2019) however, pollinated varieties and hybrids have been used to increase the yield (Jukanti et al., 2016b). The grains of the pearl millet are ovoid and 3-4 mm long (Queroz, 1991).

1.3 Composition of the legume and cereal grains

The composition of legumes varies due to the considerable number of varieties in the market. The carbohydrate content of legumes (60-70%) is lower in comparison to cereals (70-80%) containing monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Dietary fibres resist digestion and absorption by the human intestine and contain cellulose, hemicellulose, pectins, gums, mucilages, resistant starch, polysaccharides, and lignin (Oomah et al., 2011).

Cereals contribute to 50-70% of total calories in daily human nutrition. The composition of cereals depends on the variety, conditions during grain growth, husbandry, and infection (Zhou et al. 2013). The cell wall material in cereals contributes as a major source of dietary fibre and contains arabinoxylans, cellulose, hemicellulose, lignin, and β-glucans (Rosentrater et al., 2017).

The composition of commonly used cereals and legumes can be found in Table 1.2.

Table 1.2 Composition of common legumes and cereals according to the literature.

(%)	Carbohydrates	Proteins	Fat	Fibre	Ash	Moisture	literature
Legumes							
Lens culinaris (lentils; green)	63.40	24.60	1.06	10.70	2.71	8.26	(Dhull et al., 2022)
Lens culinaris (red split)	63.10	23.90	2.17	10.80	3.00	7.82	(Dhull et al., 2022)
Phaseolus vulgaris (bean)	54.30	20.90	2.49	8.55	3.80	9.93	(De Almeida et al., 2006)
Cicer arietinum (chickpea)	54.00	18.50	6.69	9.88	3.15	7.79	(De Almeida et al., 2006)
Glycine max (soybean)	30.16	36.49	19.94	9.30	4.87	8.54	(Zhou et al., 2013)
Cereals							
Pennisetum glaucum (millet)	68.05	12.99	7.96	2.83	3.21	4.96	(Hajjagana et al., 2014)
Sorghum bicolour (sorghum)	70.70	10.40	3.10	2.00	1.60	12.00	(Saleh et al., 2013)
Oryza sativa (brown rice)	50.80	7.90	2.70	1.00	1.30	12.00	(Saleh et al., 2013)
Triticum spelta (spelt)	71.90	12.80	2.17	1.96	1.86	9.30	(Biel et al., 2021)

The energy value (1.16 kcal/g) of lentils is due to high protein (≈30%) and carbohydrate contents (≈60%). The main source of energy in lentils is the starch but a part of the starch will not hydrolyse or absorb after digestion (resistant starch ≈25%) thus decreasing the number of metabolisable calories (2.5 calories/ g of resistant starch) (Kaur et al., 2010; Romano et al., 2021). However, the high protein content (≈30%) of lentils increases their energy value and makes them an ideal tool to tackle protein-energy malnutrition in developing countries where meat is expensive. Lentils contain all essential amino acids (39.3 g essential amino acids/ 100 g of proteins) but are low in sulphur-containing amino acids thus a combination of lentils with cereals is an efficient way to provide a balanced amino acid profile (Romano et al., 2021).

Lentils are a source of calcium; Ca (35-48 mg), iron; Fe (6.39-7.39 mg) and zinc; Zn (3.27-3.60 mg). Antinutrients in lentil grains such as lectins, phytates and saponins decrease the bioavailability of minerals and thus processing (i.e., de-hulling, heating, soaking in water) is needed before consumption (Kaale et al., 2022).

The consumption of lentils has been proven to lower cholesterol levels and protect against chronic diseases (cardiovascular, blood pressure and others). It could also be used to treat micronutrient malnutrition in developing countries, especially among young children. The slow digestion of lentils' carbohydrates lowers glycaemic response which is helpful for diabetics. A glycaemic index <55 is used to classify food as

low in glycaemic index. The glycaemic index of lentils was reported at 29 which is very low in comparison to bread (100) (Dhull et al., 2022).

The energy content (3.84 ± 1.6 kcal/g) of pearl millet is higher in comparison to other cereals including wheat (3.48 ± 0.3 kcal/g) (Rustagi et al., 2022). Starch is the major macromolecule (>60%) in millet and can be used as a thickener, gelling or bulking agent. The protein content is the second major component (>9%) and higher in comparison to other cereals such as maize and wheat and includes a similar profile of amino acids to wheat (Hassan et al., 2021). Millet contains a significant amount of calcium; Ca (42 mg), iron; Fe (8 mg) and zinc; Zn (3.10 mg) (Jaybhaye et al., 2014) comparable to lentils.

Pearl millet has a low glycaemic index providing healthy food for patients with diabetes. The incorporation of millet in wheat-based products (noodles) lowered the glycaemic index to 45.1 from 62.6 and from 68 to 50.8 in biscuits (Annor et al., 2017). It is also a rich source of antioxidants and fibres which lowers the risk of diseases (i.e., heart disease). Millet is a cereal free from gluten proteins and can be consumed by patients with celiac disease. Starch from millets has been applied in tablet formulations as a binder, in pharmaceutical applications (capsule) to improve disintegrant properties and could be used as an alternative to potato and maize starch due to their similar physicochemical properties (Nikita Sanjay et al., 2022).

Overall, the consumption of legumes and cereals shows many benefits to human health thus, makes them good candidates for food production.

The composition in Table 1.2 shows that starch and proteins are major components in legumes and cereals. Starch and proteins have a unique structure that determines their functional properties. For this reason, the fundamental information regarding the structure of the starch and proteins will be discussed.

1.4 Structural characterisation of the starch granules

Starch is a polysaccharide present in various storage tissues of plants (in the endosperm of seeds, stems, roots or tubers). In the grains, it is synthesised during grain development in amyloplasts and located in the endosperm (cereals) or cotyledons (legumes) in the form of starch granules. The size and shape (i.e., polygonal, oval) of the starch granules depend on the botanical source, environmental conditions, and agronomic factors (Alvarez-Ramírez, 2023). The structure of the starch granules can be found in Figure 1.1.

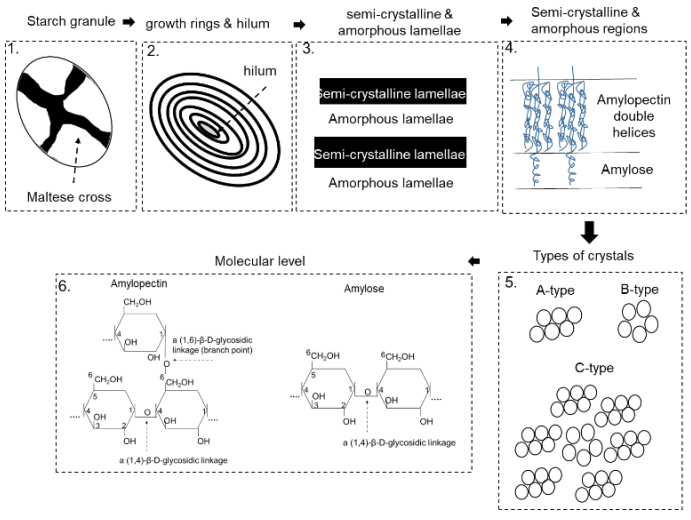


Figure 1.1 The levels of starch granules' structure starting from macro to micro scale including (1) the granule as observed under the polarised microscope (Maltese crosses), (2) lamellae and growth rings, (3) semi-crystalline and amorphous regions forming the lamellae, (4) structure of the semi-crystalline and amorphous regions of the lamellae formed by the amylopectin double helices and the amylose chains, (5) types of crystals formed by the amylopectin double helices, (6) amylopectin and amylose structure at the molecular level.

The 1st structural level of the starch granules when starting from macro to micro scale, shows a semi-crystalline nature under polarised light due to characteristic patterns called Maltese crosses. The Maltese cross pattern indicates a radial organisation within the granule. The 2D structural level shows growth rings starting from the centre or hilum of the granule and each growth ring represents a cycle of development (Hancock & Tarbet, 2000). The growth rings and 3d structural level are formed due to the presence of lamella. There are two types of lamellae, the amorphous with a thickness of 2-5 nm and semi-crystalline with a thickness of 5-6 nm. The lamella represents the 4th structural level and is composed of amylose and amylopectin packs. Each semi-crystalline or amorphous pack contains amylopectin and amylose chains, respectively. The semi-crystalline lamella is a double helix, but the structure of the amorphous is a single helix (Taggart & Mitchell, 2009). At this level, crystals are formed by the side chain branches of the amylopectin molecule (Eliasson, 2004).

Three types of crystals exist depending on the pattern of the crystallite packing and bonded water. Specifically, cereals have an A-type of crystallinity with a monoclinic conformation that is closely packed. The water molecules in this type of structure cannot be removed and are limited to eight per monoclinic unit. However, a B-type of crystallinity, observed in tubers, has a more open structure and forms a hexagonal unit. This conformation creates large a void in the centre of the unit which normally includes thirty-six molecules. A C-type of crystallinity has a core

of B-type crystallites surrounded by A-type crystallites and has been found in legumes (Alvarez-Ramírez, 2023; Enpeng et al., 2014).

The 5th structure of the starch granules represents the molecular order of the amylose and amylopectin molecules. Amylose is mostly linear and composed of around 500-2000 α-(1,4) D-glucopyranosyl units. Amylopectin is highly branched containing about 10^6 glucose units linked with α-(1,4) and α-(1,6) linkages (Hancock & Tarbet, 2000; Yin et al., 2018). Starch can be characterised depending on the amylose content from less than 15% termed waxy, 20-35% (normal) and higher than 40% (high in amylose) (Alvarez-Ramírez, 2023).

The presence of pores, channels, and cavities in the morphology of the starch granules has been observed in botanical sources with A-type crystallites including rice and maize (Alvarez-Ramírez, 2023). Protein can be found on the surface of the starch granules, sometimes associated with lipids as well as inside the pores and cavities of the starch granule's structure, these proteins are termed starch granule-associated proteins (SGAP) (Ma et al., 2022).

1.4.1 Structural characterisation of proteins

Proteins in plants are synthesised in the rough endoplasmic reticulum (ER) and are deposited within the ER in organelles or storage vacuoles called protein bodies (Shewry & Halford, 2001). The protein synthesis of wheat showed several stages of growth including the accumulation of the proteins in organelles (protein bodies) during the first months of grain development followed by the coalescence of these protein bodies into

larger protein bodies. The fragility of the membrane covering those protein bodies and the pressure exerted by the growth of the starch granules leads to the release of the protein bodies and the formation of a protein matrix (Zhou et al., 2013). In legumes, there are three stages of protein synthesis including transcription of the storage proteins' genes into RVA polymerases, translation of the genetic information into proteins and the deposition of the storage proteins into protein bodies (Millerd, 1975).

Proteins have a three-dimensional conformation divided into the primary (the amino acid sequence), secondary (local folding conformations; α-helix, β-sheet), tertiary (folding in a three-dimensional shape) and quaternary structure (the organisation of more than one polypeptide) (Buxbaum, 2015) as shown in Figure 1.2.

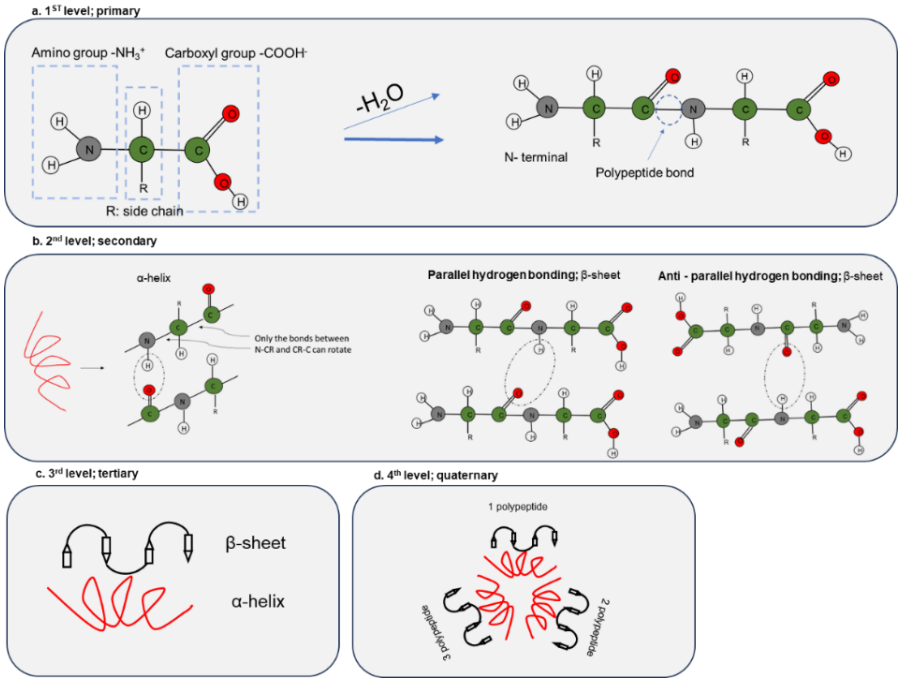


Figure 1.2 The levels of protein structure starting from the (a) primary structure (amino acid structure and polypeptide chain formation) and followed by the (b) secondary (α-helix, parallel and antiparallel β-sheet), (c) tertiary (single polypeptide) and (d) quaternary (two or more polypeptides).

The structure of amino acids contains a carbon atom linked to amine, carboxylic acid, hydrogen, and a side chain. The amino acids in the primary structure are linked by peptide bonds involving the loss of a water molecule and the reaction of the amino group of one amino acid with the carboxyl group of another amino acid as shown in Figure 1.2a. The biological function and three-dimensional structure of the proteins are defined by the sequence of the amino acids. The side chains can be acidic, basic, polar, or non-polar and define the shape of a protein, interaction with the environment and functionality (i.e., solubility).

The secondary structure involves the arrangement of the polypeptide chains formed by the amino acids in the primary structure. Those arrangements consist of the α-helix which has a right-handed and helical coil structure. Hydrogen bonding in the structure of the α-helix happens every fourth amino acid as shown in Figure 1.2b. The other arrangement in the secondary structure is named β-strand which can have a parallel or an antiparallel structure. Depending on the orientation of the C=O and N-H groups parallel or antiparallel β-sheets are formed. Both types of secondary structure exist in the globular proteins.

The schematic representation of the tertiary structure is shown in Figure 1.2c and is a three-dimensional arrangement involving a single polypeptide with secondary structures and provides the proteins' structural and functional properties. The tertiary structure contains interactions between the side chains of the amino acids including hydrophobic interactions (between non-polar amino acids), hydrogen bonding, ionic bonding (between positively and negatively charged

amino acids), Van Der Waals forces and cysteine residues (disulfide bonding). The hydrophobic amino acids are oriented within the core of the protein molecules to avoid contact with the water in globular proteins.

The quaternary structure is the final structural level of proteins and involves the association of one or more polypeptide chains through non-covalent bonds (hydrogen, Van Der Waals) into a close arrangement as shown in Figure 1.2d. Each polypeptide has a primary, secondary, and tertiary structure. (Damodaran & Parkin, 2017; Nadathur et al., 2017; Shevkani et al., 2019).

1.4.1.1 Protein classification according to Osborne and amino acid composition

There are two main types of protein, fibrous and globular. The fibrous are long and stranded in shape, have a structural role (protecting the plant tissues) and are insoluble in water. Globular proteins are spherical in shape, have a physiological role (i.e., acting as transport molecules) and are soluble in water, acids, or bases. The fibrous proteins are found in animal-based sources (collagen) and globular are found in plant-based sources (storage proteins) (Martins et al., 2018). Proteins are classified according to the source of origin, solubility, shape, physiological role, and secondary structure. Osborne's classification is used widely in the literature and divides the proteins according to solubility into four classes. Specifically, albumins are water soluble, globulins are soluble in dilute salt solutions, prolamins are soluble in 70% ethanol and glutelins are soluble in dilute alkali and dilute acid (Jarpa-

Parra, 2018; Taylor & Taylor, 2017). The composition of the proteins according to Osborne fractionation in legume and cereal grains used in this research can be found in Table 1.3.

Table 1.3 Protein composition in legumes and cereals used in this research according to Osborne fractionation.

	Water soluble	**Salt soluble**	**Ethanol soluble**	**Acid soluble**	
	Albumin (%)	Globulin (%)	Prolamin (%)	Glutelin (%)	Literature
Lentils	3.80-16.80	47.00-70.00	3.00-3.50	11.00-14.90	(Boye et al., 2010; Jarpa-Parra, 2018; Joshi et al., 2017)
Pea	20.00-50.00	24.00-72.50	4.00-5.00	3.00-4.00	(Boye et al., 2010; Martínez-Villaluenga et al., 2008; Reinkensmeier et al., 2015)
Corn	7.00-8.00	5.00-9.00	39.00-52.00 (zein)	25.00-40.00	(Anderson & Lamsa 2011; Arendt & Zannini, 2013; Shukla and Cheryan 2001)
Wheat	≈10-22.50		≈80.00 (gliadin + glutenin)	na*	(Arendt & Zannini 2013; Laze et al. 2020; Žilić et al. 2011)
Pearl millet	32.00		43.00	25.00	(Taylor & Taylor, 2017)

*na; not available

The water-soluble albumin fraction in legumes has a low molecular weight (5-80 kDa) and includes metabolic, enzymatic, and non-enzymatic proteins and anti-nutritional components including trypsin and amylase inhibitors. The salt-soluble globulin fraction in legumes is divided into 7S vicilin and 11-12S legumin in a ratio of 10.5:2. Legumins are hexamers with a molecular weight of 320-380 kDa. Legumins are composed of acidic and basic subunits linked with a disulfide bond. Vicilin is a trimer including monomers with a molecular weight of 50-60 kDa bonding with non-covalent hydrophobic interactions (Jarpa-Parra, 2018; Shevkani et al., 2019).

The 7S and 11S present structural differences between the different legumes depending on the subunit profile and amino acid sequence which affect the overall functionality of the protein. For example, the 7S globulin of soybeans comprised three types of subunits (α, α', β). The combination of those three subunits affects functionality. The core of the subunits was responsible for surface hydrophobicity and thermal stability. Similarly, the 11S legumin of soybeans is composed of three distinct types of subunits (G1, G2, G3, G4, G5) composed of acidic and basic polypeptides. Depending on the amino acid sequence of each polypeptide in the subunits, differences in gel strength were observed (Zhou et al., 2013).

The major protein class in cereal grains is prolamins, alcohol-soluble protein, which usually has a low content of the amino acid lysine but a

high content of sulphur-containing amino acids like methionine and cysteine (Nuutila & Ritala, 2004). Prolamins have different names specific for each cereal and their molecular weight varies from 10.000-100.000 Da. For example, gliadins are found in wheat, zein is found in maize, pennisetins in millet, hordein in barley, and kefarin in sorghum (Guerrieri, 2004).

There are differences in the amino acid composition of prolamins between the different cereals. Zein has a high composition of non-polar amino acids and low basic and acidic amino acids which affect solubility. Zein has a molecular weight of 44.000 Da occurs in an aggregated form and is linked with disulfide bonds (Shukla & Cheryan, 2001). Gliadins are single polypeptides divided into four groups including α, β, and γ depending on their electrophoretic mobility starting from fast to low and have a molecular weight of 30.000-80.00 Da (Guerrieri, 2004).

The water-soluble albumin proteins and salt-soluble globulins in cereals can be enzymes including trypsin and amylase inhibitors with a lower molecular weight in comparison to prolamins (<30.000 Da). The globulins have been found in the form of 11S and 12S legumins (Guerrieri, 2004).

There are differences in the amino acid composition between the lentils and the millet as can be seen in Table 1.4. Lentils are deficient in methionine and cysteine and millet in lysine (Joshi et al., 2017; Samaranayaka, 2017).

Table 1.4 Profile of essential amino acid in lentils and pearl millet compared to the profile of requirements in adults.

Amino acid	Lentils (%) (Dhull et al., 2022; Fouad & Rehab, 2015)	Pearl Millet (%) (Jukanti et al., 2016b)	Recommended amino acid patterns in adults (%) (FAO, 2013)
Methionine + Cystine	1.14 – 2.10	3.30 – 4.80	2.30
Tryptophan	0.70-1.20	1.40-4.70	0.66
Leucine	7.40-7.80	9.10-17.40	6.10
Isoleucine	3.80-4.70	3.30-5.00	3.00
Lysine	7.00-7.30	2.00-3.70	4.80
Phenylalanine	4.50-6.10	3.70-5.90	4.10
Threonine	3.00-3.80	3.40-4.90	2.50
Histidine	2.50-3.00	2.10-2.60	1.60
Valine	4.50-5.20	4.20-6.00	4.00

Due to the important role of the amino acid composition in the functional properties of the proteins their impact on the protein solubility and water absorption will be discussed next.

1.4.1.2 Protein solubility and water absorption

Solubility is an important property of proteins because it affects protein functionality and thus can be used as an indicator of protein performance (Jarpa-Parra, 2018). It is associated with emulsification, foaming and gelation properties. There are both intrinsic (surface hydrophobicity) and extrinsic factors (salt concentration in solvent, pH of solvent) affecting solubility. The solubility of proteins is hindered when hydrophobic amino acids are present on the surface but promoted with the presence of hydrophilic amino acids on the surface. Lentil proteins have a higher

solubility in alkaline pH (8.0-9.0) but low solubility at an acidic pH (4.0-5.0) due to closeness to isoelectric point (pH 4.5) and therefore absence of repulsion between charged protein molecules. Similarly, millet proteins have a higher solubility at pH 9.0 and lower solubility close to the isoelectric point (pH=4.0-5.0) (Akharume et al., 2020). Moreover, salts can screen the electrostatic repulsion between the proteins and increase solubility. However, both solubilisation and aggregation can be observed depending on the type and concentration of salt (Arntfield & Maskus, 2011; Shevkani et al., 2019).

Water absorption is also an important property indicating the ability of the proteins to prevent syneresis from a food product during storage and processing. Water absorption in proteins depends on the method of extraction, the shape and size of the protein molecules and the polarity of the surface (Jarpa-Parra, 2018). The capacity of the proteins to retain water depends on the number of polar groups (imino, amino, carboxyl, hydroxyl, carbonyl, and sulfhydryl groups) in the polypeptide chain of the protein (Shevkani et al., 2019). The presence of a higher number of charged amino acids increases binding with water and vice versa. For example, non-polar amino acids like alanine and valine tend to bind only with one water molecule but ionic amino acids like aspartic and glutamic acids and lysine tend to bind with four to seven water molecules (per amino acid). The classification of the amino acids depends on their ability to bind with water. Therefore, polar amino acids have a high binding

capacity, nonionised amino acids and intermediate and hydrophobic little to no binding capacity with water molecules (Zayas, 1996).

1.5 Morphology of the lentil and millet grains

Due to the importance of the starch and protein on the flour's functionality, their structural characteristics were discussed in the previous section. However, there is another parameter to take into consideration when researching non-fractionated flours from different families (legumes, cereals). The overall morphology of the grain and the location of the components in the grain structure impact the particle size, and the separation of components and determine the microstructure of the flour thus, the flour's properties. For this reason, the morphological characteristics of the lentils (legumes) and millets (cereals), the grains used in this thesis, will be discussed.

1.5.1 Lentil

A schematical representation of the lentil's grain morphology including microstructural images can be found in Figure 1.3. The outer layer of the legumes is called the seed coat (or testa). The inner layers include the hilum, which is part of the seed and pod connection, the micropyle opening close to the hilum, the cotyledons, and the embryo. The cotyledons constitute a major part of the grain (90%) followed by the seed coat (8%) and the embryo (2%) (Bhatty, 1988; Jennings & Foster, 2020).

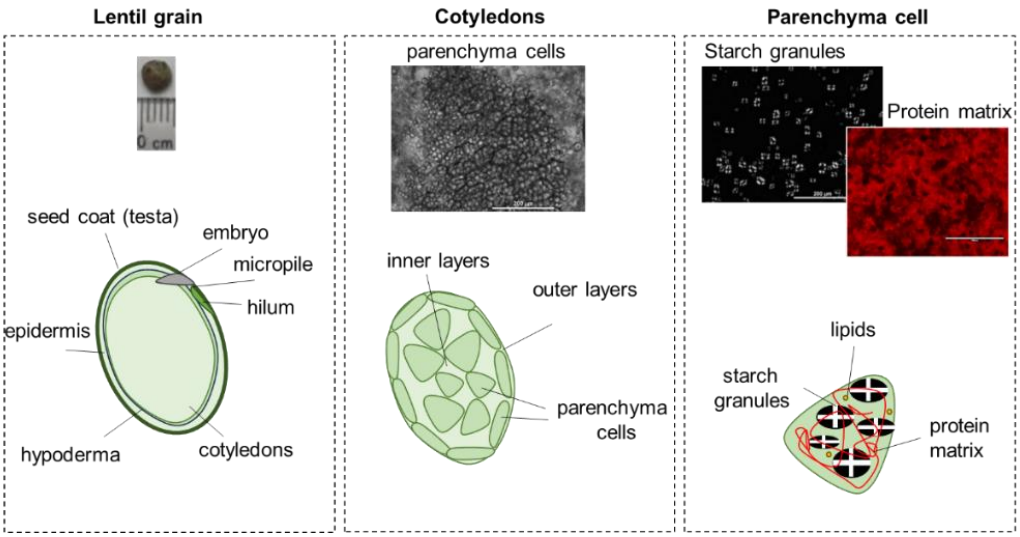


Figure 1.3 Schematic representation of the lentil grain's anatomy, the cotyledons and parenchyma cells. Microscopy images were taken to observe the parenchyma cells, the starch granules and the protein matrix using brightfield, polarised and fluorescent light (rhodamine B stain). Scale bar; 200 μm.

The seed coat of the lentils is thinner in comparison to other legumes (i.e., chickpeas, and beans) and is covered with an uneven conical papillae surface (Tripathi et al., 2019). Under the seed coat, there is the epidermis and below the epidermis, a layer of hypoderma (Erskine et al., 2009). The lentil grain has two cotyledons and little to no endosperm (Jennings & Foster, 2020).

The cotyledons of the lentils include parenchyma cells. The morphological characteristics of the cotyledons in various legumes and lentil types have shown parenchyma cells. The outer layers of parenchyma cells in the legumes have an elongated and compact arrangement in comparison to the round-shaped and loosely packed inner layers (Otto et al., 1997). The outer layers of the parenchyma cells are composed of hydrophobic cell wall materials including cellulose,

hemicellulose and pectin and the inner layers are composed of a protein-lipid membrane. The outer layers provide support and protection, and the inner layers control the ions and molecules' movement in and out of the cell. The cell wall can protect the inside of the cells up to 50 ^{o}C (Dhital et al., 2016). A middle lamella is attached to every parenchyma cell and creates intercellular spaces within the cotyledon that are larger between the parenchyma cells of the inner layer. The size, shape and thickness of the parenchyma cells vary between the different legumes and within the cell wall of a single cotyledon (Siqueira et al., 2018).

The proteins, starch and lipids can be found within the parenchyma cells. Protein is stored in small organelles called 'protein bodies' of 2-20 μm diameters, these organelles have a homogeneous or granular structure and are embedded within a proteinaceous matrix (Tully & Beevers, 1976; Zhou et al., 2013). The starch is embedded within the proteinaceous matrix forming a honeycomb structure. Lentil starch granules are oval and have a size of 10-45 μm diameter, C-crystal structure (Joshi et al., 2013a) and amylose content reported to vary between 30.6-33.9% (Kaur et al., 2010). Lipids can be found free in the form of spherosomes (Gharibzahedi et al., 2014; Siqueira et al., 2018).

1.5.2 Millet

A schematical representation of the millet's grain morphology including microstructural images can be found in Figure 1.4. The grain or kernel of the millet is called caryopsis and has four main anatomical parts The

endosperm constitutes a major part of the grain (75%) followed by the germ (16.5%) and the pericarp (8.4%) (Wrigley et al., 2017) in pearl millet and finger millet. These grains are enclosed in the pericarp and do not contain a coat thus called ‘naked’ grains (Sruthi & Rao, 2021).

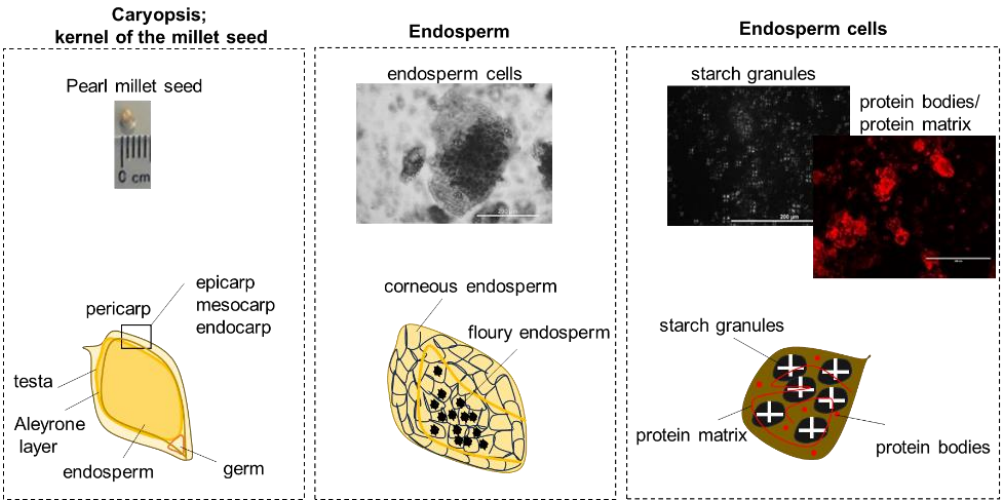


Figure 1.4 Schematic representation of the millet grain, endosperm, and endosperm cells. Microscopy images were taken to observe the endosperm cells, the starch granules and the protein matrix using brightfield, polarised and fluorescent light (rhodamine B stain). Scale bar; 200 µm.

The pericarp contains lipids and antinutrients and is divided into three sublayers (epicarp, mesocarp, endocarp) varying in thickness depending on the type of millet grain (Wrigley et al., 2017). In pearl millet, the epicarp is composed of one or two cell layers and the endocarp is composed of cross and tube cells (Queroz, 1991). Under the endocarp, there is a thin layer (0.2-0.4 µm) of coat (testa) which protects the endosperm during milling because the pericarp is loosely attached and easy to break away (Arendt & Zannini 2013). The aleurone layer can be found under the testa and consists of cells in a single layer and is 25-50 µm long (Arendt & Zannini 2013) surrounding the endosperm and germ.

It contains protein bodies, minerals, B-complex vitamins, enzymes, phytates and phenolic compounds (Queroz, 1991; Wrigley et al., 2017).

The aleurone layer and the endosperm comprise the major storage tissue (Lásztity, 1996; Queroz, 1991). The endosperm can be found under the aleurone layer and is divided into two zones, the corneous endosperm which is located closer to the aleurone layer, and the floury endosperm located under the corneous endosperm. The major components of the corneous and floury endosperms are starch and protein (Gulati et al., 2018).

The proteins of millets are located along with enzymes and minerals in subcellular and spherical in shape organelles called protein bodies (Gulati et al., 2018). Protein bodies in millets were found in two locations. Most of the protein bodies are located in the corneous endosperm comprising the protein matrix (Arendt & Zannini, 2013) and a small amount of protein bodies were free are sporadically dispersed in the floury endosperm. In the floury endosperm, a small proportion of a protein matrix was observed (Arendt & Zannini, 2013). The size of the protein bodies in the pearl millet is about 2-4 µm in diameter (Zhou et al., 2013).

The starch granules are polygonal in shape and densely packed with each other in the floury endosperm (Gulati et al., 2018). Millet starch granules are angular or polygonal and have a smaller size between 10-25 µm in diameter with pores on the surface. There are smaller starch

granules in the corneous endosperm in comparison to the floury endosperm (Gulati et al., 2018; Wrigley et al., 2017; Wu et al., 2014). Lipids are located free in the germ of the millet grain (Sruthi & Rao, 2021).

1.6 Grain processing

The production of flour from grain is conducted using wet or dry milling techniques. Extrusion has also been used but milling is the most popular method. Pre-processing methods have been used to decrease the presence of anti-nutrient factors, improve taste and palatability, ease protein and starch extraction and increase nutritional value (Dhull et al., 2022). Examples of pre-processing methods and their effect on flour characteristics can be found in Table 1.5.

Table 1.5 Examples of pre-processing methods and impact on grain characteristics.

Method	Impact	Literature
Germination	• increases antioxidant activity and protein content • reduces anti-nutrients, starch, lipid, and non-starch carbohydrate content • increases aromatic compounds due to lipid oxidation	(Arendt & Zannini, 2013; Bhati et al., 2016; Dhull et al., 2022; Fouad & Rehab, 2015)
Fermentation	• increase in phenolic compounds and amino acid composition and enhance antioxidant, antihypertensive, and hypolipidemic activity	(Annor et al., 2017; Dhull et al., 2022; Saleh et al., 2013; Sruthi & Rao, 2021)
De-hulling	• decreases anti-nutrient composition • decreases cooking time • increases protein, and starch, and reduces fibre content	(Dhull et al., 2022a; Saleh et al., 2013; Vishwakarma et al., 2018; Wang et al., 2009; Wood & Malcolmson, 2011)
Soaking	• reduces anti-nutrient compounds and minerals	(Bhati et al., 2016; Saleh et al., 2013)
Extrusion	• reduces anti-nutrients • increases starch and protein digestibility and availability of bioactive compounds	(Ek et al., 2021; Jaybhaye et al., 2014)

1.6.1 Typical lentil processing steps

Lentils are usually consumed as whole or de-hulled cooked seeds but also as ingredients in the form of flour, isolated protein, and starch in food products. Processing of the lentil grains includes dry processing (<10% moisture) (milling) to produce flours that can be used in dough-based products (i.e., snacks) and high moisture (>10%) thermal treatments (cooking) to sell as ready-to-eat products (i.e., in cans and jars). The grain of the lentils can also be pre-processed, as described in Table 1.5.

Dry processing involves the cleaning of the grain followed by de-hulling (separation of the hull from the cotyledons) and splitting (separation of the cotyledons) if needed. Red lentils are usually consumed after de-hulling the grain and splitting the cotyledons. Milling of the lentil grain follows as a standard method to prepare flour and reduce the particle size of the grains. Milling efficiency can be affected by various parameters including seed size, shape and weight, moisture content, composition, and de-hulling characteristics (Thakur et al., 2019).

The seed size and shape of different legumes are attributed to genotypic characteristics and affect milling efficiency. The shape of legumes can vary from cylindrical to pyramidal, oval, and kidney shaped. For example, small seeds and seeds with sharp and angular edges break easily and are difficult to de-hull. The hull does not separate from the cotyledons, the macronutrients within the cotyledon cells are protected and more difficult to separate (Vishwakarma et al., 2018). In round types of seeds, there is an increase in the contact of the mechanical forces with the

surface of the seed resulting in larger flour yields and lower coarse seed parts of the seed (Wood & Malcolmson, 2011).

The rupture force is reported to be higher for green whole lentils in comparison to red whole lentils which was attributed to the higher protein and starch content in the green whole lentils (Gharibzahedi et al., 2014).

Flour composition and milling efficiency have been correlated with the fibre content from the hull and the cotyledons' cell walls. The structure of lentil grains was discussed in Section 1.5.1. The hulls (outer layer) bind to the cotyledons (inner layer) through gums. The gums are comprised of a cellulosic microfibril surrounded by a network of non-starch polysaccharides and proteins. The quantity and nature of those gums determine the strong or loose attachment of the hull and the cotyledons. Arabinogalactan is a type of gum associated with easy-to-mill legume grains but pectin type of gum with difficult-to-mill legume grains (Vishwakarma et al., 2018).

In easy-to-mill legumes, the hulls are easily removed and leave the cotyledon cells unprotected and exposed to the mechanical forces of a mill (Vishwakarma et al., 2018). Hulls impact the flow and physical properties of the raw grains during milling. Therefore, decreasing milling efficiency prevents the separation of the protein matrix from starch granules. Whereas de-hulled grains had a lower particle size after milling with enhanced protein-starch separation (Saldanha do Carmo et al., 2020).

The cell wall thickness, rigidity and cell-to-cell adhesion could explain differences in the particle size and fragmentation between legume flours (Gharibzahedi et al., 2014). For example, a greater cell wall thickness produces a higher particle size when an impact mill is used on legumes (Thakur et al., 2019).

The storage of legumes after milling could impact the quality of the food products. The cultivar, storage time and processing affect the flavour of the legumes due to the oxidation of unsaturated fatty acids. However, there is still little understanding of the volatile compounds responsible for this off-flavour of commercial legumes including lentils, peas, and chickpeas (Rajhi et al., 2021). The storage of whole and de-hulled flours from legumes for a month did not show significant changes in the pasting properties. The colour of whole legume flours was darker after storage due to changes in the chemistry and structure of polyphenols found in the hulls of the grains. These changes increased bitterness in bread formulation (Sopiwnyk et al., 2020).

An extra step involving fractionation of the flours using air classification and sieves to prepare protein-rich, starch-rich, or fibre-rich materials can be applied for food product application. Extraction or isolation of the flour components can be achieved by applying wet processing methods with water, alkaline, and acid solutions and ultrafiltration (Joshi et al., 2017; Wood & Malcolmson, 2011).

1.6.2 Typical millet processing steps

Millet is used as a coarse flour in porridge and as a fine flour in bread, snacks, and infant foods in many African and Asian countries (Jaybhaye et al., 2014). Processing of millets improves the nutrient availability and organoleptic characteristics of the final products (Rani et al., 2018; Saleh et al., 2013). The grain texture and hard seed hull creates difficulty in processing the grain. The presence of anti-nutrient factors decreases the bioavailability of the protein and mineral availability. Pre-processing methods as detailed in Table 1.5 are used to enhance the nutritional profile of the millets (Jaybhaye et al., 2014).

De-hulling using abrasive, disk mills or mechanical de-hullers is used to remove the outer layers of the grains, improve bioavailability, and reduce the cooking time (Rani et al., 2018) like in legumes. This process is also used to produce white flour by excluding the dark-coloured outer layers of the cereal seed which is a barrier to consumer acceptability (Rosentrater & Evers, 2018).

Millet flour is mostly traditionally produced using household methods like cracking by hand or by using electrical devices like mills (Saleh et al., 2013) but there is still a lack of large-industrial-scale processing technologies for food production (Jaybhaye et al., 2014). The milling methodology of millet is not well established mostly due to a lack of industrial (large-scale) utilisation. Milling has been used to produce meals (coarse particles), and flour (fine particles) and remove the bran (grain, germ, and pericarp) (Wrigley et al., 2017). Flour from millet has

been incorporated into wheat flour in the production of extruded products (chapatti), bread, pasta, and snacks. The substitution of wheat flour with millet decreased the volume of the bread but did not change the colour and crumb taste (Jaybhaye et al., 2014).

Millet grain can be stored for a long time but, the flour has a short life due to oxidation. Oxidative and hydrolytic changes that unsaturated fatty acids undergo during storage cause rancidity and loss of flavour in millets (Bhati et al., 2016; Kapoor & Kapoor, 1990; Yadav et al., 2012). For this reason, most research on millet flour has been focused on pre-treatment methods. A combination of methods has been applied to increase storage stability and flour quality by combining, wet heat treatments followed by drying, de-hulling and grinding. De-hulling followed by autoclaving and milling has also been applied (Rani et al., 2018; Sruthi & Rao, 2021).

Overall, the literature discussed here showed that milling is the primary step for both the lentil and the millet grains prior to further processing thus, a further discussion on milling will follow.

1.7 Milling

The term milling is used to describe the decrease in grain's particle size and flour production or botanical tissue separation (seed coat, endosperm, cotyledons, germ) (Norman & Evers, 1994; Oghbaei & Prakash, 2016). There is a variety of milling equipment including hammers (Loubes et al., 2018a), pins, jets (Drakos et al., 2017) and ball

mills (Huang et al., 2008) which employ forces like impact, compression, shear, and attrition.

Milling is a mechanical process that promotes bioavailability by removing the outer layers of the grain (i.e., seed coat) and increasing the available surface area of the components (starch, proteins) for reaction (i.e., water binding) (Alldrick, 2002). For example, lower particle size and higher available surface increase the water absorption, swelling and hardness in biscuits from sorghum (Dayakar Rao et al., 2016). Similarly, de-hulling and milling of cowpeas had the same effect on the functional properties of the flour including higher water absorption and protein solubility (Kerr et al., 2000).

When milling breaks the grain converting it into a mixture of particles varying in size and composition including whole cells or cell clumps, fragments of the cells and fragments/ clusters of starch and/or protein components (Norman & Evers, 1994). These changes in the structure of the grain and the frictional heat and mechanical energy have been found to induce physical and chemical changes to the components' structure including damage to the crystallinity and morphology of the starch granules (He et al., 2014; Huang et al., 2008; Palavecino et al., 2019) but scarce evidence exists on plant-based proteins. The impact of milling on the starch and protein structure and functional properties will be discussed later in this chapter in Sections 1.7.1.1 & 1.7.1.2.

The impact of milling on the physical and chemical properties of unfractionated flour affects the texture and structure of the final product.

Milling was reported to increase the crunchiness and crispiness of bread (Sivakumar et al., 2022) and enhance the springiness of egg white protein gels (Wu et al., 2022). One exception is a cryogenic type of milling which has shown a lower level of structural damage (Huang et al., 2008). Cryogenic mills have been found to decrease heat generation during milling (Hasjim et al., 2013). However, it has been observed that heat generation can also be controlled by including pause intervals during the mill operation (González et al., 2018).

This thesis focuses on one type of mill, the ball mill. Most research has been conducted on the ball milling of fractionated material (isolated starch and protein) which could be used as a basis for understanding the impact of ball milling on non-fractionated flours, thus it is discussed here.

1.7.1 Ball milling

The ball mill has been characterised as a highly efficient and environmentally friendly method to modify whole flours (Palavecino et al., 2019; Tian et al., 2022), starch (Juarez-Arellano et al. 2021; Tian et al., 2022) and protein isolates (Liu et al., 2017). The ball mill uses collision, friction and shear forces resulting from both the grinding balls and containers when in operation (Liu et al., 2017). The motion of the balls creates kinetic energy acting on the material, breaking down the chemical bonds and reducing particle size. Ball mills are capable of breaking, rearranging, and deforming the particles of the material (Bangar et al., 2023).

Physical modification by ball milling has been applied mostly on isolated starch to increase the starch solubility in ambient temperature by destroying the crystalline regions of the starch granules' ordered structure and increasing the amorphous domains (Bao et al., 2018). During ball milling the collision, friction and shear have also been found to denature proteins, and expose the hydrophobic amino acids buried in the protein structure which increases the protein hydrophobicity and changes the functional properties of proteins (Liu et al., 2021).

Various ball milling operation principles have been applied to isolated flours from legumes and cereals. However, there is not an established method, yet. The different operational principles applied in isolates, whole flours and parts of the flours can be found in Table 1.6.

Table 1.6 Examples of ball mill operation principles for starch, protein, fibre isolates and whole flours from legumes and cereals according to recent literature.

plant material	container-ball material	operation	literature
potato starch	• silicon nitride, stainless steel, and tungsten carbide balls	• 600 rpm • Various milling times	(Juarez-Arellano et al., 2021)
corn, pinhao starch	• stainless steel	• 30 min • 30 Hz	(Beninca et al., 2020)
corn, potato, tapioca starch	• steel balls	• Various timings	(Dome et al., 2020)
soy protein isolate	• stainless steel	• Various times • 10 Hz	(Liu et al., 2017)
wheat gluten protein	• aluminium oxide	• 400 rpm • Various time	(Liu et al., 2021)
sorghum flour	• zirconium oxide	• 400 rpm • Various treatment time	(Palavecino et al., 2019)
wheat endosperm flour	• na	• 170 rpm • Various times	(Tian et al., 2022)
mung bean flour	• na	• 35 hours • 455 rpm	(Yu et al., 2023)

*na; not available

1.7.1.1 The effect of ball mill on starch granules

The impact of the ball mill has been used as an alternative physical method inducing changes to the structure and functional properties of isolated starch granules from various cereal and legume sources including maize, wheat, and mung bean (Bangar et al., 2023). The mechanical stress of the ball mill disrupts the hydrogen bonds within the molecular chains of the starch molecules. The crystal structure is disrupted, observed as a decrease in the crystalline region and an increase in the amorphous region. Moreover, ball milling changes the characteristics of the starch granule's surface, with an increase in surface area due to fragmentation (Hu et al., 2016).

The surface morphology of isolated maize, potato, and corn starch granules, observed using SEM (scanning environmental microscope), changed after ball milling. The untreated starch presented uniform and smooth surfaces. The ball-milled starches presented a rough surface with small particulate aggregates attached. Damaged starch granules formed clumps with each other and/ or adhered to the surface of starch granules with larger particle sizes contributing to the rough surface morphology (He et al., 2014; Liu et al., 2020; Liu et al., 2011).

The amylose content of the starch granules increases upon ball milling. It has been suggested that breakage of α-1,6 branched points in amylopectin followed by the release of long branch chains of the amylopectin, which are subsequently recorded as amylose (Bangar et al., 2023). An apparent increase in the amorphous content of isolated

starch granules has been observed using X-ray diffraction analysis (He et al., 2014). Fourier transform infrared (FT-IR) analysis showed a decrease in the intensity of the bands corresponding to the crystalline state (1047 cm^{-1}) and an increase in the bands corresponding to the amorphous state (1018 cm^{-1}) indicating that the ordered structure of the starch granules was destroyed. Moreover, the ratio of 1047/1018 cm^{-1} which is used to quantify the degree of ordered structure in starch showed a lower value for the ball-milled starch in comparison to the untreated starch (0.75) (Liu et al., 2011).

Juarez-Arellano et al., (2021), described the three levels of starch damage on isolated potato starch granules. The first level included a loss in birefringence under polarised light, loss of crystallinity and decrease in viscosity and at a secondary level partial agglomeration of damaged starch granules occurred. A higher milling severity (longer milling times or higher speed) and lower particle size increased agglomeration. Starch agglomeration has been observed in maize starch too (Liu et al., 2011). At the third level increasing the ball milling severity further destroyed the granular structure and crystallinity of the starch granules leading to the formation of amorphous agglomerates and loss in the ability to gelatinise and form a viscous paste (Juarez-Arellano et al., 2021).

The damage to the starch granules' structure by milling was associated with lower gelatinisation temperatures, pasting properties, swelling and cold-water solubility (González et al., 2018; Liu et al., 2011; Martínez-Bustos et al., 2007; Moraes et al., 2013) and some examples can be seen in Table 1.7.

Table 1.7 The impact of ball milling on the functional properties of isolated starch granules from various sources including pasting, gelatinisation, water absorption and solubility.

Botanical source	**Operating parameter**	**observation**	**literature**
A-and B-types from wheat	• 0-60 min	• decrease in thermal stability • increase in solubility	(Han et al., 2022)
A-(corn), B-(potato), C-(mung bean) type	• 1 hour	• decrease in solubility and swelling • decrease in pasting values • decrease in initial gelatinisation temperature	(Liu et al., 2020)
Rice (A-type)	• 0.26-4.08 kj/g	• increase in water solubility • decrease in gelatinisation enthalpy • decrease in pasting values	(González et al., 2018)
Maize (A-type)	• 5 hours	• increase in cold-water solubility • decrease in water-holding	(He et al., 2014)

Starch is the major component in cereal and legume flours thus any changes in the structure of the starch granules by ball milling will affect flour functionality. However, in the legume and cereal non-fractionated flours other components are present, such as proteins. The impact of ball milling on proteins should also be considered to understand any changes in the properties of the end product. For example, ball milling of wheat flour showed a decrease in dough development which was linked to the lower solubility of the proteins after ball milling and a reduction in their ability to hydrate and form a strong protein network during kneading (Hackenberg et al. 2019; Vogel, Scherf, and Koehler 2019).

1.7.1.2 The effect of ball mill on proteins

The native form of plant proteins is associated with low functionality due to low solubility which limits food applications. Plant proteins can be found in grains in protein bodies, or as a protein matrix and their bioavailability is restricted by cell wall material as shown in Section 1.5. Changes in the morphology and particle size distribution, structural conformation and consequently the functional properties of the proteins including water absorption, water solubility, emulsification, foaming and gelation observed after physical treatments on plant proteins (Manzoor et al., 2022; Mirmoghtadaie et al., 2016; Nikbakht Nasrabadi et al., 2021).

The morphology of protein isolates after ball milling was observed under a scanning environmental microscope (SEM). Native proteins present a

spherical shape with a smooth surface but after ball milling showed irregular, disordered, and flaky protein fragments with a rough surface and increased porosity (Liu et al., 2021; Manzoor et al., 2022).

In general, the physical modification of proteins showed the disruption of intermolecular interactions in the protein structure including (hydrophobic, electrostatic, hydrogen bonding, disulfide bridges and Vader Walls forces) (Nikbakht Nasrabadi et al., 2021). The structural conformation of the proteins has been evaluated using Fourier transform infrared spectroscopy (FTIR) which indicated changes in the secondary protein structure after ball milling. High contents of α-helix and β-sheet represent a highly ordered structure in native proteins. Ball milling decreased the α-helix and β-sheet and increased the random coil and β-turn content suggesting the transformation of the β-sheet and α-helix to β-turn and random coil (Liu et al., 2021; Ramadhan & Foster, 2018). The conformation of the proteins' secondary structure upon ball milling has been monitored using circular dichroism spectra which showed a decrease in the intensity of the bands corresponding to α-helix (195, 208 and 222 nm) and an increase in the intensity of the peak at 218 nm corresponding to β-turn content confirming a loss in native order (Manzoor et al., 2022).

A change in the structure of the proteins by ball milling has been found to expose the hydrophobic amino acids buried in the structure of the proteins which has been linked to reduced protein solubility. Surface hydrophobicity has been used to evaluate the interfacial behaviour of the ball-milled proteins. Ball milling has been shown to change the tertiary

structure of the protein molecules by unfolding parts of the polypeptide chain exposing the hydrophobic amino acid groups buried in the proteins' globular structure and increasing hydrophobicity (Liu et al., 2021; Vogel et al., 2018). Lentils include about 38% of non-polar amino acids (i.e., valine, isoleucine, phenylalanine) that are hydrophobic in nature thus the exposure of those amino acids would increase hydrophobicity and decrease solubility.

Protein solubility depends on the structure of the protein. An increase in protein solubility has been attributed to lower particle size or protein fragmentation which increases the available surface area of proteins to interact with water (Mirmoghtadaie et al., 2016). A decrease in protein solubility after ball milling could be attributed to the formation of insoluble protein aggregates due to interactions between newly exposed hydrophobic amino acids (Liu et al., 2021).

The thermal properties of native and ball-milled proteins showed differences in the thermal stability, associated with protein structure changes. Ball milling decreases thermal stability by reducing denaturation temperature and enthalpy. A lower degree of α-helix and β-sheet conformation indicated the disruption of intermolecular bonds in the secondary structure of the protein molecules which explained the decrease in the denaturation temperature (Manzoor et al., 2022).

Ball milling increased cold gelation properties including gel strength and water-holding capacity of soybean protein isolates. It was hypothesised that both the exposure of the hydrophobic amino acid and the decrease

in particle size were responsible for the formation of a more dense and homogeneous gel network (Liu et al. 2017).

The impact of ball milling on fractionated starch and proteins was discussed here. Although this literature is important and gives evidence on the impact of ball milling on the structure and functionality of starch and protein, there is still a lack of information on the impact of ball milling on non-fractionated flours. To the author's knowledge, there is only one research addressing the impact of ball milling on both the starch and the protein from non-fractionated wheat flour (Tian, Wang, Ma, et al., 2022; Vogel et al., 2018). Moreover, non-fractionated flours present a higher particle size in comparison to fractionated flours because starch granules are in clumps or embedded in a protein matrix and cell wall material. The presence of non-starch components and particle size in non-fractionated flours could act as a barrier to heat transfer created during ball milling. Also, it has been suggested that non-starch components (protein, cell wall material) act as a cushion to the mechanical forces of the ball mill, and starch granules in whole meal flours might experience a lower degree of damage (Enpeng et al., 2014; Hasjim et al., 2013). It is, therefore, the aim of the first experimental chapter to address this gap.

Another important research gap was found in the application of ball-milled materials. Also, there is no information linking the impact of ball milling on non-fractionated flours with the gel formation and gel properties. As the second experimental chapter addresses this gap, the

fundamentals of gels and the gelation of starch, proteins and starch-protein systems were reviewed.

1.8 Gelation

1.8.1 Definition of gelation and gel classification

Gelation refers to the formation of a continuous three-dimensional network that holds substantial amounts of water (hydrogels), oil (oleogels) or air (aerogels). The three-dimensional network formed at the end of the gelation process is a gel and is composed of polymer molecules called gelling agents (i.e., polysaccharides, protein). The term gel refers to a material in an intermediate state with both elastic (solid) and viscous (liquid) characteristics leading to a semisolid texture. Gels (hydrogels) entrap and immobilise water and other food components in their structure (Joshi et al., 2013a). The first step in the gelation process involves the unfolding or dissociation of the gelling agents to expose reactive sites which can be reversible. The second step involves the interactions between the unfolded gelling agents to make complexes with a higher molecular weight and is not reversible (Nath et al., 2023; Siddiqui et al., 2022).

A gel network is formed by cross-linking and usually involves weak interactions including hydrogen bonds, electrostatic interactions, and hydrophobic interactions. Hydrogen cross-linking refers to the gel formation when hydrogen bonding occurs between oxygen atoms and hydrogen atoms. Ionic cross-linking occurs by the pairing of anions and cations within the polymer structure or between polymer anions and metal cations. Hydrophobic association occurs by hydrophobic

interactions of hydrophobic groups leading to aggregation. Chemical cross-linking occurs through intermolecular disulfide bonds, Maillard reactions and enzymes (Liu et al., 2022). There are two categories of cross-linking driven by physical forces including heat and pressure or driven by chemical processes including acidic, ionic, and enzymatic processes (Nath et al., 2023). The polymer chains form extended junction zones when a gel is formed in the absence of chemicals in contrast to the formation of single covalent bonds found in chemically cross-linked gel networks (Nunes, Raymundo, & Sousa 2006).

A feature commonly observed is that the preparation of a gel in a container will give a specific shape which is retained after the removal of the container. Gels are deformed when stress is applied, store elastic energy and recover to their original shape after removing the stress. A part of the gel cannot recover after deformation and like a liquid, new bonds take the position of the broken bonds and at high stress, gels fracture (Morris, 2007).

Gel microstructures present in food products include jams, jellies, desserts, and yoghurts, formed using various polysaccharide and/or protein gelling agents. The type of gel structure depends on the structure and physicochemical properties of the gelling agents (dimension, surface activity, polarity, thermal stability) and the environmental conditions during gelation including pH, temperature, water activity, shear, and ionic strength (Banerjee & Bhattacharya, 2012; Nazir et al., 2017).

For example, the amylose/ amylopectin ratio has been found to influence the hardness of starch-based gels. Amylose is important for the formation of a gel network and low amylose content formulations create softer gels. Softer gels are formed in lower amylose concentration because the branched structure of the amylopectin leads to a less frequent cross-linking resulting in the formation of aggregates instead of a continuous network (Berski & Ziobro, 2018).

Another example is the effect of pH on the formation of protein gels. Heat-induced gelation of globular proteins involves disulphide, Van der Waals, hydrophobic and electrostatic interactions. However, the extent of those interactions depends on extrinsic factors such as the pH. Proteins have no net charge at their isoelectric point and thus no electrostatic repulsion which enhances protein-protein interactions and leads to the formation of aggregates which associate with larger agglomerates and connect to form a three-dimensional network. However, a pH far from the isoelectric point increases the surface charge and electrostatic repulsion between the protein molecules therefore, preventing protein-protein interactions and the formation of a three-dimensional network (Singh et al., 2014; Totosaus et al., 2002).

The number of the gelling agents involved in the gel formation, the gelation mechanism, the structure, and morphology are used in gel classification as seen in Figure 1.5.

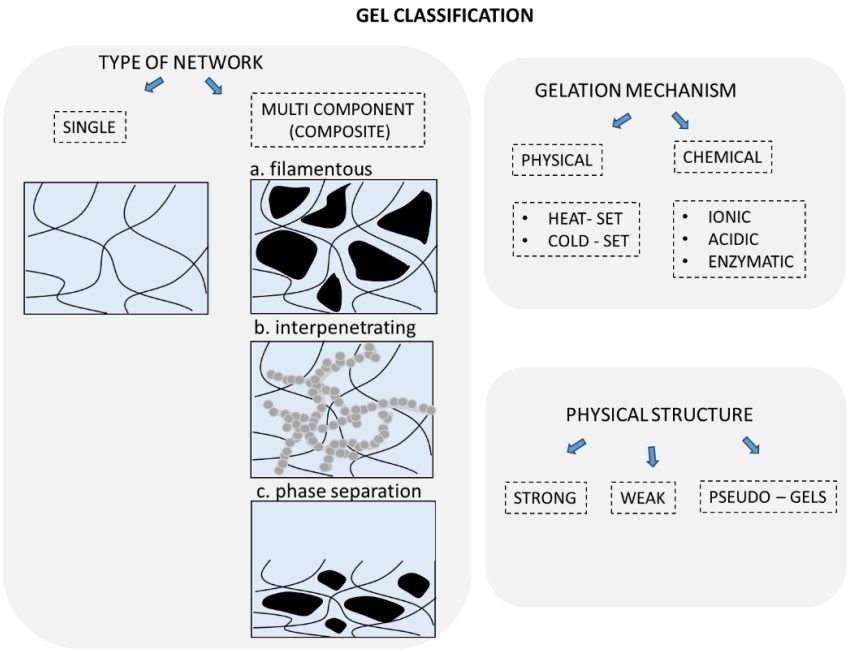


Figure 1.5 Gel classification according to the type of network, gelation mechanism and physical structure. The schematic on the type of network shows only a few examples and does not cover all range of different networks.

The type and number of gelling agents are factors used in gel classification (Banerjee & Bhattacharya, 2012). There are two distinct types of gels based on the number of gelling agents including single and multi-component gels. The single networks are formed by a single gelling agent such as proteins or starch as seen in Figure 1.5. In single types of gels, the rheological properties and textural performance are constructed by one component however, the multi-component or mixed gels are formed by the combination of more than one component and therefore the properties and behaviour of the gels depend on more than one component (Zha et al., 2021). The multi-component gels can be formed by the combination of a gelling agent with a non-gelling agent or two gelling agents and are distinguished into various categories depending on the morphology of the gel structure (filamentous, interpenetrating, phase separation) (Joshi et al., 2014).

There are two types of interactions occurring when proteins and polysaccharides are combined including associative phase separation (thermodynamic compatibility where starch and protein form complexes) which leads to a three-dimensional gel network (filamentous or interpenetrating) or segregative phase separation (thermodynamic incompatibility where starch and protein do not form any complexes) (Kazemi-Taskooh & Varidi, 2023).

A filamentous multi-component network has been characterised as a network where one component forms a continuous network and the other acts as a filler in the space of the continuous network as seen in Figure 1.5a. Components can act as active fillers thus contributing to the rheological, structural and textural properties of the gel or as inactive fillers without playing any role in the properties or behaviour of the gel.

Another type of multi-component or mixed gels is the interpenetrating network as seen in Figure 1.5b. In this case, both components gel independently, and do not form any covalent bonds with each other but are partially interlaced on a molecular scale. Various methods have been used to form that type of gel including heating, ionic and enzymatic cross-linking (Banerjee & Bhattacharya, 2012). The interpenetrating networks are considered to have a better mechanical performance and textural properties in comparison to the conventional single types of gels which has been attributed to the chain entanglement and non-covalent interaction (i.e., hydrogen bonds) between the gelling agents and the interactions between the hydrophilic groups (i.e., hydroxyl, carboxyl, amino) of the gelling agents and water. Moreover, the properties of the

interpenetrating types of gels can be manipulated by internal (i.e., the molecular weight of the polymers) and external factors (i.e., pH, temperature) (Du et al., 2021).

However, a mixture of uncharged or similarly charged polymers can also lead to phase separation as seen in Figure 1.5c. Phase separation of composite mixtures has been found to occur when the total polymer concentration exceeds 4% depending on external factors (pH, ionic strength) (Kasapis, 2008). For example, in a protein-polysaccharide mixture when the pH<pI of protein, electrostatic interactions occur between the positively charged proteins and the negatively charged (anionic) polysaccharides forming a composite gel. However, at pH>pI proteins create negative repulsions between proteins and polysaccharides resulting in phase separation (Kazemi-Taskooh & Varidi, 2023).

According to the gelation mechanism gels can be classified into physical where gelation occurs using physical forces (heat, pressure) and chemical-induced gels where gelation occurs using chemical processes (acidic, ionic, enzymatic). Temperature is an important parameter in gelation that classifies gels into two categories heat-induced gels and cold-induced gels. In heat-induced gels, the gelation mechanism occurs in two steps. The first step includes the dissociation or unfolding of the molecules due to an increase in the temperature. The second step involves the association or aggregation and gelation of the molecules when temperature is decreased. However, in cold-induced gels, the mechanism is slightly different. The first step involves the heating of a

solution to promote dissociation or unfolding of the molecules and obtain protein aggregates and a cooling step follows. However, gelation does not take place on the cooling step but is followed by another step which includes the addition of chemical means (i.e., alkali, acid) that promote gelation at ambient temperature (Yang et al., 2021).

The unfolding of polysaccharides usually occurs at 50-90 ^{o}C and for proteins at higher temperatures usually from 80-95 ^{o}C because higher energy is needed to expose the hydrophobic amino acids buried in the core of the protein which is important because the hydrophobic amino acids interact with each other, form aggregates and subsequently a protein gel network (Du et al., 2021).

Chemically induced gels can be formed using ionotropic, acidic, and enzymatic processes. Ionotropic hydrogels are an example of hydrogels formed through cross-linking in the presence of polyelectrolytes. Changes in the gelation mechanism (i.e., the temperature of gelation) and structure (i.e., the gel stiffness) of the network are facilitated by the selection of ions (Djabourov et al., 2013). This technique has been mostly applied for the encapsulation of drugs and biomolecules (Abitbol et al., 2021). Acid-induced gelation has been used in cold-set gelation processes to reduce the electrostatic repulsion between the protein aggregates at room temperature and induce gel formation (Kharlamova et al., 2018). Enzymatic hydrolysis has been applied to plant-based proteins to increase solubility, and surface hydrophobicity, reduce the molecular weight and expose their sulfhydryl content. A low degree of hydrolysis (<6%) on peanut proteins showed a significant increase in

their gelling capacity and hardness compared to their non-enzymatically treated counterparts (Chen & Campanella, 2022).

Polymer networks can be categorised according to their physical structure in strong, weak and pseudo gels. Strong gels are usually chemically induced cross-linked gels which cannot be reformed when broken. However, when cross-links can be broken the gels are characterised as weak and such gels can be colloidal or biopolymer gels. Pseudo-gel is usually entangled polymer systems which exhibit gel-like properties because the entanglements of the polymer chains imitate chemical cross-links over a range of time scales. However, constant applied stress on a pseudo gel will show a liquid-like response (Djabourov et al., 2013).

The strength of a gel depends on the density of the cross-links with a higher density of cross-links forming strong gels. The spacing between the cross-links, the molecular weight of the polymers and the amount of solvent (water) affects the strength of a gel. Increasing the spacing and strength of a gel can be reduced by increasing the spacing between the cross-links or by increasing the molecular weight of the polymer chain (Grillet et al., 2012).

1.8.2 Mechanism of gelation in single component gels

1.8.2.1 Starch-based gels

Starch gels are formed after gelatinisation a process that starch undergoes in excess water and elevated temperature (exact temperature varies between starches from different botanical sources). In temperatures lower than the onset temperature of gelatinisation (between 50-75 ^{o}C) the changes in the structure of the gel are reversible upon drying with the starch granules returning to their native form. However, above the onset temperature of gelatinisation, the changes are irreversible (Donmez et al., 2021).

During gelatinisation, starch granules swell, rupture, and undergo an order-to-disorder phase transition (Djabourov et al., 2013) but starch gelation describes the process where free amylose or amylopectin that has been liberated from the granule forms an ordered structure. At a critical concentration and a certain degree of cross-linking the starch solution will transform into a gel with a stable network. The key factors for gel formation are the composition of starch and the conditions of gel formation (Nath et al., 2023). A schematic representation of the hydrothermal changes starch undergoes during gelatinisation and gelation can be found in Figure 1.6.

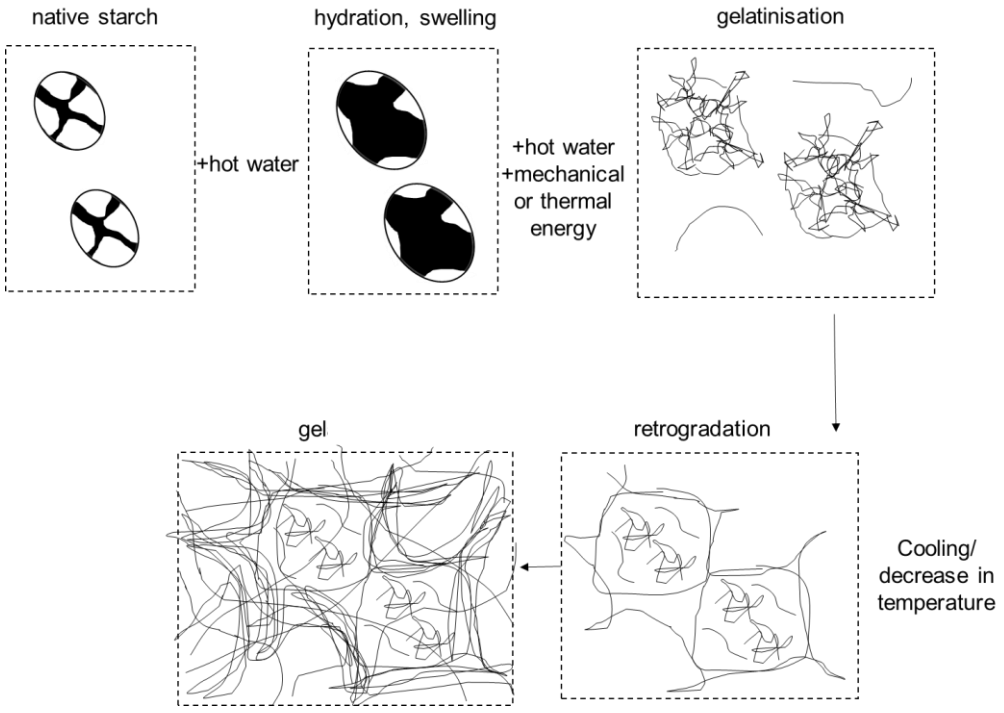


Figure 1.6 Hydrothermal changes of starch granules in temperature and excess of water starting from the starch in the native structure and presenting birefringence under a polarised microscope. During the heating step the temperature increases, starch hydrates, swells and loses its birefringence. The disruption of the starch granules leading to gelatinisation and the formation of a paste follows. At the cooling step when temperature is lowered and during storage, retrogradation occurs due to the association of the amylose molecules (and amylopectin molecules) forming a gel. Adapted from Goesaert et al., (2005).

Upon gelatinisation, the hydrogen bonds of the amylopectin's double helices are disrupted and followed by the leaching of the amylose from the swollen starch granules (Acevedo et al., 2013b). Gelatinisation can be monitored by changes in crystallinity which can be observed using a polarised light microscope (loss in birefringence), by using the X-RAY diffraction resulting from the parallel packing of the amylopectin double helices or by changes in the enthalpy and temperature of gelatinisation. Gelatinised starch does not present any X-RAY pattern and has been characterised as amorphous (Sopade et al., 2006).

However, A- or B-type crystallinity might develop with time depending on the circumstances. Although in the native starch granule, the

amylopectin constitutes the crystalline domains in the gelatinised form both the amylopectin and the amylose can form crystalline domains. The formation of double helices by the amylose and amylopectin after gelatinisation is called retrogradation and can be seen by changes in the rheological, textural and macroscopical properties of the gels (Eliasson, 2010). Amylose has a short re-crystallisation time starting from minutes after gelation to hours in comparison to amylopectin's recrystallisation time (hours to days) (Acevedo et al., 2013b).

In many food products at the end of gelatinisation and in the final gel structure there are remnants of starch granules which can be observed under the fluorescent microscope as swollen and fragmented starch granules within the continuous gel network. Those remnants are called starch ghosts and are molecules leached from the starch granules during gelatinisation that occur by an incomplete dissolution of the starch granules during gelatinisation. Complete gelatinisation has been observed only in severe conditions like elevated temperatures exceeding 100 ^{o}C (Eliasson, 2010).

1.8.2.2 Protein-based gels

Protein gelation requires denaturation before gelation and is performed under heat, and pressure, using chemical means (acidity, ionic strength) or biological means (enzymatic methods). The first step in protein gelation is denaturation which involves the unfolding or dissociation of the protein molecules leading to the exposure of reactive sites. The second step of protein gelation involves interactions between the

unfolded molecules leading to aggregation (Aguilera & Rademacher, 2004; Prameela et al., 2018).

There are various extrinsic and intrinsic factors affecting the gelation of proteins as can be seen from Table 1.8.

Table 1.8 The effect of extrinsic and intrinsic factors on the formation of protein gel according to Totosaus et al., (2002).

Extrinsic factor	**Action**
Protein concentration	Minimal protein concentration is needed to form a gel network
pH	>pI*< (high charge, prevention of gel formation) pH = pI (low charge, gel formation)
Temperature	Denaturation (heat-set gels, cold-set gels) and gelation (heat-set gels)
Ionic strength	<ionic strength (fine-stranded gels) >ionic strength (gel network)
Intrinsic factor	
Hydrophobicity	Affects protein-protein and protein-solvent interactions
Electrostatic interactions	Affects protein-protein and protein-solvent interactions
Disulfide bonds	Increases the molecular weight thus gel strength
Amino acid composition	>31.5% hydrophobic AA* (opaque gel) <31.5% hydrophobic AA (translucent gel)
Molecular weight	Affects gel strength

*pI; isoelectric point, AA; amino acid

There are two types of protein gels depending on the gelation mechanism, heat-induced and cold-induced. In heat-induced gelation, the protein molecules unfold due to increased temperature and form aggregates by decreasing the temperature. The rate of heating affects the size of the aggregates. Fast heating of a protein solution induces the formation of aggregates with small particle sizes and vice versa (Aguilera & Rademacher, 2004; Munialo et al., 2017).

Chemical solutions are used for the formation of a cold-set protein gel. Cold gelation involves the heating of a protein solution to promote denaturation and exposure of the proteins' reactive groups like heat-induced gelation. However, protein aggregation is not induced by a change in the temperature. A change in the pH (acid-induced gelation) or ionic strength (salt-induced gelation) of the cooled denatured protein solution leads to aggregation and the formation of a cold-set protein gel network (Nazir et al., 2017). Acid-induced gelation requires the addition of acetic, lactic, citric, or tartaric acids to reduce the pH towards the isoelectric point and thus reduce the electrostatic repulsion between the protein aggregates leading to gel formation (Prameela et al., 2018).

Various forces including disulphide bonds, hydrophobic, electrostatic, and ionic interactions, and hydrogen bonding play a significant role in the ability of the proteins to form a gel. The interactions and bonding of the globular proteins affect the mechanical, rheological, and viscoelastic properties of the protein gel (Aguilera & Rademacher, 2004; Shand et al., 2007).

The supramolecular structure of the protein gels is classified as a cross-linked network or a network consisting of stands and/ or clusters of aggregated protein particles. It has been reported that globular proteins like legume proteins form strings of beads (fine-stranded network) or clusters (particulate network) of aggregated protein particles with a diameter of more than 1 μm (Figure 1.7) (Aguilera & Rademacher, 2004; Shand et al., 2007).

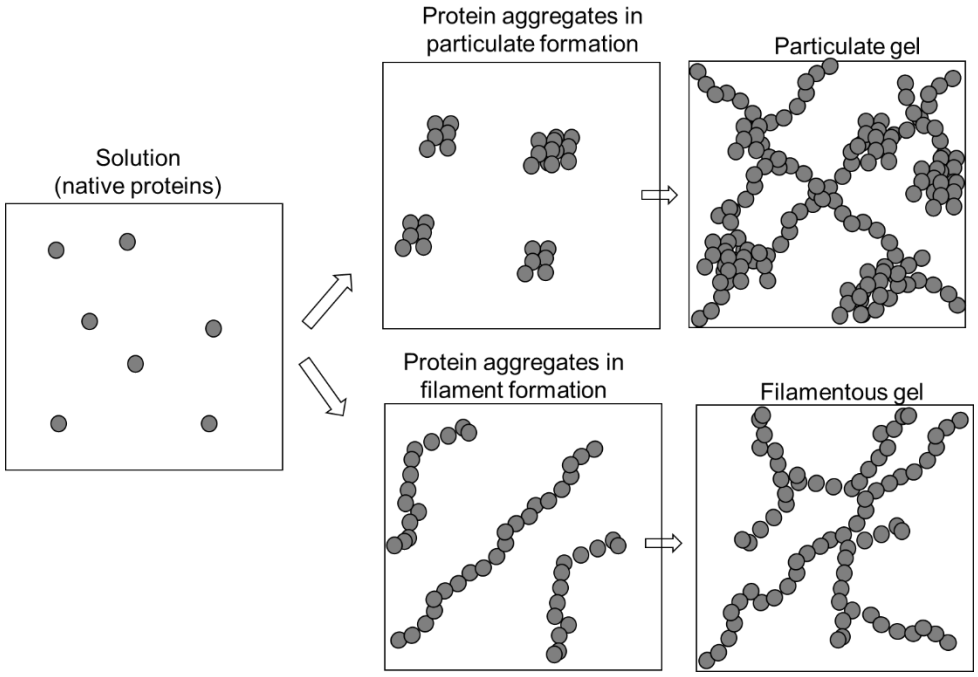


Figure 1.7 Schematic representation of the gelling process (from liquid dispersion to aggregation and gel formation) in globular proteins. Aggregation of protein molecules into a particulate and a filamentous type of network.

An increased ionic strength promotes the formation of particulate gels due to the decrease of electrostatic repulsion between the protein molecules but at low ionic strength, proteins form fine-stranded gels. Particulate gels have been observed to be opaque and have poor water holding but fine stranded are translucent, with a larger water holding capacity (Prameela et al., 2018; Zha et al., 2021).

1.8.3 Mechanism of gelation in mixed component gels

Understanding the behaviour of mixed gels is important because the behaviour of the polymers is different in comparison to their behaviour in the absence of each other (Prameela et al., 2018). The majority of protein and polysaccharide mixtures lead to phase separation as the components are thermodynamically incompatible and cannot form any complexes at neutral pH. The gels resulting from the combination of

incompatible polymer mixtures can lead to composite gels with various structures depending on the gelling mechanism of each polymer, the composition of the polymers and the gel formation conditions (Li et al., 2007).

In binary systems where proteins and starches are combined, the mechanism of gelation and gel properties are widely determined by the dominant fraction (Acevedo et al., 2013; Joshi et al., 2014). Starch gelatinisation occurs at 50-75 ^{o}C thus at lower temperatures than protein denaturation (>80 ^{o}C) therefore, the starch granules are expected to form a gel network faster than the proteins. The starch network will dominate over the weak and slow-forming protein network and proteins will not be able to penetrate the well-developed starch network (Joshi et al., 2014). However, the concentration of starch and protein content in binary gels affects the structure and mechanical properties of the gel network.

At neutral pH (7.00) a mixture of starch granules and whey protein formed two different networks depending on starch/ protein composition. High protein content (>50%) formed a continuous network including a dispersed starch phase, but low protein content (<50%) formed a continuous starch network. It was suggested that the presence of proteins hinders the association of the starch granules and the formation of a continuous gel network prior to protein denaturation (Sopade et al., 2006). The same observation has been made in the formation of gels from starch granules and soy protein and mixtures of starch protein from lentils. Starch granules function as fillers to the protein network when the

starch content is low (>23%), enhance the gel hardness and form a denser network (Joshi et al., 2014; Li et al., 2007). However, increasing starch concentration (<37%) occurs in the breakage of the protein network by the swollen starch granules with the starch forming the continuous network and protein acting as a filler (Li et al., 2007; Yu et al., 2020a).

The ability of the starch granules to put pressure on the walls of the protein gel network during gelatinisation depends on the starch granules' swelling ability (Kong et al., 2016). Cross-linked cassava starches were used to enhance the properties of surimi gels. Cross-linking increased the swelling ability of the starch granules increased viscosity and enhanced the G' referring to the elastic behaviour of the gel (Kong et al., 2016). Similar findings have been found with the addition of modified (NaCl, pH) potato and tapioca starch in myofibrillar protein gels (Wu et al., 2018a), cross-linked tapioca starches in whey protein isolate (Ren & Wang, 2019).

A higher concentration of polysaccharides increases the water-holding capacity of protein-based gels because it induces the formation of hydrogen bonds with amide, carboxyl, amino, and hydroxyl groups of proteins. However, at low polysaccharide concentration, the structure can be easily disrupted either when a force is applied (i.e., centrifugation) or by particle rearrangement (i.e., starch retrogradation) which decreases the water-holding capacity of the gel. Additionally, in low polysaccharide concentration, a higher amount of free-bound water is

expelled when a force is applied to the gel (Kazemi-Taskooh & Varidi, 2023).

2 Chapter: Materials and methods

2.1 Materials

2.2 Raw materials

Multiple batches of the raw materials were purchased (September 2019). The raw materials used for this research included green whole lentils (GWL) (Tesco, Nottingham, UK), red split lentils (RSL) (Tesco, Nottingham, UK), red whole lentil grains (RWL) (TOPTEN Wholesales, California, USA), pearl millet (PM) (whole millet Bajri seeds, Jalpur, Leister, UK), maize seeds (M) (Natco Popping corn, Sainsbury's, Nottingham, UK), wheat flour (W) (Sainsbury's, Nottingham, UK), yellow pea seeds (YP) (dried yellow split peas, Sainsbury's, Nottingham, UK).

Starches were purchased from various manufacturers; potato starch (S-A) (Native potato starch, Healy Group, Coalville, UK), maize starch (S-B) (Native maize starch, National starch, Slough, UK) and pea starch (S-C) (Native pea starch, Roquette, Corby, UK).

A yellow pea protein isolate (YPPI) was also used (Pea Isolate protein, My Vegan, Manchester, UK).

2.2.1 Chemicals

Hydrochloric acid (HCl, 37%, Fisher Scientific, Loughborough, UK), sodium chloride (NaCl, Fisher Scientific, Loughborough, UK) and sodium hydroxide (NaOH, Honeywell, Raunheim**,** Germany) were used for extraction, neutralisation and pH control in various experiments.

Rhodamine B (0.01 g/L, 0.10 ml, 546λ/568λ), fluorescein isothiocyanate (0.05 g/L, 0.10 ml, 495λ/513λ) and calcofluor white (1 g/L, dilution 1:4 w/w

with H_2O) purchased from Sigma Aldrich (Dorset, UK) were used for fluorescent microscopy.

1-anilinonaphthalene-8-sulfonate (ANS) (Thermo Scientific, Loughborough, UK) and 0.01 M Phosphate buffer (PBS) (Boster Biological Technology, Pleasanton CA, USA) were used to measure protein hydrophobicity.

2.3 General Methods

2.3.1 Milling (grain and flour)

Milling is a versatile method used for reducing particle size, separating the botanical tissues (i.e., hull, cotyledons or endosperm), and physical transformation of the structure of the components i.e., starch damage. Factors affecting the particle size reduction and fragmentation include composition, macro/microstructure of grains and mill design (Thakur et al., 2019) as detailed in section 1.7.1.

An overview of the milling methods used for each sample in this thesis can be seen in Table 2.1.

Table 2.1 Overview of the milling processing used for each sample in this thesis.

	Hammer milling	Ball milling
Green whole lentils	✓	✓
Red split lentils	✓	✓
Red whole lentils	✓	✓
Yellow pea	✓	✓
Pearl millet	✓	✓
Maize	✓	✓
Wheat		✓
Starch A- (wheat), B-(potato), C-(pea)		✓

2.3.2 Laboratory hammer mill

A laboratory hammer mill is considered a low-cost and simple method of milling. It uses impact force to crush the sample through the impact of metal hammers attached to a spinning rotor. The hammer mill has three main parts; the feeder (entry point of the sample), the grinding chamber (the sample is crushed and passed through a sieve) and the discharge opening (milled sample exit point) as seen in Figure 2.1.

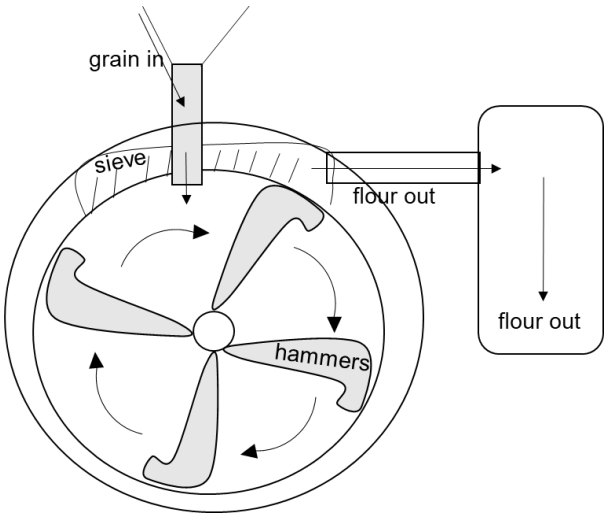


Figure 2.1 Schematic representation of the hammer mill's structural figures.

A laboratory hammer mill (3100, Perten Instruments, Hägersten, Sweden) performing at 16800 rpm and equipped with a sieve (between 1-2 mm) was used to produce fine flour (labelled as the control flour) from the lentil and cereal grains (GWL, RSL, RWL, PM, YP, M) at a milling rate of 50 g/min. The presence of a sieve ensured that all flours would have a more uniform particle size.

2.3.3 Pulverissette ball mill

The pulverissette ball mill can be used for mixing, milling, and physical transformation of components. The ball milling is equipped with a supporting disc and four positions for the grinding jars. The grinding jars and supporting discs operate in different directions. Thus, the action of the centrifugal forces follows the same and opposite directions. In the grinding jars, the balls can move in the same or opposite direction to the grinding jars and friction is created between the balls and the walls of the jar (Fritsch GmbH. Instruments, 2003) as seen in Figure 2.2.

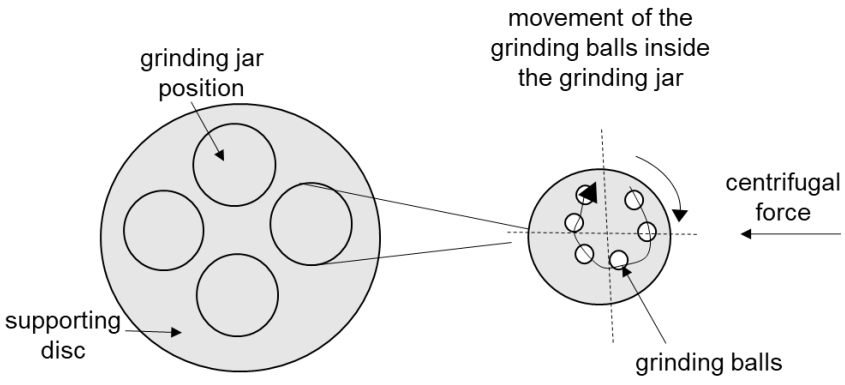


Figure 2.2 Schematic representation of the pulverisette ball mill's structural figures.

A planetary ball mill (Planetary mill pulverisette 5/4, Fritsch Gmb dH, Idar-Oberstein, Germany) was used for 2 and 4 hours (labelled as BM2

and BM4 respectively) to physically modify the control flours including hammer milled and non-hammer milled flours/starches (Table 2.1).

Preliminary experiments showed non-significant changes in the birefringence of the starch granules' samples under a polarised light microscope when ball-milled for less than 2 hours or at 3 hours, hence 2 hours and 4 hours were used. Flour (40 g) was put in zirconium oxide jars (250 ml) (Fritsch Gmb dH, Idar-Oberstein, Germany) with 15 balls (20 mm in diameter) (Fritsch Gmb dH, Idar-Oberstein, Germany) at a fixed speed of 300 rpm. The ball-to-powder ratio was selected using the manufacturer's recommendations. The ball milling configuration included 15 min of grinding and pause intervals of 1 min to reduce heat generation.

2.3.4 Chemical analysis of the control flours

The chemical composition of the control flours (GWL, RSL, PM) was conducted by Eurofins Food Testing (Wolverhampton, UK). The referencing methods included the combustion method of Dumas analysis for crude protein using the nitrogen-to-protein conversion factor of 6.25, the AOAC 9941.43 method for fibres, for ash (550 ^{o}C) and fat (using an organic solvent) gravimetry analysis and carbohydrate was estimated by difference.

2.3.5 Moisture content of the control and ball-milled flours

The moisture content of the control and physically modified flours (GWL, RSL, PM) was determined using an MB90 Moisture Analyzer (Ohaus, 90 g x 0,001 g; capacity x readability, Nänikon, Switzerland). The flour

sample was subjected to 105 ^{o}C until complete drying (10 min approximately). Three replicates of all samples were used.

2.3.6 Particle size analysis

Particle size can influence the properties and functionality of flours, for example, small particles can hydrate faster. The particle size analyser was used to detect any differences in the particle size between the control and physically modified flours (GWL, RSL, PM).

The particle size analyser is equipped with a source of illumination (laser beam) which passes through the sample, the sample measurement region, a Fourier lens, beam processing optics, a multi-element detector to measure the pattern of the scattered light and a data system to deconvolute the scattering data into the size distribution (in volume). The total angular intensity (both direct and scattered light) is collected by the multi-element detector. The light intensity changes depending on the size of the particles. Particles are separated into two categories: large which scatter light at small angles relative to the laser beam and small which scatter the light at large angles. The Fraunhofer theory is used to describe the scattering of light. Fraunhofer theory assumes that particles are spherical, opaque, scatter equivalently at wide or narrow angles and interact with light differently than the medium (Figure 2.3). Finally, the scattering pattern is converted to a particle-size distribution by deconvolution.

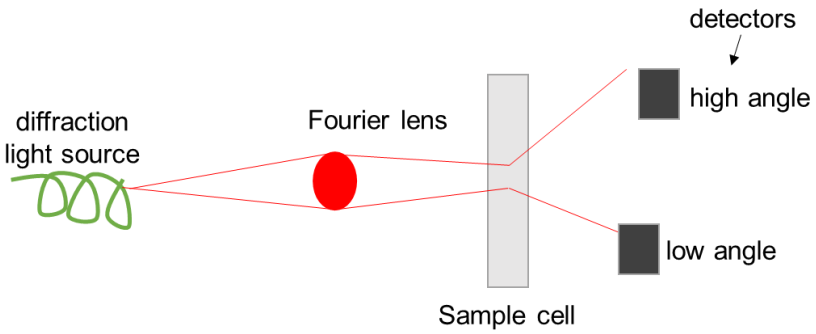


Figure 2.3 Schematic representation of the particle size distribution principle.

The particle size distribution of the control and physically modified flours (GWL, RSL, RWL, PM) was measured in the Tornado Dry Powder System (DPS, LS 13320, Beckman Coulter, High Wycombe, UK) using a Laser Diffraction Particle Size Analyser (LS 13320, Beckman Coulter, High Wycombe, UK). The Fraunhofer theory based on the spherical approximation of the particles was used as the optical model. Data were analysed using the instrument's software. The graphical representations of the volume-based size distributions are presented. Three replicates of all samples were used.

2.3.7 Water absorption (WA) and water solubility index (WSI) of the flour

The water absorption and solubility followed the method of Du et al., (2014) with some modifications. The control and physically modified flours (GWL, RSL, PM) were dispersed in water (1:20 w/v), stirred in a vortex mixer (SA 8, Biocote, Coventry, UK) for 5 sec and then placed for 1 hour at 60 rpm on a roller mixer (Stuart, SRT9D, Biocote, Coventry, UK) at ambient temperature. The dispersions were subjected to centrifugation (Rotina 380R, Hettich Zentrifugen, Tuttlingen, Germany)

for 30 min at ambient temperature and 4,863 RCF. The supernatant was poured into pre-weighed aluminium pots and dried in the vacuum oven (OVA03100, Gallenkamp, Cambridge, UK) for 24 hours at 60 ^{o}C until constant weight. Equation 2.1 & Equation 2.2 were used for the calculation of the WA and WSI, respectively. Three replicates of all samples were used.

Equation 2.1: Water absorption

$$WA\ (g/g) = \frac{Pellet\ after\ centrifugation\ (g)}{flour\ (g)}$$

Equation 2.2: Water solubility index

$$WSI\ (\%) = \frac{dried\ solids\ from\ supernatant\ (g)}{flour\ (g)\ x100}$$

2.3.8 Microscopy

Microscopes can give valuable information about the microstructure of both the flours and the gels. The microstructure of foods can be challenging due to the complexity of the material. Therefore, a combination of microscopy techniques can be conducted to differentiate between artefacts and structural features (Aguilera & Stanley, 1990). In this thesis, different microscopy methods (bright field, polarised, fluorescent light) were used. Microscopy was used as a qualitative analysis to differentiate the microstructural differences between the flours and observe the impact of the ball mill on the flour's microstructure.

2.3.8.1 Bright-field and polarised light microscopy

Bright-field microscopy is used to enhance the observation of a specimen's microstructure in microns which cannot be visualised by the naked eye. Bright-field microscopy was combined with a polariser to identify the starch granules. Specifically, starch presents birefringence a property depending on the direction of light. Birefringence in starch can be observed under polarised light in the form of the characteristic 'Maltese cross'. The 'Maltese cross' represents the radial organisation within the starch granules' structure. Changes in the shape of the 'Maltese cross' have been linked to changes in the radial organisation of the starch granules i.e., gelatinisation. In food, only components with asymmetric crystals present birefringence (i.e., starch, lipids) (Alvarez-Ramírez, 2023; Ratnayake et al., 2002). Techniques including Differential scanning calorimetry and X-ray diffraction were used to confirm any changes in the structural organisation of the starch granules. The principle of a brightfield and polarised microscope can be seen schematically, in Figure 2.4.

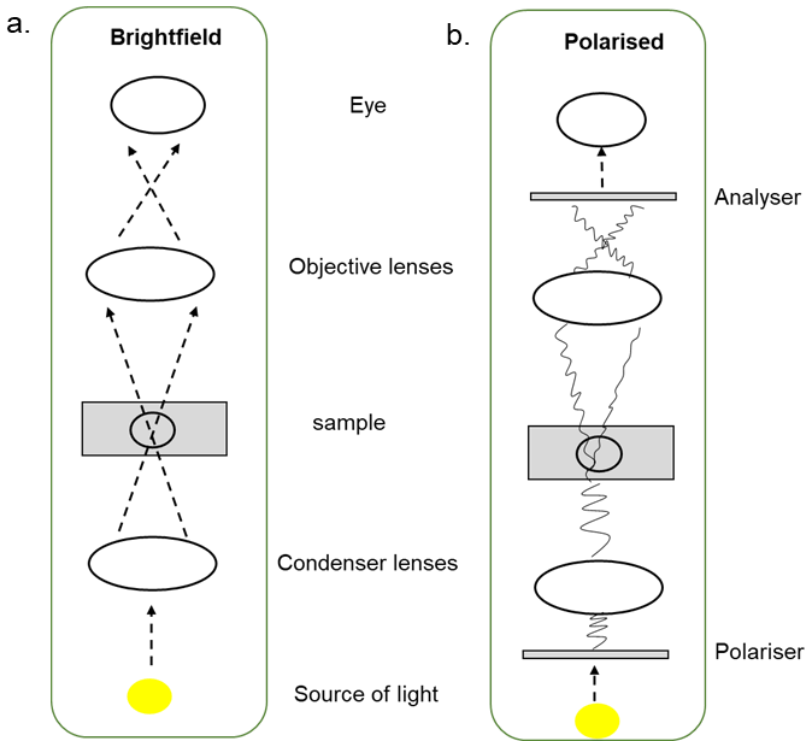


Figure 2.4 Schematic of (a) Brightfield and (b) polarised light microscopy.

The light source of the brightfield microscopy can be found both on the top or bottom of the microscope. The brightfield microscope includes a source of transmitted light, a set of objective lenses and an eyepiece. The light vibrates in various directions, is collected by the objective lenses and is visible by the eyepiece (Figure 2.4a). For polarised microscopy, the direction of light changes to one direction using a polariser which is collected by the objective lenses, an analyser and finally observed through the eyepiece (Figure 2.4b).

A bright field light microscope (Eclipse ci, Nikon, UK) was used to capture the brightfield and polarised micrographs of the control and physically modified flours (GWL, RSL, PM). 0.10 ml of H_2O was added to the flour, observed under the bright field, and polarised light.

2.3.8.2 Fluorescent light microscopy on flours and gels

Fluorescent microscopy was used to identify and differentiate between the various components within the microstructure of the flours and the gels. Fluorescence microscopy is based on the fluorescent property of a specimen to absorb and re-emit light in specific wavelengths. Chemical compounds in stains (fluorophores) bind with the different components and are observed in different wavelengths (Figure 2.5).

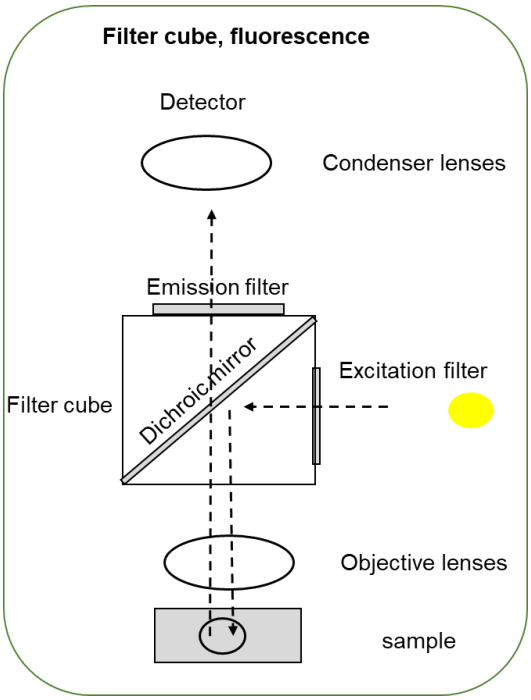


Figure 2.5 Schematic representation of a fluorescence light microscopy principle.

Fluorescent microscopy was used to visualise both control and physically modified flours and gels. A microscope (EVOS, Thermo Fisher Scientific, Loughborough, UK) equipped with LED light cubes including an RFP red-light cube, 542 λ_{em}/593λ_{ex}, GFP green-light cube, 482 λ_{em}/524 λ_{ex} and a DAPI blue-light cube, 360 λ_{em}/447 λ_{ex}. 0.10 ml of Rhodamine B was used to stain the proteins, 0.10 ml of fluorescein isothiocyanate was used to stain the starch granules and 0.10 ml of

Fluorescein isothiocyanate (FITC) was used to stain the fibres in the samples. The scale bar in all micrographs presented represents 200 μm.

2.3.9 Rapid visco analysis (RVA)

In an RVA the sample is placed in an aluminium canister with the solvent. A paddle operated by a stirring motor in the RVA's tower is placed in the canister. The canister is pushed down to the machine and a copper block clamps around the canister. The canister and the block are closed ensuring constant and reliable heating and cooling of the sample in the canister (Crosbie & Ross, 2007).

The RVA was used in this thesis both on the control and ball-milled unfractionated (GWL, RWL, RSL, YP, PM, M, W) and fractionated starch flours (S-A; wheat, S-B; potato, S-C; pea flours). The methodology used to characterise the pasting properties of the legume and cereal flours is the most common method used by other researchers in the literature (Hasjim et al., 2013; Loubes et al., 2018a; Nilsson et al., 2023; Sun & Xiong, 2014). The same method was used with slight modifications to prepare the heat-set gels and the details will be found later in this chapter.

To prepare a suspension with constant weight, the moisture content of the samples was taken into consideration. The control and ball-milled flour suspensions were prepared using water or a NaCl solution (1 to 8 M); for GWL and RSL. The final suspension was calculated to contain 10.6% solids accounting for the moisture content of each sample.

The dispersions were manually stirred in the canister with a spatula for 4 min until homogeneous. Also, the flour dispersions were stirred by the RVA machine for the first 10 sec of the measurement, at 50 ºC and 960 rpm. Each measurement was completed in 13 min.

A Rapid Visco Analyser (RVA 4800, Perten Instruments, New South Wales, Australia) connected to a water bath (Fisher Scientific, Ince model 3016S, Massachusetts, USA) was used according to AACC International Method 76-21.01 (Method, 1960) (Figure 2.6).

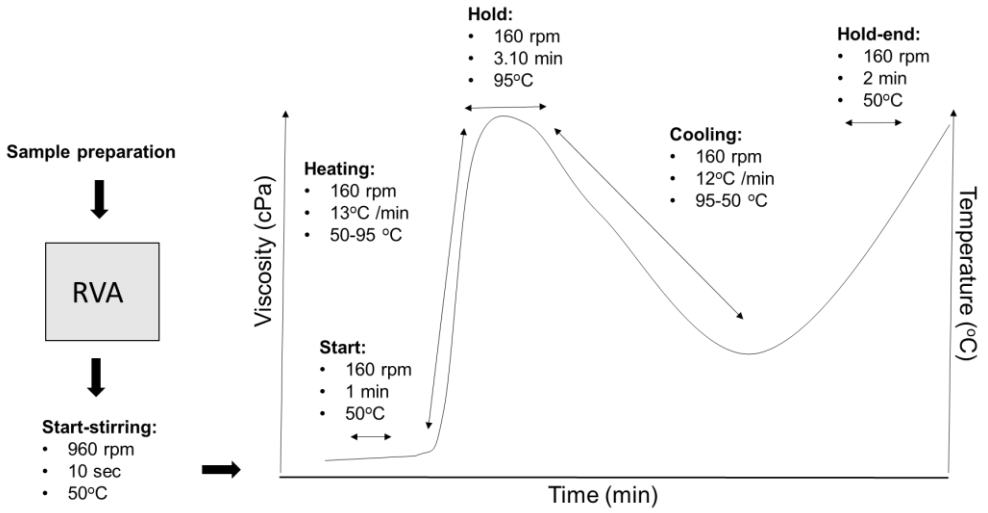


Figure 2.6 Schematic representation of the RVA methodology on an RVA graph.

2.3.9.1 Parameters calculated during RVA

Each sample was analysed three times and the peak viscosity (PV), break down (BD), trough viscosity (TV), set back (SB), final viscosity (FV), pasting time (Pt) and pasting temperature (PT) were recorded by the TCW (Thermocline for Windows) software. Those parameters are measured during a heating and cooling cycle with an intermediate isothermal step. A representation of the parameters on an RVA graph of

a starch-rich sample, which is a key material studied by RVA, can be seen in Figure 2.7.

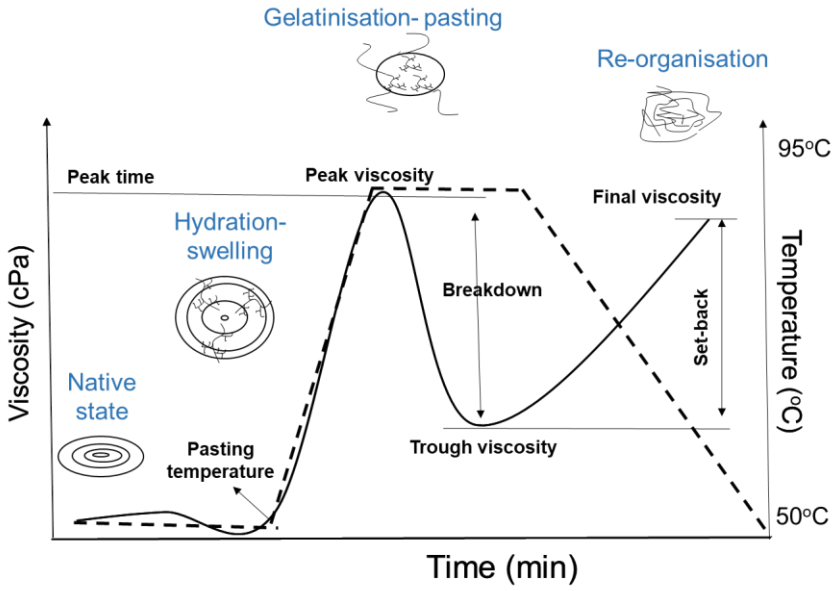


Figure 2.7 Schematic representation of a standard RVA curve of starch-rich samples.

For a starch-rich material, starch granules are in a native state (semi-crystalline, low water solubility, low water absorbance) at the beginning of the heating step but start to hydrate as the temperature increases. Starch granules start to swell which creates a restriction of the water-starch mixture to flow, therefore increasing the viscosity. As the temperature continues to increase starch granules are gelatinised and the peak viscosity is measured. Peak viscosity is described as an indication of the water-binding properties of the starch and the highest viscosity has been reached.

After heating the temperature remains high for some minutes which causes the disruption of the starch granules and the leaching of the amylose due to the continuation of the shearing action. This property is measured by the trough viscosity.

Disintegration of starch granules followed by leaching of the amylose as the starch granules cannot withstand heat and shear. This is depicted with a decrease in viscosity and is measured by the Breakdown. The breakdown can be also used to measure the ability of the sample to withstand shear and heating.

After the breakdown and the end of the isothermal step, the cooling cycle starts. The temperature decreases and viscosity starts to increase again as the leached amylose molecules start to retrograde. This property is measured by the setback. At the end of the cooling cycle, the final viscosity is measured. The final viscosity reflects the re-crystallisation of the amylose upon cooling (Balet et al., 2019).

2.3.10 Control and ball-milled flour characterisation methods

2.3.10.1 Protein solubility

The proportion of nitrogen content within the soluble phase of a protein-based food product has been described as protein solubility (Zayas, 1997). Protein solubility is a functional property determining the application of proteins in food systems. High protein solubility is linked to high dispersibility and the creation of dispersed colloidal systems. The electrostatic and hydrophobic interactions between the protein molecules of a solvent affect solubility. Temperature, ionic strength and pH are the most important extrinsic factors affecting protein solubility (Soderberg, 2013).

In this research, pH was used to change the solubility of proteins. At the isoelectric point (no net charge), pH 4.0-4.8 in legumes, attractive forces

dominate and the protein molecules associate resulting in insolubility. The solubility of pulse proteins as a function of the pH presents a u-shape (bell curve) with the minimum point of the curve corresponding to the isoelectric point. Above or below the isoelectric point the repulsion between the proteins is high and protein-H_2O interactions are enforced, enhancing solubility (Zayas, 1997).

Protein solubility of the control and physically modified flours (GWL, RSL, PM) were measured following the method of Carbonaro et al., (1997). The flours were dispersed in 1:20 w/v water and placed on a roller mixer (SRT9D, Stuart, Biocote, Coventry, UK) for 1.5 h at 25 ^oC. The pH was adjusted to 8.5 using a pH meter (Mettler Toledo Ltd., Leicester, UK) for the GWL and RSL (Arntfield & Maskus, 2011; Boye et al., 2010) and to pH 9.0 for the PM (Akharume et al., 2020) using 0.1 M NaOH. The pH was checked every 30 min and adjusted to 8.5 or 9.0 when needed. After 1.5 hours, the samples were subjected to centrifugation (Rotina 380R, Hettich Zentrifugen, Tuttlingen, Germany) for 30 min in ambient temperature at 4,863 RCF and then the pellet and the supernatant were freeze-dried (Edwards Modulyo K4 Freeze Dryer Lab, Burgess Hill, UK) until the samples were completely dried with moisture <10%, before being subjected to elemental analysis.

Protein composition is usually determined using the Kjeldahl or the Dumas combustion method. The Dumas method is considered low-cost, simple, safe and fast in comparison to the Kjeldahl method. The principle includes the combustion of the samples at a very high temperature (around 950 ^oC) in the presence of oxygen. H_2O, CO_2, SO_2, NO_x and N_2

are formed after combustion with the CO_2 and SO_2 being removed. The total N_2 is measured by reducing NO_x and using a detector for thermal conductivity. A nitrogen-to-protein factor is used to calculate the crude protein in the sample (Moore et al., 2010).

A CHNS-0 Analyser, (CE Instruments, Wigan, UK) was used and a small quantity (50 ± 10 mg) of the freeze-dried control and modified flours prepared as described above, weighed into tin pressed capsules (CE Instruments, Wigan, UK). 25 ± 10 mg of standard sulphanilamide (CE Instruments, Wigan, UK) was used as the reference sample. The protein content was calculated using Equation 2.3 and the nitrogen-to-protein conversion factor of 6.25 was used in line with the literature (Carbonaro et al., 1997; Dalgetty & Baik, 2003a; Saldanha do Carmo et al., 2020). The protein solubility was expressed as a percentage of the flour's total protein content. Three replicates of all the samples were used.

Equation 2.3: Nitrogen-to-protein conversion

$$N \times 6.25$$

Where: N=nitrogen content

2.3.10.2 Protein hydrophobicity

Hydrophobicity is a function related to the structure of the proteins specifically the size and shape of the proteins, the composition and sequence of amino acids and molecular interactions (Jiang et al., 2015).

Protein surface hydrophobicity can be measured using fluorescent probes. One of the most common probes used is the amphiphilic 1-

anilinonaphthalene-8-sulfonate (ANS). ANS probe binds to hydrophobic amino acids (glycine, alanine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan). The methionine and tryptophan contents are low in lentils (>1.40%) but lentils contain higher proportions of the amino acids leucine (≈7%), isoleucine (≈4%) and phenylalanine (≈5%) (Dhull et al., 2022). Once the hydrophobic amino acids are bound to the ANS an increase in the fluorescent intensity of the sample is observed, which is measured using a spectrophotometer.

A suspension of the control or physically modified flour (GWL, RSL, RWL) with water (1:20 w/v) were placed on a rolling mixer (SRT9D, Stuart, Biocote, Coventry, UK) at 60 rpm, in ambient temperature, for 2 hours. The dispersions were then placed in a centrifuge (Rotina 380R, Hettich Zentrifugen, Tuttlingen, Germany) for 30 min at 4,863 RCF, ambient temperature and the supernatant was freeze-dried (Edwards Modulyo K4 Freeze Dryer Lab, Burgess Hill, UK).

The protein content of the freeze-dried supernatant was measured as per the method described in Section 2.3.10.1. A protein dispersion (protein content; 1 mg/ml) was then prepared with phosphate buffer (0.01 M pH=7.0) to maintain constant pH. The dispersion was subjected to dilution in various concentrations (ranging from 0-1 mg protein). Each concentration (1.8 ml) was mixed with 9 µl of 1-anilinonaphthalene-8-sulfonate (ANS), stirred in a vortex mixer (SA 8, Biocote, Coventry, UK) for 5 sec and 200 µl of this mix were added in a cell culture microplate (Greiner bio-one, Frickenhausen, Germany).

The fluorescent intensity of the samples was measured in a UV-Vis plate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany) at $365\lambda_{em}/484\lambda_{ex}$. The fluorescent hydrophobicity was plotted against the protein concentration as seen in Figure 2.8 and the initial slope was calculated as the index of hydrophobicity (H_o) (Wagner et al., 2000).

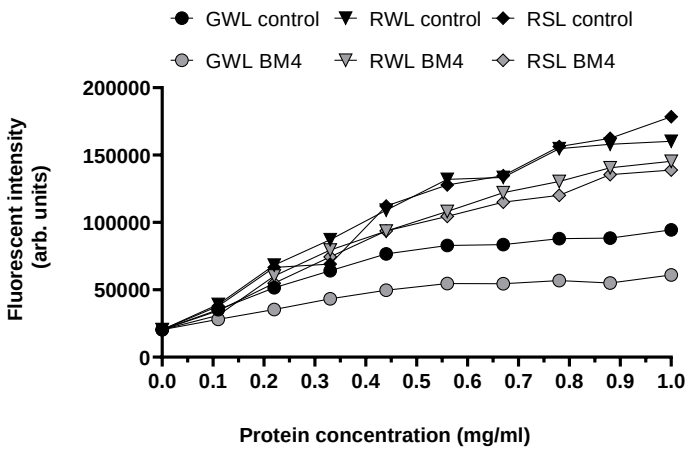


Figure 2.8 Fluorescent intensity versus protein concentration (%) in control and BM4 lentil flours. Control: native, BM4; 4 hours of ball milling

2.3.10.3 Protein and starch extraction from the RSL control flour

Protein and starch from flours can be extracted using dry or wet methods. Wet extraction is a conventional method that typically extracts a minimum of 70% of the protein content (Eze et al., 2022; Ma et al., 2022). For example, alkaline extraction whereby proteins are extracted through pH manipulation. Sodium hydroxide or potassium hydroxide is used to increase the pH between 8.0-11.0 enhancing the protein charge and therefore protein solubility. Following centrifugation, the starch and fibers co-settle at the bottom (pellet) forming a white brown-ish layer and the supernatant is acidified with HCl close to the isoelectric point (pH 4.0

to 5.0) leading to a reduction in electrostatic repulsion between the protein molecules and precipitation.

Proteins from RSL were extracted using an alkali extraction method based on previously published literature (Joshi et al., 2014; Tarahi et al., 2022). The RSL flour was placed in water (1:20 w/v) and the pH was adjusted to 8.5 using NaOH (1 M) to solubilise the proteins. The choice of pH was explained in Section 2.3.10.1. The suspension was stirred using a roller mixer (Stuart, SRT9D, Biocote, Coventry, UK) at room temperature for 2 hours and the pH was checked every 30 min. It was then placed in a centrifuge (Rotina 380R, Hettich Zentrifugen, Germany) for 30 min, at room temperature at 4,863 RCF.

Following centrifugation, the starch and fibers were separated from the proteins by co-settling at the bottom of the centrifuge tube (pellet) and further washed with H_2O (1:3 w/v) until neutralisation was achieved. The supernatant was collected as the protein-rich fraction and subjected to acidic precipitation to pH 4.5 (isoelectric point) using HCl (0.1 M). Following centrifugation (Rotina 380R, Hettich Zentrifugen, Germany) for 30 min at room temperature at 4,863 RCF/g the protein settled at the bottom of the centrifuge tube was collected and further washed with H_2O (1:3 w/v) until neutralisation was achieved.

The protein and starch/fibers-rich fractions were finally centrifuged (Rotina 380R, Hettich Zentrifugen, Germany) for 30 min, at room temperature at 4,863 RCF/g prior to freeze-drying (Edwards Modulyo K4

Freeze Dryer Lab, Burgess Hill, UK). The purity of the fractions was not evaluated.

2.3.11 X-RD diffraction

Starch crystallinity is determined by the double helices packing of the amylopectin starch molecule. The interaction between the X-rays and the arrangement of atoms in materials presenting crystallinity is studied using X-RD. The X-rays are focused onto a sample at an angle (θ). The wavelength of the X-rays and the distance between crystal layers are comparable. For this reason, incident X-rays will be transmitted through the sample or diffracted as seen in Figure 2.9. The intensity of the diffracted X-rays is recorded by the X-RD. The angle of the detector above the source of the X-rays always remains 2θ. Over time the incident angle is increased to produce all possible diffracted beams from the sample. The unit cell dimensions of the crystal are used to determine the 2θ values. Sharp diffraction peaks are not produced by amorphous materials, only by crystalline materials (Dean, 2017).

The X-ray was used to study the crystalline nature of the control and physically modified flours.

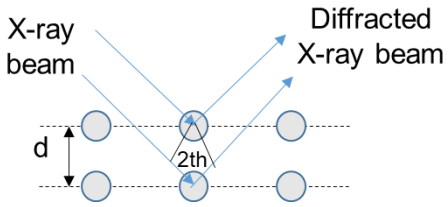


Figure 2.9 Schematic representation of the X-ray principle.

The X-ray diffraction patterns of the control and physically modified flours were determined using an X-ray diffractometer (D8 advance, Bruker,

Coventry, UK) following the methodology of Pozo et al., (2018). The degree of crystallinity was calculated as the ratio of the crystalline area to the total area, following Equation 2.4.

Equation 2.4: Degree of crystallinity

$$DOC = \frac{A}{A+B}\ x\ 100$$

Where: DOC= degree of crystallinity, A= diffraction crystalline peaks, B= amorphous phase

2.3.12 Differential scanning calorimeter (DSC)

Differential scanning calorimetry (DSC) is a quantitative analysis measuring the changes in the physical or chemical state of a sample. The method is used to detect heat absorption (endothermic) or liberation (exothermic) that occurs under various temperatures in H_2O (Aguilera & Stanley, 1990). The principle of thermal analysis includes the measurement of the heat flow difference between the sample and a reference (Figure 2.10). The analysis was used to provide insight into the overall structure of the control and physically modified flours.

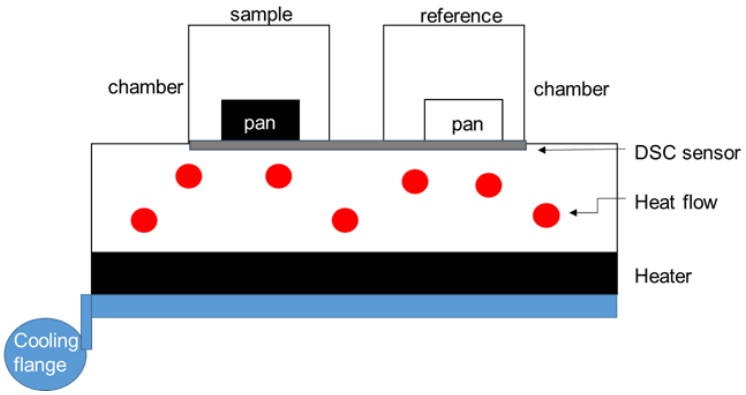


Figure 2.10 Schematic representation of a differential scanning calorimeter (DSC).

The thermal behaviour of the control and physically modified flours (GWL, RSL, PM) followed the methodology of Nagaprabha, Devisetti & Bhattacharya, (2018). The flours were weighed in stainless steel pans (1:3 w/v; flour to water) and sealed hermetically. The dispersions were left to hydrate overnight at 4 ^{o}C on a roller mixer (SRT9D, Stuart, Biocote, Coventry, UK) at ambient temperature. The next day the measurements were performed using a DSC^{+3} (Mettler Toledo, Leicester, UK) from 20-120 ^{o}C at a 10 ^{o}C/min^{-1} heating rate. The calorimeter was calibrated with indium and an empty pan was used as a reference. The T_o (onset), T_p (peak), T_c (end set) and the enthalpy ΔE were computed by the software STARe (Mettler Toledo, Leicester, UK). The analysis was conducted three times for each sample.

2.4 Heat set gel preparation and characterisation methods

2.4.1 Heat-set pastes/gels preparation

An RVA was also used to form heat-set pastes/gels from the control and physically modified flours of the GWL, RSL, and RWL. The methodology included the dispersion of the lentil flours in water (10.6% solids). The flour dispersions were manually stirred in the canister with a spatula for 4 min until homogeneous. The flour dispersions were then transferred to an RVA and heated up to 95 ^{o}C. The details of the method can be seen in Figure 2.11.

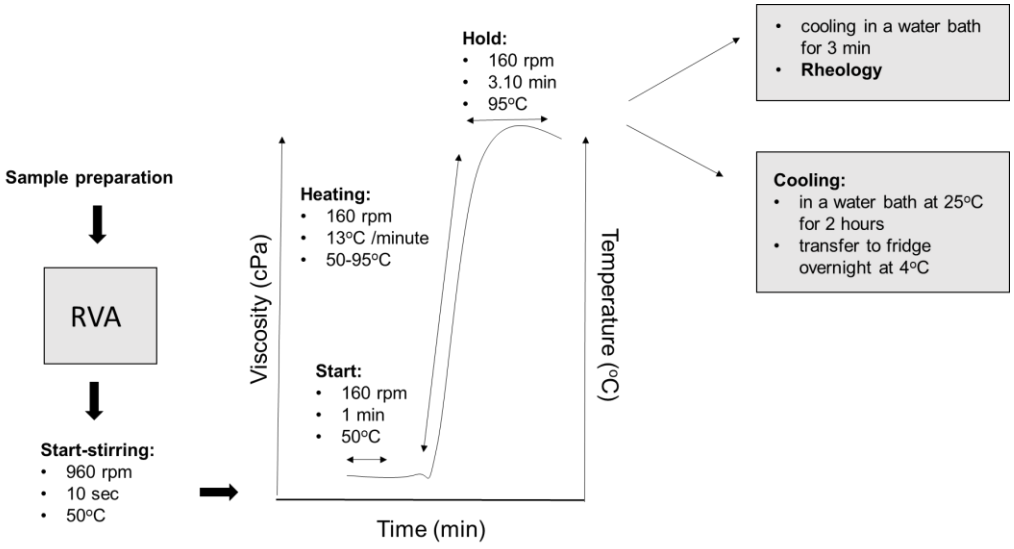


Figure 2.11 Heat-set lentil paste/gel preparation under a controlled environment using an RVA and a water bath.

Following heating in the RVA, the samples were processed and analysed as follows:

For rheological experiments, the pastes were immediately transferred to a water bath (Nickel Electro™ Clifton™ Unstirred Digital Water Bath, Fisher Scientific, Loughborough, UK). The temperature of the paste was checked after 1.5 min and 3 min until the temperature reached 75 °C. The pastes were then immediately transferred to the rheometer and the methodology described in 2.4.2 was followed.

For texture analysis, water capacity and microscopy experiment the pastes were transferred to 25 ml tubes (25 mm diameter x 90 mm height) which were placed immediately in a water bath at 25 °C (Nickel Electro™ Clifton™ Unstirred Digital Water Bath, Fisher Scientific, Loughborough, UK) for gel formation and then transferred to the fridge. All formed gels were analysed the next day.

2.4.2 Rheology

Rheology is used to study the deformation and flow of matter. Rheology comes from the words ‘ρέω’ and ‘λόγος’ in Greek. Food rheology is useful in terms of understanding how food structure responds to applied force and deformation. For this research, the rheological experiments have been used to collect further insight into the strength and internal structure of the gels.

To understand the flow behaviour of a material the two-plate model can be used as seen in Figure 2.12. When a material is found between two plates, a force (F) moves the plates in opposite directions and velocity (v) is created on the top plate (Mezger, 2014).

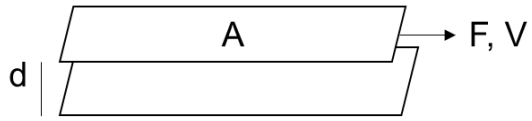


Figure 2.12 Schematic representation describing the flow behaviour of a material between two plates.

The thinner the material the farther and faster the plate will be moved and vice versa. Shear stress is measured by the force applied to the area of the plate (F/A). The shear stress is proportional to the velocity gradient (speed or shear rate) which is measured by the ratio of velocity to the sample’s thickness (Equation 2.5) (Hall et al., 1996).

Equation 2.5: Velocity

$$\sigma = \eta \frac{V}{d}$$

Where: σ= shear stress, η= coefficient of viscosity, V= velocity, d= sample’s thickness

The structural changes that occur in food processing can be monitored using small-amplitude oscillatory techniques, alongside changes in the physical state of food from liquid to solid and vice versa with temperature changes. In oscillatory experiments, the two-plate model can be applied, with the upper plate moving around a rotational axis in oscillation without completing a cycle in one direction and the lower plate not moving. The stress response resulting from the torque when shear strain is applied is measured. Torque refers to the resistance of the materials when shear is applied as seen in Figure 2.13.

Gels are some of the materials that exhibit a viscoelastic behaviour. Viscoelasticity is the property of a material to exhibit both viscous (liquid-like) and elastic (solid-like) behaviour. The difference between viscous and elastic materials is found in response to a strain when stress is applied. Elastic materials can deform to a certain strain when a constant stress is applied, which does not change except when the stress is removed. However, the strain of the viscous materials continuously increases when constant stress is applied. Rheological properties of the gels are examined using oscillatory tests and the data are reported in terms of storage (G') and loss (G'') moduli representing the elastic and viscous behaviour respectively (Mezger, 2014).

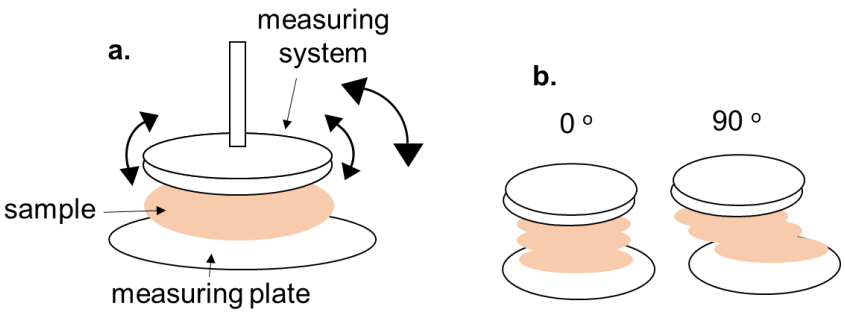


Figure 2.13 Schematic representation of the oscillatory measurement principle of the two-plate model where the upper plates deflect on angles 0^o and 90^o. Adapted by Ramli et al., (2022).

A temperature sweep test was used in this thesis to understand the gel formation prepared using the control and physically modified lentil flours (GWL, RWL, RSL). Followed by a frequency test to gather more details about the structure of the gels. Before conducting any temperature or frequency sweep tests an amplitude test was used to determine the non-destructive deformation range of the samples and the linear viscoelastic range (LVR) showing the behaviour of the sample under various strains. Moreover, another parameter called the tan δ was calculated by the ratio of the viscous and loss modulus (G'/G''). tan δ is described as the tendency of a material to dissipate or store energy (damping behaviour) (Rao, 2013).

2.4.2.1 Sample preparation

A Rheo Compass (301 rheometer, Anton Paar, Austria) connected to a water bath at 30 ^oC (Grant, gr150, UK) was used to determine the rheological properties of the pastes/gels. The pastes were prepared following the method described in Section 2.4.1. Each paste was placed on the lower serrated plate with a spatula and the upper plate (PP50/P2, 40 mm, Anton Paar, Austria) using a 1 mm gap between the plates. The exposed paste was trimmed and covered with silicon oil (Sigma Aldrich, St.Loius, USA) to prevent dehydration. Five replicates were performed for each paste/gel.

The linear viscoelastic region was conducted using an amplitude sweep test strain (γ) (0.1-100%), performed at 25 ^{o}C at frequency (ω) (10 rad/s).

2.4.2.2 Temperature sweep test

Pastes were transferred to a pre-heated plate (70 ^{o}C) and cooled down from 70 to 25 ^{o}C with a cooling rate of 0.5 ^{o}C per minute to form gels. During cooling the G' and G'' was recorded at ω (10 rad/s), γ (0.1%).

2.4.2.3 Frequency sweep test

Upon completion of a temperature sweep test (sample cooled to 25 ^{o}C), a frequency sweep test was performed on the gels at 25 ^{o}C and over a frequency range of ω (0.1-10 rad/s), γ (0.1%). The dependence of G' and G'' on frequency was recorded within the linear viscoelastic region.

2.4.3 Water retention of gels

The water retention of the gels was measured by the water-holding capacity of the gels which was quantified by assessing the amount of liquid expelled (syneresis) from the control and physically modified gels. The liquid expelled from the heat-induced gels was quantified using the centrifugation method (Wu et al., 2018b). 5 g of each gel was transferred in pre-weighed 50 ml centrifuge tubes and centrifuged for 30 min at 5.000 rpm in ambient temperature (Rotina 380R, Hettich Zentrifugen, Germany). The centrifuge tubes with the gels were inverted, after 15 min the supernatant was discarded and the gels in the centrifuge tubes were weighed again. Each value represents a mean of three replicates. The WHC (%) of the gels was calculated using Equation 2.6.

Equation 2.6: Water holding capacity

$$WHC\ (\%) = \frac{gel\ after\ discarding\ liquid\ (g)}{gel\ (g)}\ x100$$

2.4.4 Texture analysis

The textural properties of food can be defined as a group of physical characteristics arising from the structural elements of food, primarily sensed by the touch, associated with the deformation, disintegration and flow of the food when force is applied and measured using mass, time and distance functions. Texture analysis is useful in determining large deformation behaviour and was developed in 1960 to imitate the movement of the human jaw when consuming food (Bourne, 2002).

A texture analyser TA.HD.plus (Stable microsystems Ltd., Godalming, UK) equipped with the Exponent connect software (Stable Microsystems Ltd., Godalming, UK), was used to characterise the mechanical properties of the control and ball-milled lentil gels under force.

Two types of uniaxial compression were used in this thesis including sample deformation using a non-destructive compression test and a sample deformation using a destructive compression test (Figure 2.14). Starch-gel food systems have been found to collapse between 20-50% of compression. A strain where gels collapse could produce invalid parameters at the second cycle of the TPA measurement (M. Huang et al., 2007). At a high extent of deformation (>50%) gels break into pieces and the fracture ability can be measured. The extent of deformation was selected at 25% and 80% strain after conducting preliminary tests with various compression percentages.

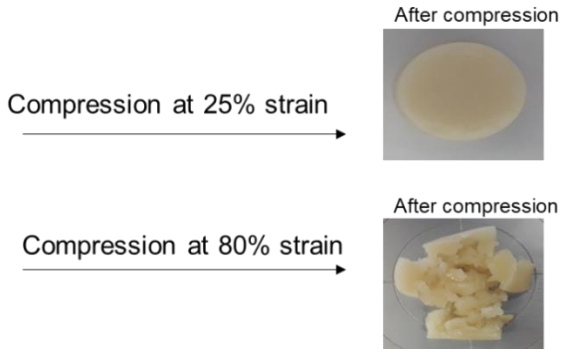


Figure 2.14 Heat-set gel prepared from control RSL flour under non-destructive (25% strain) and destructive force (80% strain).

2.4.4.1 Sample preparation

Five independent gels were prepared for each type of gel in tubes as described in Section 2.4.1. Each gel was cut into five cylindrical pieces (diameter 22 mm x height 10 mm) using a stainless-steel cutter. Each batch of five cylindrical pieces was kept within a Petri dish to avoid water loss. The total number of texture analysis measurements per type of gel was 25 (5 repetitions per gel sample). The experiment took place at ambient temperature. The cylindrical gels were lubricated with sunflower oil (Sainsbury's, Nottingham, UK) to decrease the friction between the sample and the probe used for the compression test.

2.4.4.2 Uniaxial compression test of gels: non-destructive

To understand the behaviour of a sample when it is deformed under a compression force a two-bite test is used, called Texture Profile Analysis (TPA). The TPA test has been used widely in the literature for the characterisation of gels. However, there is a variety of methods using different settings and probes. For this reason, the type of food, the sample and probe geometries and the parameters relevant to the type of food were taken into consideration before starting the TPA test.

A 100 mm cylindrical probe was used for this test to ensure the whole surface of the sample was in contact with the probe during the measurement. The probe was larger than the sample to ensure that the forces recorded through the measurement would be primarily due to uniaxial compression. A probe smaller than the sample was not selected because the forces would derive primarily from puncture which is a combination of compression, and shear. The same principle applies to a probe with the same size as the sample.

The probe was lowered to the sample to ensure the measurement would start from the same point for all samples (10 mm ± 1mm). This was a way to control the initial sample contact with the probe and reduce the generation of variable results. Trigger force was set at automatic at 5 g.

The measurement was conducted using a crosshead speed of 1 mm/sec, in line with previously published literature (Fan et al., 2017a; Johansson et al., 2022; Yu et al., 2020b; Zheng et al., 2019). The same speed for compression (test speed) and withdrawal (post-test speed) were used.

As previously discussed, the testing protocol involves the application of strain in 2 cycles and 5 sec waiting time between cycles was set, based on previous literature (Zheng et al., 2019), as well as aligning with the estimated speed of human chewing.

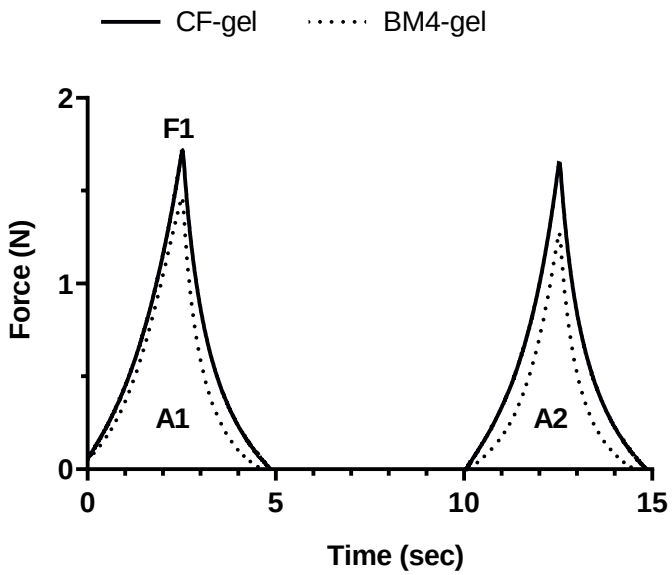


Figure 2.15 Example of the compression tests graph at a non-destructive strain (25 %) on the heat-induced gel prepared with a control (continuous line) and four hours ball-milled (dotted line) GWL flour. CF; control flour, BM4; 4 hours ball milling.

The hardness of the gels is measured as the maximum force (F1) obtained on the first deformation cycle of the TPA test and is described as the resistance of a material to deformation. Cohesiveness is the ability of the gel's internal structure to withstand a compression force and was evaluated by dividing the area of the second cycle divided by the area of the first cycle (A2/A1) as seen in Figure 2.15.

2.4.4.3 Uniaxial compression test: destructive

The fracture of a gel is the ability to break/rupture/fail when force is applied. The fracture of a gel is measured at the point of the plot's first significant peak (where the force falls off) (Burey et al., 2008).

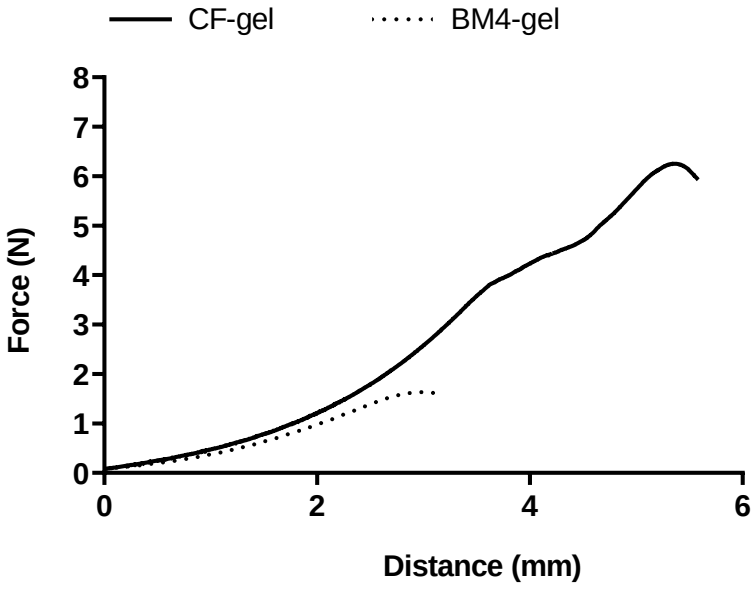


Figure 2.16 Example of the compression tests graph at a destructive strain (80%) on the heat-induced gel prepared with a control (continuous line) and 4 hours ball-milled (dotted line) GWL flour. CF; control flour, BM4; 4 hours ball milling.

A new set of lentil gels was prepared before the uniaxial destructive compression test. The fracture of the lentil gels used 80% compression, a 100 mm probe, 1 mm/s crosshead speed and automatic trigger force at 5 g The fracture/ brittleness of the gels was defined from the highest peak in the force deformation curve before the total rupture of the gels as seen in Figure 2.16.

2.5 Statistical analysis

The SPSS Statistics 27 software was used to conduct the statistical analysis. The mean values and standard deviation were determined and presented next to the values in the tables or as error bars in the figures. One-way ANOVA (analysis of variance) was used for the evaluation of the statistically significant differences and Tukey's multiple comparison tests at ($p<0.05$) significance level was used for comparing the means

between the results. Pearson’s correlation was also used to evaluate the impact of composition on the pasting properties of the control flours.

3 Chapter: The impact of the ball mill on the pasting and physiochemical properties of lentils and pearl millet

3.1 Introduction

There is a great interest in using sustainable sources such as pulses and underutilised cereals in food production including high-moisture meat analogues, extruded snacks (Marchini et al., 2021; Mefleh et al., 2022; Vasilean et al., 2018), ready-to-eat popped products (Cabrera-Ramírez et al., 2021; Kumari et al., 2019), bread (Čukelj Mustač et al., 2020) and pasta (Tyl et al., 2020).

The first stage for using pulses and cereals in food production is converting the grains into flour using milling machines (i.e., hammer, jet, pin mills) (Norman & Evers, 1994). The bread and flour regulations (1998) in the UK do not specify the flour's particle size thus several types of mills have been used in the literature. Milling changes the particle size of the grains and affects flour functionality and final product microstructure (Marchini et al., 2021). For example, the ball milling type has been found to increase the content of the amorphous phase, hydration, and pasting properties of isolated starch (Dome et al., 2020; Juarez-Arellano et al., 2021; Tian, Wang, Wang, Sun, et al., 2022; Vogel et al., 2018). A soy protein isolate (>90%) (Yang et al., 2020) and concentrated oat bran protein (<90% protein) (Ramadhan & Foster, 2018) also showed changes in the secondary structure and an increase in hydrophobicity indicating protein denaturation after ball milling.

The structural changes of the flour's macromolecules (starch, protein) demonstrated that ball milling can be used as a method of physical modification for food production however, there is still a lack of information regarding the effect of ball milling on lentil and millet flours.

3.2 Results and discussion

This experimental chapter aimed to address the impact of ball milling on flours from green whole lentils (GWL), red split lentils (RSL) and pearl millet (PM) before (control) and after physical modification (ball milling in dry conditions). Other samples including red whole lentils (RWL), yellow peas (YP), maize (M) and wheat (W) were used for comparison. To address this aim research has been conducted on the following objectives:

- Understanding the role of composition and microstructure on the pasting properties of the control flours.
- Evaluating the impact of ball milling on the particle size, microstructure, water-related properties and pasting of the legume and cereal flours.
- Discussing the role of composition and microstructure on the flour's pasting properties after ball milling.

3.2.1 Control flour composition

The composition of legume and cereal control flours, investigated in this thesis are presented in Table 3.1.

Table 3.1 Control flour; pre-ball-milled, composition of the legume (GWL; green whole lentils, RSL; red split lentils; RWL; red whole lentils, YP; yellow pea) and cereal flours (PM; pearl millet, M; maize; W; wheat) analysed by Eurofins collection (Wolverhampton, UK) see Section 2.3.4 for measurement details.

Analyte (g/100g of sample)	GWL	RSL	RWL	YP	PM	M	W
Crude protein (Nx6.25)	23.80	25.70	30.00	13.50	9.80	9.70	9.90
Carbohydrates	51.90	59.20	54.20	71.70	68.20	64.30	74.90
Total Fat	0.90	0.60	0.70	1.30	4.20	3.60	0.90
Crude Fibre	10.70	4.30	5.40	4.90	7.10	12.20	2.70
Ash	2.70	2.70	2.40	1.70	1.60	1.00	0.70
Moisture	10.90	8.00	7.20	6.90	10.00	9.30	10.80
Total	100.90	100.50	99.90	100.00	100.90	100.00	99.90

The protein content in legumes varied from 13.5% to 30% with YP having the lowest protein composition and the RWL the highest. All cereals (PM, M, W) had a similar protein composition (≈9.80%) lower in comparison to the legumes. Cereals had a similar carbohydrate content (68.20% to 74.90%) to the YP (71.70%) and higher than the lentils (51.90% to 59.20%). Total fat was low for all legumes (>1%) and W but was higher for the PM and M (≈4%). GWL and M had the highest crude fibre content (≈11.50%) and W the lowest (2.7%) in comparison to the other cereals and legumes. The crude fibre content was two times higher for the GWL in comparison to the other legumes. M had almost two times higher fibre content in comparison to PM (7.1%). Ash content was higher for the lentils (≈2.5%) and lower for the M and W (>1%).

There were differences in the composition of the legumes tested which can be attributed to differences in the species, cultivar, environmental factors during plant growth and differences in the maturity level of the grains during harvesting (Hall et al., 2017). Also, the process of removing the seed coat (de-hulling), decreases the fibre content in the flours (Dhull et al., 2022) explaining the lower crude fiber content of the YP and RSL in comparison to the GWL and RWL.

The proximate composition of legumes and cereals was in line with other researchers (Amadou et al., 2013; Arendt & Zannini, 2013a; De Almeida et al., 2006; Hajjagana et al., 2014; Joshi et al., 2017; Oomah et al., 2011).

3.2.2 Pasting properties of the control legume and cereal flours

The pasting properties of the control (hammer milled, pre-ball-milled) flours from legumes and cereals were characterised using a rapid visco analyser (RVA). RVA viscosity profiles are widely used to track the changes in the starch granule properties under heat, and continuous shearing. Composition (starch content and non-starch content) and microstructure are factors that affect paste-forming properties in flours.

The pasting time (Pt) and temperature (PT) values where viscosity started to increase and the pasting properties through heating (PV; peak viscosity), isothermal (TV; through viscosity, BD; break down) and cooling (SB; set back; FV; final viscosity) conditions were recorded. Starch was the major component in all flours and therefore it was expected to mainly contribute to viscosity however, flours are composed of other components such as proteins and fibres that can contribute to viscosity (Table 3.1) RVA was used as a method to understand the impact of composition and microstructure on viscosity.

3.2.2.1 Pasting properties of the control flours

The pasting properties of the control GWL, RWL, RSL, and YP can be found in Figure 3.1. The paste-forming properties of all legumes showed the same pattern with a viscosity increase within three minutes followed by a continued increase until the end of the profile in contrast to the typical pasting curves of starch-rich materials.

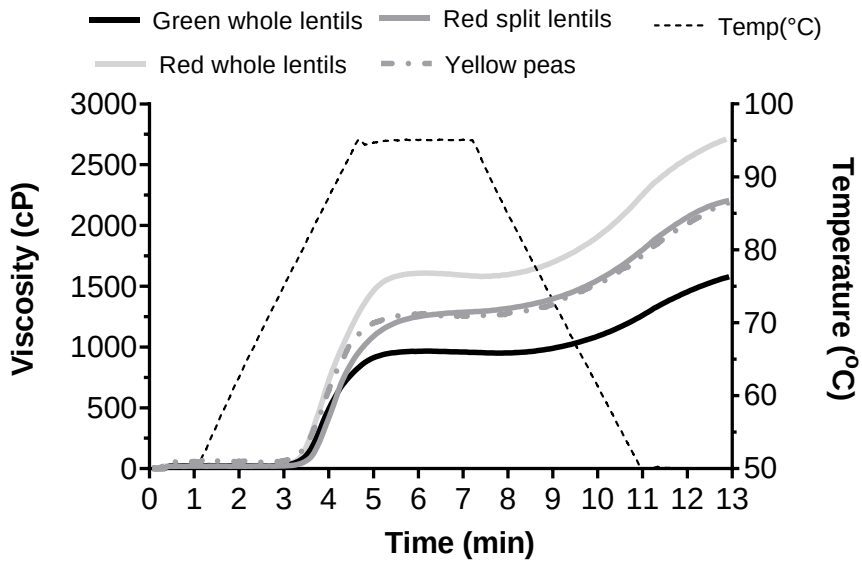


Figure 3.1 Rapid visco analysis (RVA) profile of the control legume flours under heating and cooling for thirteen minutes in excess of water (10.6% solids) and continuous shear (160 rpm).

The absence of a sharp peak in viscosity of various lentil cultivars pasting curves was also previously found using a similar technique of micro visco amylograph in the literature (Ek et al., 2021). Similarly, in various chickpea cultivars, only a slight peak in viscosity was observed (Meares et al., 2004).

For starch-rich materials, the typical viscosity response to heating and shear is a sharp peak as the starch granules hydrate and swell followed by a decrease in viscosity as the swollen starch granules are broken down by the shear force applied, as can be seen in Figure 3.2 for pearl millet (M) and wheat (W).

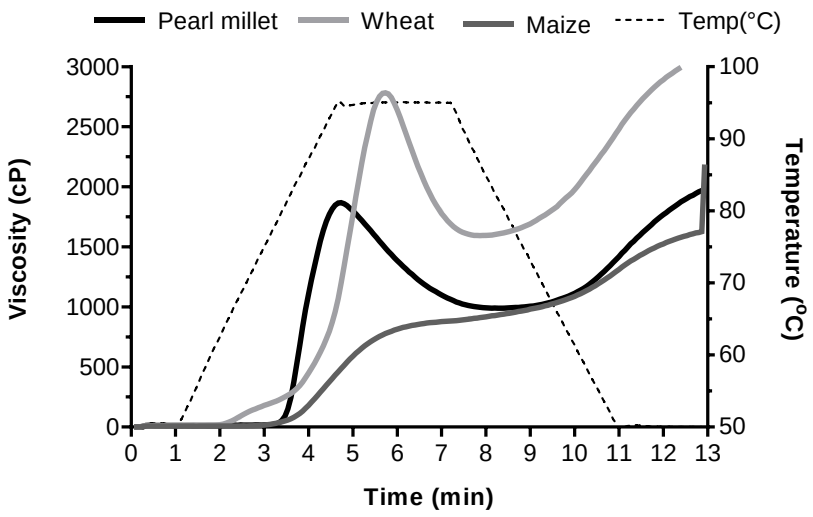


Figure 3.2 Rapid visco analysis (RVA) profile of the control cereal flours under heating and cooling for thirteen minutes in excess of water (10.6% solids) and continuous shear.

Starch granule hydration occurred in the W sample first, followed by PM and M however PM reached peak viscosity faster. Viscosity started to increase sooner for the W (2 min) but later for the PM and M (3 min). The pasting curves of the viscosity in the W flour were highest, followed by PM and M.

To understand whether the composition is related to the PV and the shape of the pasting curve, the composition of the legume and cereals samples was correlated to the PV using a linear regression statistical analysis as can be seen in Figure 3.3.

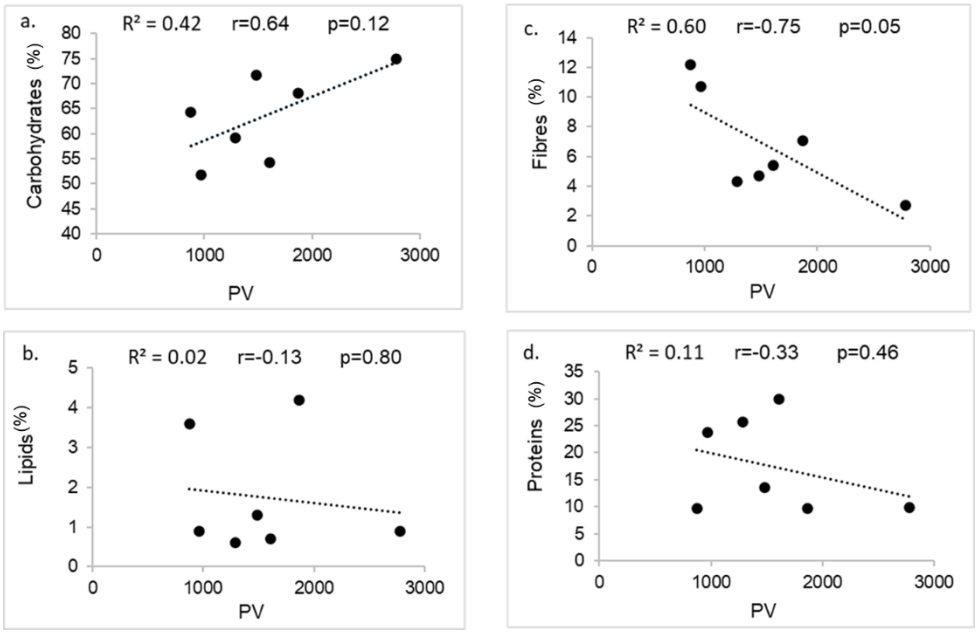


Figure 3.3 Correlation between the (a) carbohydrates, (b) lipids, (c) proteins, (d) fibres composition and the peak viscosity (PV) of the legume and cereal samples. R2=coefficient of determination, r=degree of correlation, p=Pearson's correlation coefficient.

The correlation analysis of the carbohydrate content and PV in the legumes and cereals samples was not significant as seen in Figure 3.3a. Ragaee and Abdel-Aal, (2006) observed differences in the pasting peaks of various cereal grains and attributed higher viscosity to higher starch content. However, YP had a similar carbohydrate content to cereals, still did not show a sharp pasting peak and followed the same viscosity profile as the lentil's samples. The swelling and disruption of starch granules account for the major changes in viscosity measured by RVA and yet a common sharp pasting curve was not seen in legumes or maize samples in this thesis with a carbohydrate content of 51.90-71.70% and 64.30% respectively.

The lipids composition was also not found to significantly correlate with PV as seen in Figure 3.3b. In contrast, lipids in millet have been found to form complexes with the amylose, with those complexes limiting

starch swelling and resisting damage due to heat and shear (Srichuwong et al., 2017).

There was a significant correlation between the fibre composition and the PV as shown in Figure 3.3c, with higher fibre content presenting the highest PV. The lowest recorded value of PV in this sample set was from the GWL and M samples, which could be attributed to the higher fibre composition in comparison to the other legume and cereal samples. Similarly, the highest PV of the W could be related to the very low fibre composition.

Fibres are used in the food industry as thickeners increasing the viscosity of a starch-based system, however, Qiu et al., (2015) reported that low molecular weight gum fibre found in the cell walls of maize flour could decrease PV. This phenomenon was attributed to chain entanglement between the gums and the amylose or short amylopectin chains inhibiting further swelling of the starch granules. Published studies on the effect of fibres are contrasting, with isolated soluble fibres from peas, lentils and chickpeas not increasing pasting viscosity (Dalgetty & Baik, 2003a).

It should also be taken into consideration that the data analysed is of a limited number of samples and is evenly distributed creating additional uncertainty to this proposed hypothesis. Further investigation with a greater number of samples is required to evaluate the effect of fibres on pasting.

A non-significant correlation was found between the protein composition and the PV, as presented in Figure 3.3d. A lower viscosity has been observed when proteins are added to a starch-based system (Akinwale et al., 2017; Enpeng et al., 2014; Joshi et al., 2014) due to water competition. Sun & Xiong, (2014) observed the absence of a sharp peak and a lower viscosity when pea starch and protein isolates were combined, and the composition of the protein isolate increased. It was hypothesised that the replacement of starch with protein decreased the amylose content (starch dilution) in the canister and decreased the swelling power of the starch resulting in a lower viscosity. There was a clear difference between the protein content in the lentil flours (13.50-30%) and cereals (9.70-9.80%) contributing to starch dilution and thus could explain the lower viscosity. To investigate this hypothesis the impact of proteins on the pasting behaviour of the lentils was further evaluated.

3.2.2.2 Effect of protein on pasting properties of legume flours

As discussed in the previous section 3.2.2.1, the protein content of the legume samples investigated varies between 13.50-30% and represents the second largest component after carbohydrates (Table 3.1).

Globulins are the main storage proteins in lentils (47-70%) along with albumins (3.8-16%) (Jarpa-Parra, 2018; Joshi et al., 2017). Ghumman, Kaur & Singh, (2016), compared the rheological properties of starch, starch-albumin and starch-globulin blends isolated from lentils, finding a higher restriction of starch swelling when globulins were added

compared to albumin. It is therefore hypothesised that the presence of protein, particularly globulins in the legume flours inhibits starch granules hydration and swelling, explaining the lack of a sharp/high peak viscosity. To test this hypothesis NaCl was added to the GWL and RSL flour suspensions before RVA analysis to solubilise the globulins in the continuous phase.

As can be seen in Figure 3.4, the pasting curve's overall shape did not change significantly for GWL with the addition of NaCl and there was no starch swelling peak nor breakdown observed, as seen in starch-rich materials. However, a more prominent pasting peak appeared for RSL with NaCl addition, indicating an increase in the swelling ability of starch as seen in Figure 3.4. This result was in line with Joshi et al., (2014) who showed an increase in viscosity of isolated starch and protein composites by adding 0.60 M NaCl.

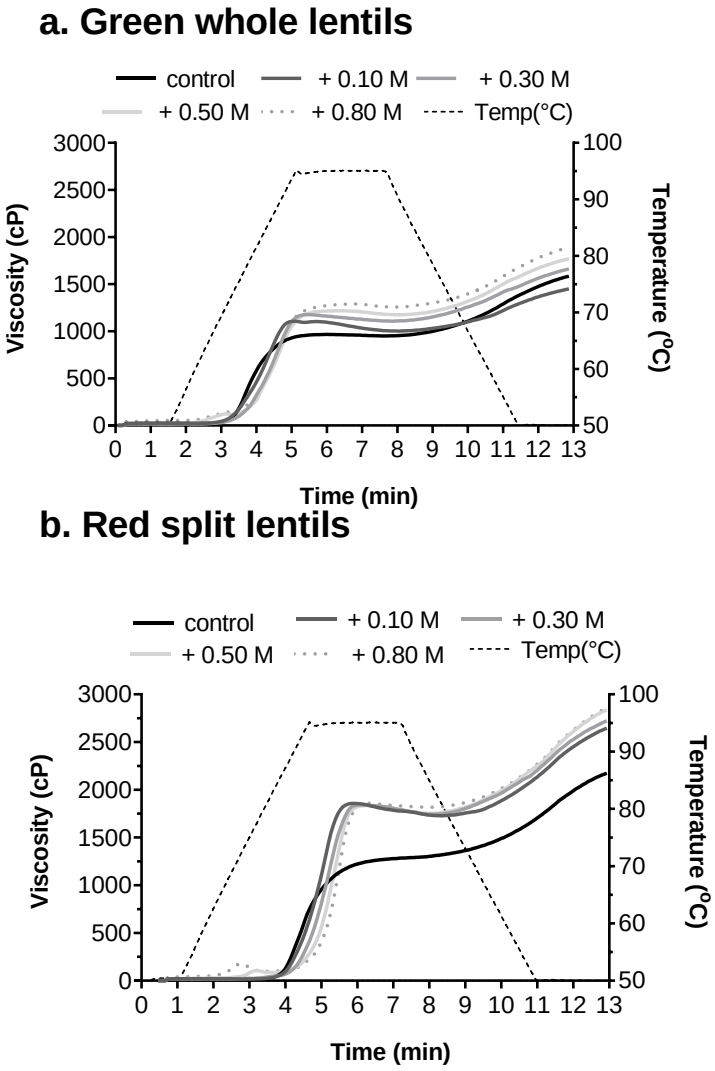


Figure 3.4 Rapid visco analysis (RVA) profile of the control (a) Green whole lentil and (b) Red split lentil flours under heating and cooling for thirteen minutes in excess of water and continuous shear with the addition of NaCl (0.10-0.80 M).

In an aqueous solution, NaCl is dissociated and Na^+ interacts with the COO^- groups and Cl^- interacts with the $NH3^+$ amino acid groups of the protein's structure (Li & Xiong, 2021). The addition of NaCl ions induces protein-protein interactions and increases the water availability in the bulk (Hansen et al., 2016), therefore potentially increasing starch hydration and viscosity.

The pasting parameters after NaCl was added to the suspension of the flours can be found in Table 3.2. The PT of the GWL did not change by increasing NaCl concentration but the PT of the RSL increased at 0.30 M and then decreased significantly at 0.50 M and 0.80 M. A significant increase in the PV of the RSL and GWL was observed after the addition of salt. FV was only increased for the RSL in all NaCl concentrations but increased only after the addition of 0.30 M for the GWL. GWL showed a lower affinity to increased ionic strength in comparison to the RSL.

Table 3.2 The pasting parameters of the control lentil flours after NaCl addition (0.00-0.80 M). GWL; green whole lentils, RSL; red split lentils, PT; peak temperature, Pt; peak time, PV; peak viscosity, BD; break down, TV; trough viscosity, SB; set-back, FV; final viscosity. One-way ANOVA was conducted for each lentil separately and different letters in each column represent a significant difference at $p<0.05$.

NaCl	PT (ºC)	Pt (min)	PV (cP)	BD (cP)	TV (cP)	SB (cP)	FV (cP)
GWL							
0.00 M	79.10±0.05^{a}	6.00±0.08^{b}	966.70±9.90^{d}	16.70±4.50^{d}	950.00±5.70^{e}	634.30±4.10^{a}	1584.30±6.60^{d}
0.10 M	78.60±0.40^{a}	5.10±0.03^{d}	1107.00±9.90^{c}	105.00±0.80^{a}	1002.00±10.70^{d}	447.70±17.40^{d}	1449.70±25.95^{e}
0.30 M	81.80±1.00^{a}	5.50±0.08^{c}	1177.00±21.70^{b}	70.30±5.55^{b}	1106.70±16.10^{c}	554.70±16.30^{c}	1661.30±32.40^{c}
0.50 M	80.70±6.90^{a}	6.30±0.20^{a}	1218.00±6.20^{b}	43.00±2.95^{c}	1175.00±9.10^{b}	595.30±2.60^{b}	1770.30±6.90^{b}
0.80 M	74.50±2.80^{a}	6.90±0.09^{a}	1290.30±20.20^{a}	33.00±5.90^{c}	1257.30±24.10^{a}	632.70±3.40^{a}	1890.00±23.80^{a}
RSL							
0.00 M	80.70±0.10^{b}	7.00±0.00^{a}	1287.70±5.60^{b}	26.00±3.45^{c}	1261.70±8.00^{c}	946.30±16.80^{c}	2208.00±11.40^{d}
0.10 M	81.50±0.10^{b}	5.50±0.05^{b}	1857.30±2.50^{a}	130.30±9.65^{a}	1727.00±12.00^{b}	974.00±10.70$^{c, b}$	2701.00±15.30^{c}
0.30 M	84.05±0.04^{a}	5.70±0.00^{b}	1842.00±17.70^{a}	99.70±2.60^{b}	1742.30±16.80^{b}	1033.30±29.80^{b}	2775.70±28.20^{b}
0.50 M	69.40±0.04^{d}	5.90±0.06^{b}	1837.00±7.80^{a}	85.00±5.10^{b}	1752.00±7.80^{b}	1150.00±7.90^{a}	2902.00±8.50^{a}
0.80 M	63.20±1.06^{c}	5.90±0.08^{b}	1860.30±5.25^{a}	43.70±7.40^{c}	1816.70±6.65^{a}	1108.70±26.00^{a}	2925.30±29.35^{a}

After hammer milling, RSL was pre-processed by de-hulling and splitting, which could increase starch and protein separation. Interactions between NaCl ions and the proteins in the RSL would increase protein solubility and promote starch hydration explaining the higher viscosity of the RSL after NaCl addition.

Differences between the impact of NaCl on the two lentils could also be attributed to the type of salt-soluble globulins and their amino acid sequences. There are two globulins named 11S legumin and 7S vicilin in legumes. The ratio of 11S/7S in the different varieties of the same species can be different due to environmental and harvesting conditions (Gravel & Doyen, 2023). Kimura et al., (2008), reported that the major globulins in pea and fava beans are both 7S and 11S but in French beans, cowpeas and mung beans it is only 7S. The researchers showed that although the solubility of all proteins increased by NaCl addition, the 7S proteins from French beans had the highest solubility. Similarly, the globulins of the RSL showed a higher solubility in NaCl in comparison to the GWL in this research leaving more space for starch-water interactions and increasing viscosity.

Proteins bound with the NaCl molecules and an increase in viscosity was observed due to higher starch hydration with the available water molecules. However, the shape of the pasting curve did not change after increasing ionic strength therefore suggesting that the microstructure could also influence the pasting properties of the raw materials.

3.2.2.3 Effect of legume flour microstructure on pasting properties

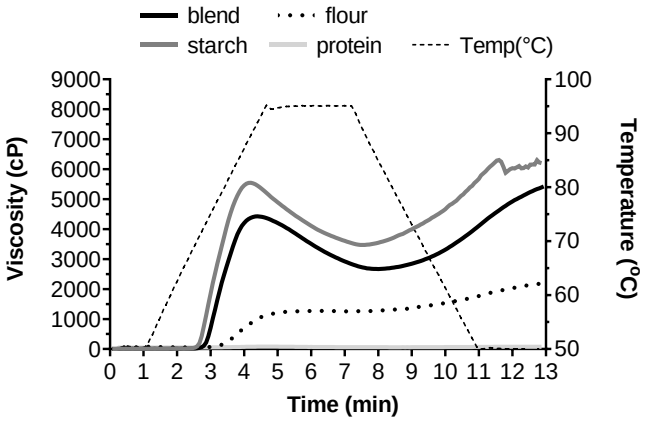
To investigate the impact of the microstructure on the pasting curve, the protein was isolated from RSL using the alkali method (Section 2.3.10.3) by increasing the solubility of proteins (pH=9). Starch is not soluble in such conditions thus the remaining material was termed ‘starch-rich’ material. The RSL was selected as it was de-hulled and presented the lowest fibre composition in comparison to the other lentils (Table 3.1) thus increasing the purity of the starch-rich material.

The pasting properties of RSL extracted protein, RSL starch-rich material, RSL flour and RSL protein-starch blend with comparable protein-starch composition to the RSL flour were evaluated. The same experiment was conducted for the YP using commercially available starch and protein fractions.

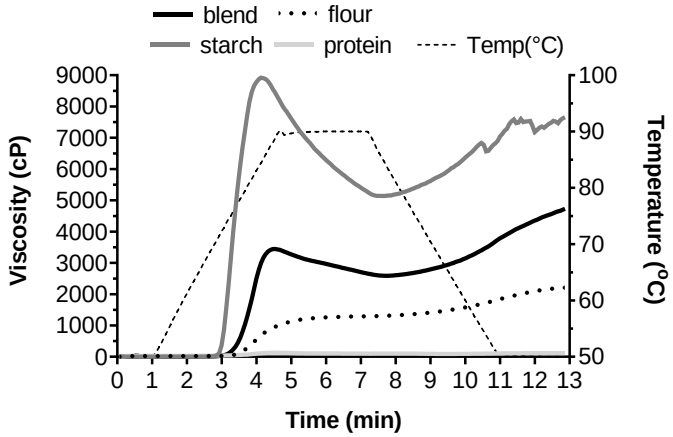
The composition of the starch-protein blend samples was formulated to mimic the flour composition. Starch accounted for 59.20% and protein at 25.70% for the RSL according to and 45.60% and 24.70% for the pea according to literature (Dalgetty & Baik, 2003a) as at the time of the experiment, the composition of the pea flour used in this thesis was not available. The actual YP composition was substantially higher in starch content at 71.70% starch and lower in protein (13.50%) than the experimental blend formulation.

The pasting curves of the YP and RSL samples can be found in Figure 3.5 and the pasting parameters for the same samples are in Table 3.3.

a. Yellow peas



b. Red split lentils



Approximate composition profiles of the YP and RSL		
	Assumed starch content (%)	**Assumes protein content (%)**
YP starch	100	0
YP protein	0	100
YP blend	46	25
YP flour	72	14
RSL starch	100	0
RSL protein	0	100
RSL blend	59	26
RSL flour	59	26

Figure 3.5 Rapid visco analysis (RVA) profile of the control (a) Yellow pea and (b) Red split lentil flour in comparison to laboratory (RSL) and industrially (pea) extracted isolated protein, starch and a protein-starch blend; formed with comparable protein and starch composition to the flours, under heating and cooling for thirteen minutes in excess of water (10.6% solids) and continuous shear.

Table 3.3 Pasting parameters of the YP and RSL flours, starch and protein isolates and starch-protein blends. YP; yellow pea, RSL; red split lentils. PT; peak temperature, Pt; peak time, PV; peak viscosity, BD; break down, TV; trough viscosity, SB; set-back, FV; final viscosity. One-way ANOVA was conducted for the YP and RSL samples separately for each column thus different letters represent a significant difference ($p<0.05$) in each column.

	PT (°C)	Pt (min)	PV (cP)	BD (cP)	TV (cP)	SB (cP)	FV (cP)
YP							
flour	80.70±0.05^{a}	7.00±0.00^{a}	1287.70±8.60^{c}	26.00±3.70^{c}	1261.70±8.10^{c}	946.30±16.80^{b}	2066.30±10.90^{b}
starch	69.80±0.80^{b}	4.30±0.03^{b}	5548.70±815.30^{a}	2079.30±365.90^{a}	3469.30±449.80^{a}	2732.30±664.50^{a}	6201.70±225.30^{a}
protein	0.00±0.00^{c}	4.90±0.03^{c}	88.30±16.35^{d}	26.30±10.65^{c}	62.00±5.70^{d}	23.70±13.00^{c}	85.70±18.70^{c}
blend	70.95±70.95^{b}	4.40±4.40^{b,c}	4426.30±65.70^{b}	1757.70±118.40^{b}	2668.70±63.50^{b}	2753.70±64.10^{a}	5422.30±29.90^{a}
RSL							
flour	75.90±0.04a^{a}	5.30±0.03^{a}	1484.30±7.85^{c}	14.70±0.40^{d}	1337.70±7.40^{c}	728.70±3.70^{c}	2208.00±11.40^{c}
starch	73.70±0.40^{b}	4.30±0.05^{c}	8938.70±727.60^{a}	3816.30±551.70^{a}	5122.30±213.30^{a}	2534.30±1046.70^{a}	7656.70±855.60^{a}
protein	0.00±0.00^{c}	4.60±0.20^{b}	135.00±6.50^{d}	55.00±3.55^{c}	80.00±9.90^{d}	36.30±28.10^{d}	116.30±38.00^{d}
blend	76.40±0.40^{a}	4.50±0.03^{c}	3444.00±145.70^{b}	854.00±42.20^{b}	2590.00±103.70^{b}	2140.00±175.20^{b}	4730.00±263.40^{b}

The starch-rich material of both legumes showed a sharp, viscosity peak, whereas the extracted protein did not demonstrate pasting and can be observed as a flat line of very low viscosity (88.30 ± 16.35, 135.00 ± 6.50 cP). RSL starch-rich material showed higher PV (8938.70 ± 727.60 cP) values in comparison to the pea starch (YP) (5548.70 ± 815.30 cP). Variation in PV could be attributed to the amylose content, crystallinity degree, starch granules' size and interactions between the amylose-amylopectin molecules within the structure of the starch granules (Wani et al., 2016). The starch-protein blends of both legumes showed also a more prominent and higher PV than the flour samples.

The same solids (10.6%) were used for the starch-rich and the starch-protein blend samples. Therefore, the lower pasting of the blends could be attributed to lower starch content. However, if the pasting properties of the flour were solely determined by composition, there would be little difference between the pasting of the blends and the flours. It should also be noted that the YP blend (46% starch) was prepared with a lower starch content in comparison to the flour (72%), as mentioned above, still the viscosity was higher for the blend. The significant differences in the pasting values of the flours and the blends can be seen in Table 3.3, indicating the microstructure of the samples has a substantial impact on pasting.

All pasting values showed the order of starch-rich material> starch-protein blend>flour>proteins. PV was significantly higher for the starch-rich material followed by the protein-starch blend in both legumes. FV followed the same trend and showed that the amylose from starch-rich

material retrograded and created a thick paste. According to the literature, proteins may hinder hydrogen bonding between the starch chains by acting as an inert filler (Sopade et al., 2006) or reduce the amount of amylose leached from the starch granules during gelatinisation by interacting with starch (Sun & Xiong, 2014) thus decreasing FV. However, the higher FV of the starch-rich material followed by the protein-starch blend and legume flours indicated that the presence of the proteins within the starch-protein blend did not hinder starch retrogradation.

The difference in the viscosity between the starch-rich and the starch-protein blend was higher in RSL in comparison to the pea. Pea protein had a lower effect on the viscosity of the starch in comparison to the RSL protein. The type, conformation and aggregation state of the proteins impact functionality. Industrially extracted proteins showed a reduction in functional performance such as solubility, in comparison to laboratory-extracted proteins (Ma et al., 2022).

Milling has been found to decrease the size of the grains but cannot separate starch and proteins effectively and passing the flour through sieves or using other means (like air classification) should be applied. In this thesis, the grain passed through the hammer mill once. Hammer milling separated the parts of the grain into flour particles to a size of <1-2 mm. Therefore, starch granules could be found free, in clusters, encapsulated by the protein matrix and within intact or broken cell structures. The presence of such structures could inhibit the starch granules' hydration and decrease the pasting viscosity of flours.

M showed a similar pasting profile to the legumes. The presence of proteins on the surface of the starch granules and in channels within the structure of the starch granule in maize using confocal microscopy has also been reported. The surface proteins in M were characterised as residues from storage proteins and the proteins within the channels as residues from enzyme complexes. The removal of the channel proteins using protease treatment increased the viscosity of the M starch granules (Bae et al., 2020; Ma et al., 2022). However, those proteins located at the surface and physically or chemically associated with the starch granules are difficult to remove by starch extraction (Tan et al., 2021). This factor was taken into consideration to understand any differences in the pasting properties between the cereals.

To conclude, microstructure impacted the ability of the legume and cereal flours to create a paste. In the next part of this Chapter the physical modification of the flours from lentils and the pearl millet was conducted using a ball milling. The impact of the ball milling on the microstructure and the properties of the flours will be evaluated next.

3.2.3 Physical transformation of control lentil and pearl millet flours

The impact of a ball mill on the microstructure of two types of lentils (red split lentil; RSL, green whole lentil; GWL) and a type of millet (pearl millet; PM) was assessed. The lentils and the PM were ball-milled for 2 (BM2) and 4 hours (BM4). These flours were selected at the beginning of this thesis before conducting any experiments on other legumes and cereal flours. However, the evaluation of the results taken from the ball mill modification created the need to include more samples in the research to ensure the repeatability of the observations.

Various techniques were used to characterise the flour including particle size analysis, moisture content, fluorescent microscopy and water-hydration properties. The birefringence, crystallinity diffraction and thermal properties of the starch within the flours were investigated after ball milling. Solubility, hydrophobicity, fluorescent light microscopy, and thermal analysis were used to identify the impact of the ball mill on proteins within the flours.

3.2.3.1 Moisture content of ball-milled flours

Vogel, Anne & Peter, (2018) reported that the friction and impact between the ball milling containers, balls and flours increased the temperature within the ball milling containers. Loubes, González & Tolaba, (2018a) showed that ball milling decreases the moisture content of the flour due to water evaporation when the ball milling containers open. Therefore, although in this thesis the ball milling method included

pause intervals to reduce heat generation, it is likely that increasing the time of ball milling increased the friction and impact resulting in higher heat generation and water evaporation when the ball milling containers open.

The impact of ball milling on the moisture content of GWL, RSL and PM can be observed in Figure 3.6.

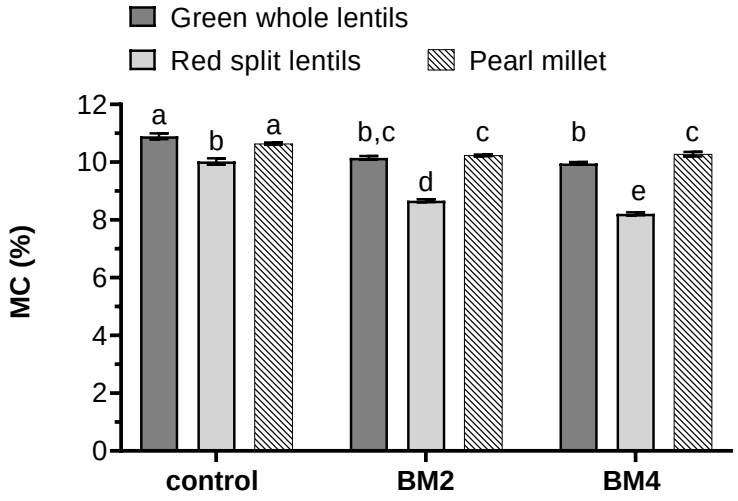


Figure 3.6 Moisture content (MC) of control and ball-milled lentils and pearl millet flours. The flours were taken immediately after ball milling to measure the moisture content and dried as per section 2.3.5. Different letters represent significant differences at $p<0.05$.

The moisture content was low in the control GWL, RSL, and PM (<10%) flours and decreased for all flours after ball milling. The decrease in moisture content of the RSL after ball milling was greater, likely to be due to the commercial pre-processing (removal of the hull and separation from the cotyledons) of the RSL increasing the surface area for moisture evaporation.

3.2.3.2 Particle size of ball-milled samples

As discussed in Chapter 1, milling is mainly used to decrease the particle size of the grains or to induce fractionation of the components thus, the impact of milling on the grains and the flours was evaluated.

The lentil and millet grains were hammer-milled to produce the control flours and the particle size analysis can be seen in Figure 3.7.

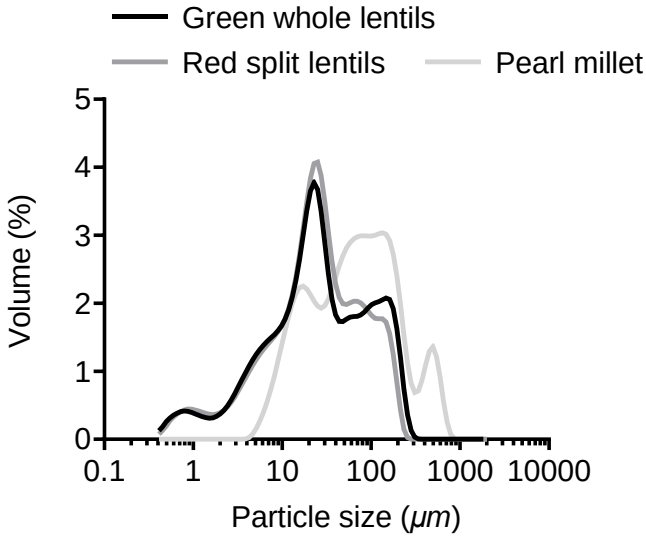


Figure 3.7 Particle size graph of the control lentil flours and the pearl millet flour. Particle size was performed on dry flour.

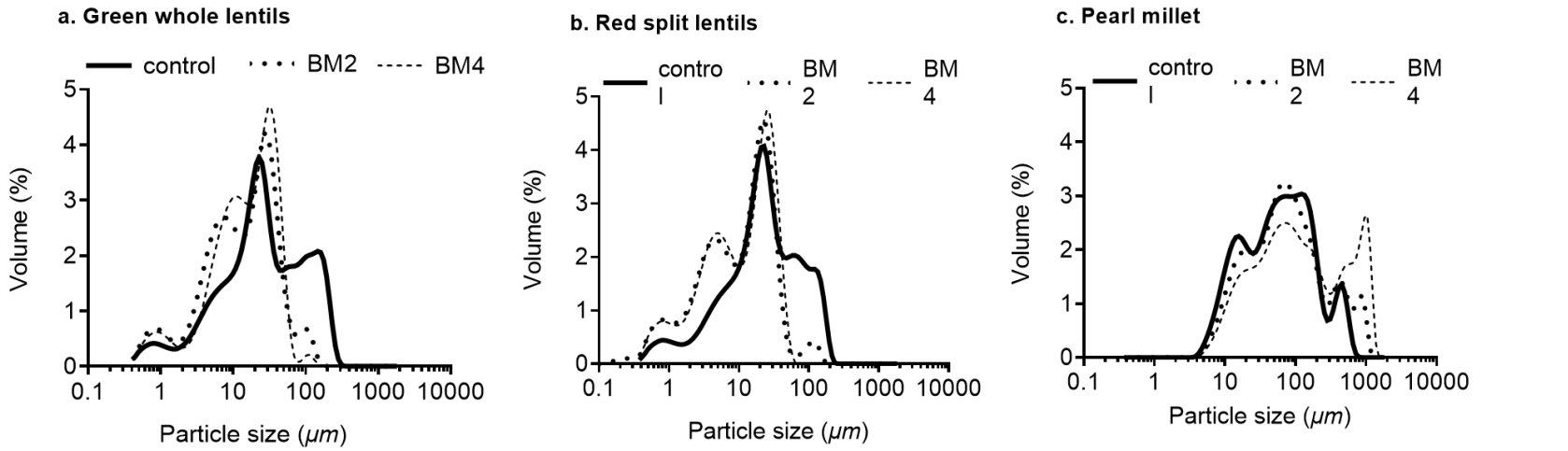
The particle size of the control flours showed a polymodal distribution. Overall particle size was higher for PM and ranged from 5-1000 µm. However, both lentils showed a lower particle size range in comparison to PM from 0.4-300 µm. The particle size range was in line with literature evidence on red split lentils (Ahmed et al., 2016), mung bean (Yu et al., 2023), de-hulled chickpea flour (Kerr et al., 2000) and sorghum; a small grain with anatomical characteristics and composition similar to PM (Palavecino et al., 2019).

In lentils, the peak between 0.4-2 μm was attributed to proteins however, none of the peaks in the PM particle size graph was attributed to the proteins. Proteins in PM have been reported to have a size >3 μm (Zhou et al., 2013). The starch granules of lentils are larger in size in comparison to PM thus the peak from 10-50 μm was attributed to the lentils' starch granules and the peaks at 4-30 μm to PM's starch granules (Zhou et al., 2013). The peak from 50-300 μm in the lentils was attributed to dissociated flour particles including intact or broken cell structures or cell wall material in line with Gharibzahedi et al., (2014) & Joshi et al., (2013b). The peaks from 30-1000 μm were attributed to cells, cell wall material and parts of the endosperm in PM in line with Arendt & Zannini, (2013a) & Gulati et al., (2018).

Differences in the particle size between the lentils were attributed to differences in the composition. RSL grain was de-hulled with a lower composition of hull fibres in comparison to the GWL. The GWL hull fibres increased the proportion of the coarse fraction (Thakur et al., 2019) and accounted for the higher volume of the peak at 150-300 μm in GWL.

Moreover, in Table 3.1 of this thesis and according to Moldovan et al., (2015) and Chávez-Murillo et al., (2018), the starch content is higher in RSL in comparison to GWL. RSL showed a higher volume within the 10-50 μm region attributed to starch granules.

The impact of the ball mill on the particle size of the GWL, RSL and PM after BM2 and BM4 can be seen in Figure 3.8. Ball-milled flours showed a polymodal distribution as was observed for the control.



	Green whole lentils		
	control	**BM2**	**BM4**
d90	147.68±1.32	42.42±0.30	40.16±0.94

	Red split lentils		
	control	**BM2**	**BM4**
d90	108.18±3.64	33.91±0.37	35.04±0.01

	Pearl millet		
	control	**BM2**	**BM4**
d90	260.75±43.30	403.65±233.21	459.88±170.30

Figure 3.8 Particle size analysis graphs and d90 values showing the impact of ball milling on (a) Green whole lentils, (b) Red split lentils and (c) Pearl millet flours.

A shift in the lentils' distribution to lower particle sizes was observed after BM2 and BM4 in the particle size distribution. However, a shift of PM to a higher particle size was observed after ball milling and was likely a result of particle agglomeration. The decrease in d90 values for lentils and the increase in d90 values for PM confirmed the finding that ball milling had a different impact on the size of the flour particles.

Ball milling of lentils reduced the particle size of the lentils. BM2 decreased the population of the particles with size >100 µm promoting an increase in the volume of the peaks between 10-70 µm and <10 µm for both lentils. The particle size of both lentils was further decreased by increasing the ball milling time. BM4 decreased the particle size of the RSL to <100 µm. BM4 further decreased the population of particles with >100 µm size for the GWL. A decrease in particle size due to an increase in the ball milling time has been also observed for other legumes in the literature (Yu et al., 2023).

Ball milling had the opposite effect on the PM in comparison to the lentils. The particle size (5-1000 µm) of PM increased to 6-2000 µm after ball milling and was accompanied by a decrease in the volume of the particles with a size <400 µm. This observation was also confirmed by the d90 value. The damage and fragmentation of PM flour particles by the ball milling process promoted agglomeration with other flour particles and increased particle size. Loubes, González & Tolaba, (2018b) & Juarez-Arellano et al., (2021) attributed similar starch agglomeration of isolated rice starch to the cleavage of the glycosidic bonds in starch structure and creation of hydrogen bonds by free hydroxyl groups. This

hypothesis was suggested after observing changes in the morphology of the starch granules from polyhedral-shaped particles and smooth surfaces to flat-shaped particles and rough surfaces. An increase in the particle size of cereal flour (wheat) after ball milling was also reported by Vogel, Anne & Peter, (2018) due to the hydrogen bonding of starch granules but also crosslinking between the proteins located on the surface of flour particles.

The changes in the particle size of the lentils and the millet after ball milling suggested changes in the microstructure of the flour. For this reason, fluorescent microscopy was used to visualise the distribution of the starch, protein and fibres within the flours.

3.2.3.3 Ball-milled flour microstructure observed under fluorescent light

The observation of flour particles (GWL, RSL and PM) under fluorescent light microscopy was conducted after the addition of a water droplet and compared to the control samples, micrographs are presented in Figure 3.9.

The fluorescent microscopy reveals only the stained surface of the starch granules, not any internal cavities. The magnification of the microscope did not allow any observation of the starch granules' cross sections. The black or darker parts of the starch granules represented a higher thickness that light could not pass through.

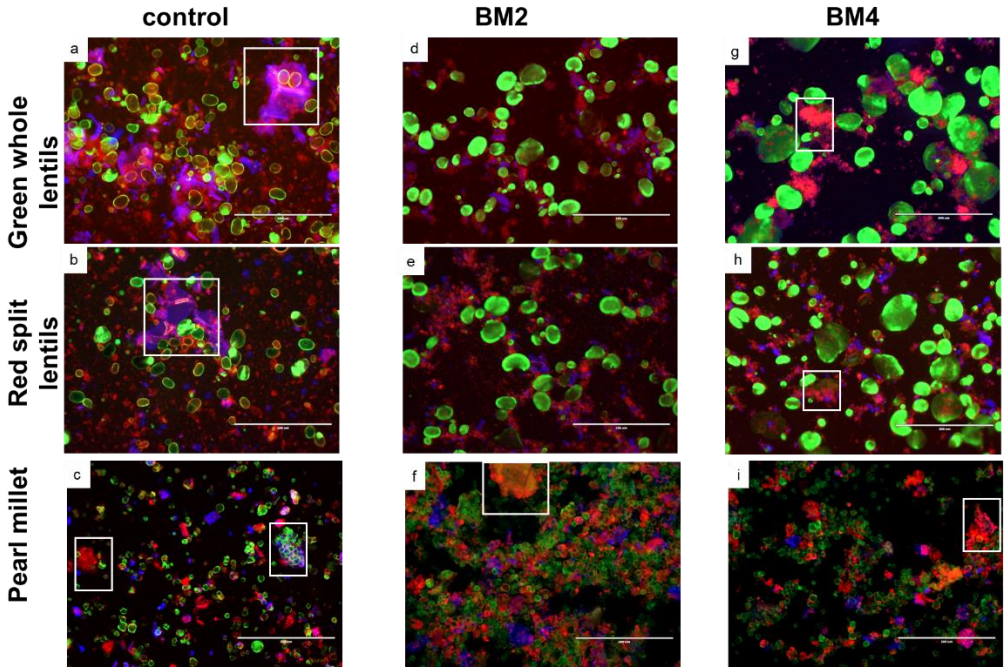


Figure 3.9 Fluorescent light micrographs of the control and ball-milled lentils and the pearl millet flour. All micrographs were taken after adding a droplet of water and at 25 ^{o}C. Scale bar: 200 µm. The white boxes show (a, b) intact cell wall material from the cotyledons, (c) intact parts of the endosperm, (g, h) aggregated proteins and (f, i) agglomerated particles. Fluorescent staining identified starch as a green colour, proteins as a red colour and fibres as a blue colour.

Oval-shaped starch granules within the range of 10-50 µm were observed for the control lentil flours and polygonal-shaped granules within 4-30 µm for the control PM flour. These observations were in line with the particle sizes presented in Figure 3.7 and attributed to starch granules for all flours.

Free protein bodies were observed in both the lentils and the PM control flours in various shapes and sizes >3 µm. The higher number of proteins observed in the lentil samples compared to the PM was in line with the composition in Table 3.1. It was not clear whether there was a separation of any starch granule-associated proteins. Intact cells within the control lentil flours appear pink/ blue in colour within the white boxes in Figure 3.9a,b with a size of >100 µm confirming observations made from the

particle size analysis. The same observation was made for the PM where starch in clusters and intact parts of the endosperm represented a particle size >100 μm in Figure 3.9c.

Ball milling increased the size of the stained protein entities, which can be observed in white boxes at Figure 3.9g, h, in the lentil flours indicating a change in the structure and aggregation. Denaturation followed by aggregation has been reported in the literature due to changes in the secondary structure of the proteins; increased number of α-helixes and decreased number of β-sheets, and exposure of the hydrophobic amino acids buried within the interior of the proteins' structure (Liu et al., 2017; Liu et al., 2021).

Broken cells observed in the lentil control flours' micrographs (white boxes in Figure 3.9a, b disappeared after the application of ball milling due to fragmentation in Figure 3.9d, e, g, h. The fragmentation of whole cells and fibres was also evidenced by a decrease in the volume of the peaks between 100-1000 μm in Figure 3.8.

Ball milling promoted the agglomeration of PM's proteins and starch granules as can be seen in Figure 3.9f, i which was previously identified by the increased particle size of PM flour observed in Figure 3.8.

An increase in the swelling ability of the starch granules by increasing the time of ball milling was observed for the GWL and RSL in Figure 3.9d, e, g, h. Native starch granules are not soluble in water under room temperature conditions and thus cannot swell without a temperature increase. However, ball milling disrupts the semi-crystalline structure of

the starch allowing water molecules to enter the interior of the starch granules even at room temperature conditions (Bangar et al., 2023). The stains used for the observation of the starch granules in the flours under fluorescent light contain water. It was hypothesised that ball milling disrupted the structure of the starch granules causing an increase in their swelling capacity under room temperature conditions. To confirm this hypothesis, the control and BM4 transmitted light micrographs of the GWL and RSL under dry and wet conditions were observed and can be found in Figure 3.10.

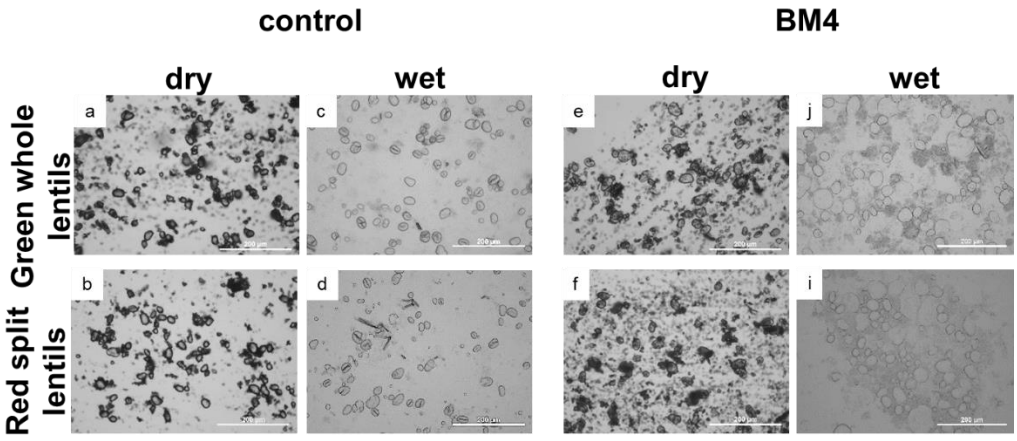


Figure 3.10 The control and ball-milled lentil flours under transmitted light using dry and wet conditions. Scale bar; 200 µm. dry; flour, wet; flour with a droplet of water. Control; prior to ball milling, BM4; after 4 hours of ball milling.

The starch granules of the control flours did not swell upon dry as can be seen in Figure 3.10a, b or wet conditions as can be seen in Figure 3.10c, d. Ball milling did not change the behaviour of the starch granules under dry conditions as shown in Figure 3.10e, f. However, swollen starch granules were observed for the ball-milled flours under wet conditions confirming that ball milling impacted the starch granule's structure in Figure 3.10j, i.

Having observed microstructural differences between the control and ball-milled starch granules of the lentil flours further evaluation was needed. Polarised light microscopy and X-RD diffraction were used next to identify any changes in the semi-crystalline structure of the starch granules of the flours after ball milling.

3.2.3.4 Starch granule microstructure observed under transmitted and polarised light

A combination of transmitted and polarised light microscopy was used to observe starch granules of control and ball-milled GWL, RSL and PM samples, as presented in Figure 3.11.

A polarised light microscope improves contrast by changing the perpendicular direction of light. Native starch granules can be identified under polarised light due to birefringent crystalline regions formed by radially arranged amylopectin molecules. The combination of crystalline and amorphous regions in the starch granules' structure is observed in the form of a cross under polarised light called the Maltese cross (Xiao et al., 2020).

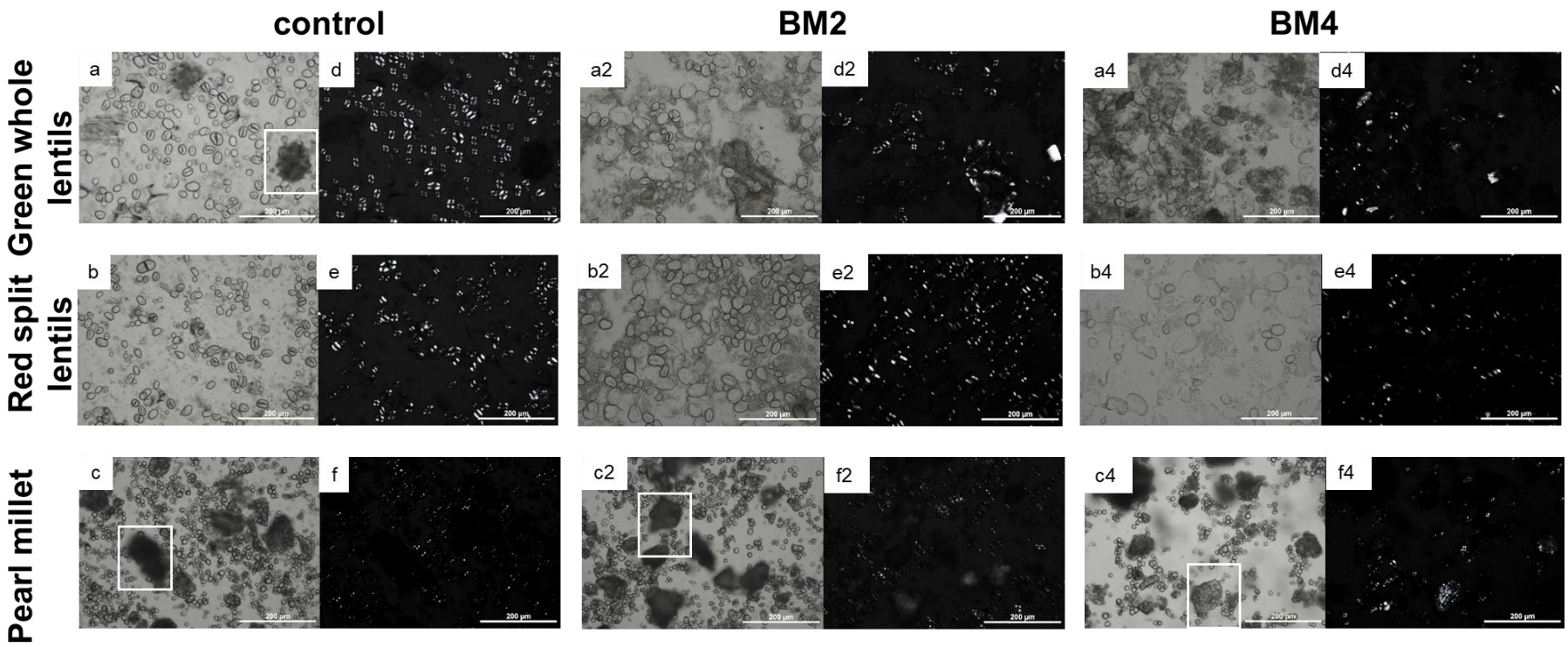


Figure 3.11 Bright-field and polarised light of the control (a-f), BM2 (g-i) and BM4 (m-r) flours from the lentils and pearl millet flours. The micrographs were taken after adding a droplet of water to the flour at 25 ^{o}C. Scale bar; 200 µm. White boxes in (a) show cells, (c) parts of the endosperm and (i, o) starch agglomerates. BM2; 2 hours ball milling, BM4; 4 hours ball milling.

Bright-field light of control lentils and PM flours revealed the same shape and size of starch granules as observed under the fluorescent light in Figure 3.9a, b, c. Lentils presented oval-shaped starch granules (10-50 μm) and PM polygonal-shaped starch granules (4-30 μm). Cell structure was observed for the GWL and can be seen in a white box in Figure 3.11a and parts of the endosperm for the PM and can be seen in a white box in Figure 3.11c.

The control GWL, RSL, and PM showed birefringence and characteristic 'Maltese crosses' indicating the position of the hilum in the centre of the starch granules. Birefringence confirmed the presence of crystalline material and radial orientation indicating a native starch granules structure. Differences in the intensity of birefringence are attributed to the granules' size, relative crystallinity, microcrystalline orientation, and the position of the starch granules to the direction of the light beam (Chakraborty et al., 2020; Kumar et al., 2016). The weaker birefringent pattern of RSL in comparison to GWL indicated a lower degree of molecular orientation. Differences in the intensity of birefringence between different beans (Ambigaipalan et al., 2014) and types of wheat (Kumar et al., 2016) have been observed previously.

A decrease in birefringence intensity and development of voids starting from the centre of the lentils; and starch granules by increasing the time of ball milling as seen in Figure 3.11j, k, m, p indicated changes in the starch granule's structure. Ball milling breaks the intermolecular bonds of starch molecules allowing hydrogen bonding with the water molecules

under ambient temperature. Amylopectin loses radial orientation thus, birefringence (He et al., 2014).

The birefringence of PM did not change indicating that ball milling could not change the structure of the starch granules. To further examine the impact of ball milling on the semi-crystalline structure of the GWL, RSL, and PM starch granules X-RD (diffraction analysis) was conducted.

3.2.3.5 X-RD diffraction patterns and crystallinity

Differences in the crystallinity between control and ball-milled GWL, RSL, and PM flours were identified by observing the diffraction patterns using X-RD, presented in Figure 3.12.

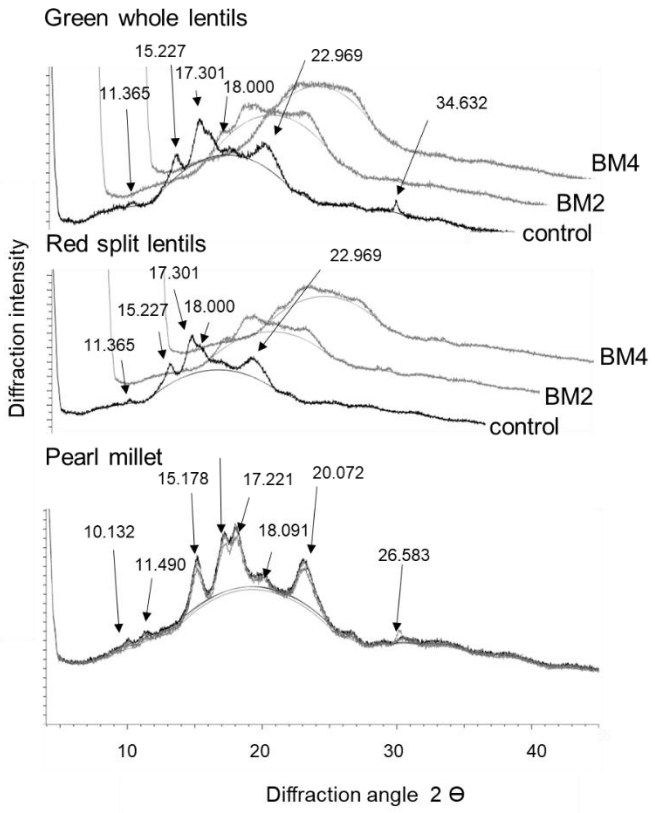


Figure 3.12 X-RD diffraction patterns of the control and ball-milled lentils and the pearl millet flours. BM2; 2 hours of ball milling, BM4; 4 hours of ball milling.

The control samples from lentils exhibited strong diffraction peaks at 15º and 23º, a double peak between 17º and 18º and a weak peak at 11º for both lentils. Those peaks are typical of C-type starch found in lentils (Joshi et al., 2017). An extra peak appeared for the GWL at 34º which did not correspond to any typical A-, B- or C-type of starch. This peak could be attributed to GWL hull fibres like cellulose and hemicellulose (Keskin et al., 2022; Yadav et al., 2019). The PM control exhibited strong diffraction peaks at 15º, 17º, 18º, 20º and 23º typical for A-type cereal starches in line with Olamiti et al., (2020).

The intensity of the diffraction peaks can be used as an indication of the ordered structure of the starch granules. A gradual decrease in the intensity of the diffraction peaks in GWL and RSL was observed by increasing the time of the ball mill. Crystallinity decreased for the GWL from 12.50% (control) to 8.60% (BM2) and 5.20% (BM4). Similarly, crystallinity decreased for the RSL from 13.30% (control) to 6.6% (BM2) and 5.00% (BM4). Decreased crystallinity indicated the disruption of hydrogen or glycosidic bonds within the amylopectin structure leading to a transition from a semi-crystalline to an amorphous state (Dome et al., 2020). The PM flour showed a slight decrease in crystallinity after ball milling from 18.20% (control) to 16.60% (BM2) and 15.50% (BM4).

Microscopy revealed changes in the appearance of starch granules in lentil flours and a further analysis was conducted using polarised light microscopy and X-RD diffraction analysis confirming a disruption in the semi-crystalline structure of the starch granules. However, microscopy also revealed an increase in the size of the proteins in the lentil flours

that was attributed to protein aggregation. The functional properties of the proteins are impacted by changes in their structure thus, the protein solubility and hydrophobicity of the proteins in the flours were evaluated.

3.2.3.6 Protein solubility at pH=9 and pH=6.5

Solubility is a functional property of proteins described as the amount of protein present in solution under specific conditions. Proteins can be soluble, partly soluble or insoluble in water. Environmental factors including ionic strength, solvent type, pH, temperature, and processing conditions also play a role in protein solubility (Zayas, 1997). Alkali pH has been found to favour protein solubility thus it is used in plant protein extraction. In pulses maximum protein solubility in alkali pH is estimated at 8.0-10.0 (Kiosseoglou & Paraskevopoulou, 2011) in millet was reported at 9.0 (Akharume et al., 2020).

Changes in the pH lead to electrostatic repulsive or attractive intermolecular forces between the amino acids within the structure of the proteins. Solubility depends on the ability of the proteins to interact with the solvent. For example, when pH is close to pI (isoelectric point), proteins have no net charge, enhancing interactions between the proteins and decreasing solubility. However, when the pH is higher or lower than pI when the proteins are charged, the electrostatic repulsion between the proteins is high thus, protein-water interactions are enhanced leading to increased solubility (Shevkani et al., 2019; Zayas, 1997).

The protein solubility of control and ball-milled GWL, RSL and PM at pH 8.5 for lentil samples and 9.0 for PM is displayed in Figure 3.13.

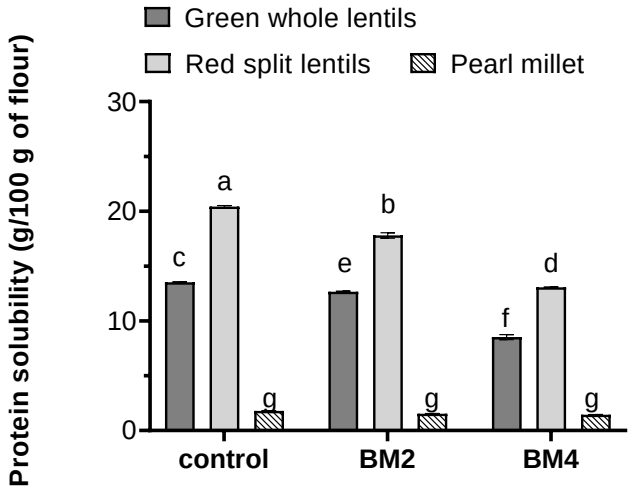


Figure 3.13 Protein solubility (100 g) of the control and ball-milled lentils at pH=8.5 and pearl millet at pH=9. Different letters represent significant differences at $p<0.05$.

Protein solubility was higher for control and ball-milled RSL compared to GWL and PM samples (regardless of processing). Increasing the time of ball milling decreased the protein solubility of both lentil samples but did not change the protein solubility of PM.

The protein composition of the GWL was 23.80% as seen in Table 3.1 with only 13% soluble at pH 8.5. However, RSL had a protein composition of 25.7% as seen in Table 3.1 of which 20.5% was soluble at pH 8.5. Differences in the protein solubility have been attributed to amino acid composition and sequence, protein molecular weight and conformation within the two lentils. Lee et al., (2021), found variations in the total and individual amino acids, polar and non-polar group content due to differences in the lentil varieties and environmental growth conditions. Control PM presented a low solubility (1.7%) at pH 9

attributed to the low content of water-soluble proteins and the high content of alcohol-soluble proteins (prolamins) (Duodu & Dowell, 2019).

In both lentil samples, soluble protein content decreased by increasing the ball milling time due to protein aggregation. The presence of protein aggregates was evidenced in fluorescent light micrographs after ball milling as seen in Figure 3.9d, e, g, h. Similarly, ball milling of soy protein isolates after four minutes showed a decrease in protein solubility (Liu et al., 2017). However, ball milling did not change the solubility of the prolamins in PM after ball milling which was in line with the absence of protein aggregates in micrographs presented in Figure 3.9f, i.

The impact of ball milling on the solubility of GWL and RSL proteins was also evaluated in water (pH 6.5) which is presented in Figure 3.14.

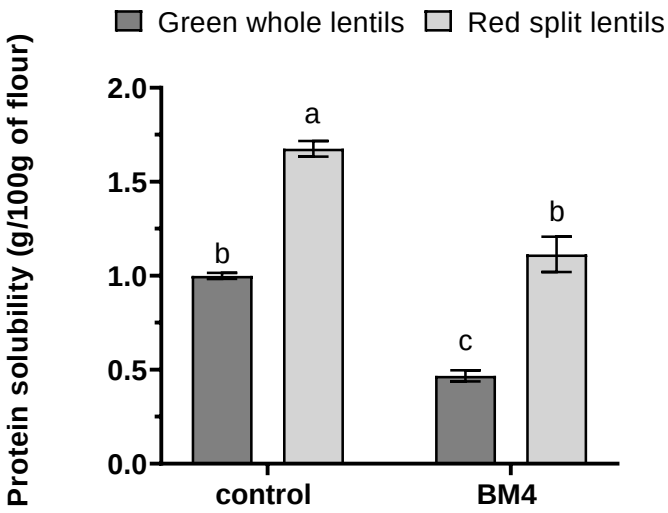


Figure 3.14 Protein solubility in 100 g of the control and ball-milled lentils in pH=6.5. Different letters represent significant differences at p<0.05.

Albumins, water-soluble proteins, contribute 10% of total protein content and are considered the second major fraction in lentils (Joshi et al., 2017). Millets mostly contain alcohol-soluble proteins called prolamins

(70-90%) followed by glutelin which is soluble in alkali and dilute acid solutions (Duodu & Dowell, 2019). Therefore, PM was not included in the experiment due to the absence of water-soluble proteins. The extremes of GWL and RSL samples were only used in this experiment.

The control RSL had only 1% (g of protein in 100 g of flour) higher content in water-soluble proteins (most likely albumins) in comparison to GWL. Due to differences in the total protein content between the lentils where RSL had 25.70% and GWL 23.80%, the results could be normalised per 100 g of total protein. In this case, RSL showed that 7 g of protein/ 100 g of total protein was soluble in water and GWL showed that 4.2 g of protein/ 100 g of total protein was soluble in water. Therefore, there was little difference between the water solubility of RSL and GWL.

Ball milling decreased the proportion of water-soluble albumin proteins in both lentils as previously found at pH 8.5, indicating that a part of the protein aggregates observed under fluorescent light in Figure 3.9 were albumins as well as globulins.

3.2.3.7 Protein hydrophobicity of the lentils in water

Surface hydrophobicity is an indication of the number of hydrophobic amino acids on the surface of the proteins. The presence of hydrophobic and hydrophilic amino acids impacts the surface hydrophobicity of proteins (Zhang et al., 2019). The protein hydrophobicity of control and ball-milled lentils in water can be seen in Figure 3.15.

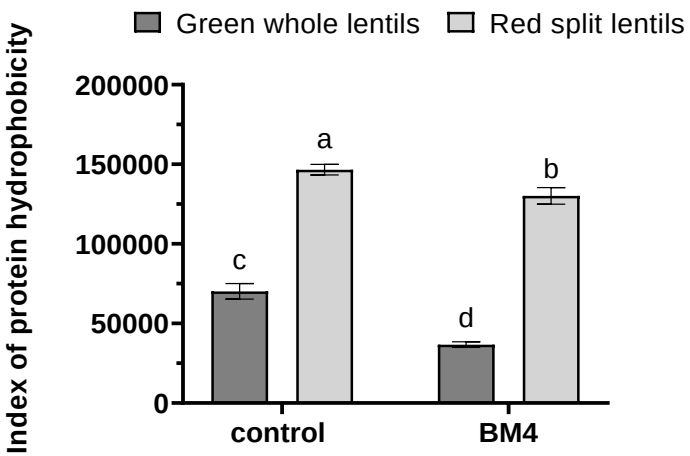


Figure 3.15 Index of protein hydrophobicity for control and ball-milled lentils at pH 7. Different letters represent significant differences at $p<0.05$.

The control GWL flour had a lower hydrophobicity in comparison to RSL. Differences in the hydrophobicity between the lentils were attributed to differences in amino acid composition and indicated that RSL proteins had more hydrophobic amino acids available to bind with the ANS (1-anilinonaphthalene-8-sulfonate) in the control samples. An amino acid analysis could be useful in identifying the proportion of hydrophobic and hydrophilic amino acids between the two lentils and better understanding the results of hydrophobicity.

Ball milling (BM4) significantly decreased the proteins' hydrophobicity in both lentils, as shown in Figure 3.15. Liu et al., (2017) & Yang et al., (2019) reported that changes in the structure of the protein due to denaturation expose the hydrophobic amino acids thus, it is expected to increase hydrophobicity. However, in this thesis protein aggregation was observed under the fluorescent microscope indicating protein denaturation and aggregation by the ball milling forces as seen in Figure 3.9d, e, g, h. Also, there was a decrease in lentil protein solubility in water

as seen in Figure 3.14. Therefore, the decrease in hydrophobicity in GWL and RSL is explained by the formation of aggregates as ANS could not bind with the hydrophobic amino acids of aggregated proteins and hydrophobicity decreased.

3.2.3.8 Water absorption (WA) and water solubility (WS)

Water absorption refers to the amount of water that can be absorbed by a sample. Water absorption is important in food materials as it provides information about the ability of the food to prevent fluid leakage during storage or processing (Lee et al., 2021). Water absorption is affected by the composition of the flour. A higher water absorption has been observed with a higher content of hydrophilic components in flour such as polysaccharides and proteins (polar amino acid residues with high water affinity) (Godswill et al., 2019).

The control and ball-milled water absorption and water solubility of GWL, RSL and PM can be observed in Figure 3.16.

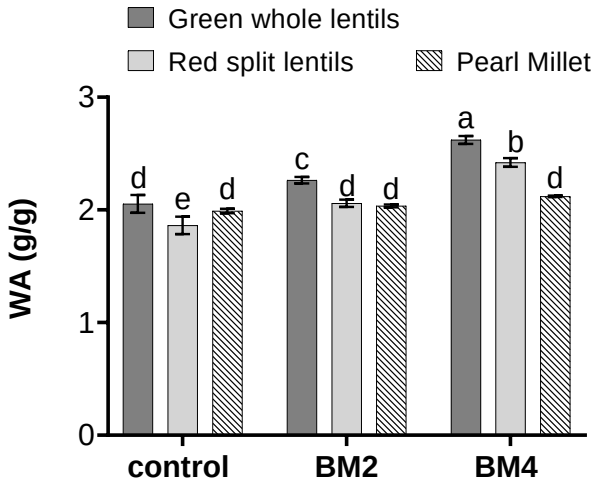


Figure 3.16 Water absorption (WA) (g/g) of control and ball-milled lentils and the pearl millet flours in 1:20 w/ v at 25 ^{o}C. BM2; 2 hours ball milling, BM4; 4 hours ball milling. Different letters represent significant differences at $p<0.05$.

The water absorption of control flours followed the order of GWL=PM>RSL. As it was previously discussed any differences in the water absorption of the flours could be attributed to composition. According to Table 3.1, the carbohydrate content in all three flours is the highest. The fraction of carbohydrates was considered to contain mainly starch and sugars because crude fibres were calculated as a separate fraction. Starch was the main component in all three flours with the PM containing about 10-18% more in comparison to lentils. Intact starch granules were reported to have a small contribution to water absorption (about 0.5 times its dry weight) and the major contribution in water absorption is expected to occur from other components in the flour (i.e., fibres, proteins) rather than starch (Godswill et al., 2019).

Proteins affect water absorption depending on their quantity, composition, and packing in the grain. The polar hydrophilic groups (i.e., amino, and carboxyl) are related to the binding of water to proteins. For example, aspartic acid, glutamic acid and lysine tend to bind four to seven water molecules per amino acid in comparison to non–polar amino acids like alanine and valine which bind only one water molecule per amino acid (Zayas, 1996). PM contains a higher amount of glutamic acid (22%) in comparison to lentils (7%), but lentils have a higher amount of lysine in comparison to PM (3%) (Dhull et al., 2022; Jukanti et al., 2016a). As there are differences in the amino acid composition between the different cultivars and grains reported in the literature and the grains used in this research the role of the proteins in the water absorption of the flours was not clear.

According to the literature, the cell wall material of legumes includes insoluble dietary fibres (IDF) (10-15%) consisting of cellulose, hemicellulose, and lignin along with soluble dietary fibres (SDF) (0.6-2.4%) such as gums and mucilage. IDFs are mostly located in the hulls of legumes (Tiwari & Cummins, 2011). Therefore, the exclusion of the hulls in the RSL sample due to pre-processing possibly decreased IDF and certainly decreased the overall fibre content in RSL, as evidenced in Table 3.1 (4.3%) in comparison to GWL (10.7%) and could explain the slightly higher water absorption of the GWL.

PM contains approximately 5% IDF and 3% SDF and most fibres are hemicellulose, lignin, and cellulose located in the tough fibrous seed coat of the grain (Krishnan & Meera, 2018) which is resistant to milling. The overall composition and structure of the PM remained the same as there was not any significant impact of the ball milling on its particle size as seen in Figure 3.8 or microstructure as seen in Figure 3.9 thus, the ability of the flour to absorb water was not affected by the ball milling.

Water absorption of GWL and RSL samples increased by ball milling attributed to the loss of the starch granules' birefringence (Figure 3.11) and crystallinity (Figure 3.12). Water absorption was evaluated at room temperature (25^{o}C) in which the native starch granules are insoluble due to their rigid double-helical conformation. However, ball milling reduced the rigidity of the amorphous rings and entanglement with the crystalline regions creating a space for hydrogen bonding between the hydroxyl groups of the amorphous regions and water molecules (Li et al., 2014) thus increasing water absorption of starch granules in 25 ^{o}C as seen by

the swollen starch granules observed in room temperature in Figure 3.10.

Additionally, it is likely that heat produced during ball milling also disrupted the structure of IDFs and increased their porosity and hydration hence promoting water absorption (Tiwari & Cummins, 2011). Whole cells in both legumes were absent after ball milling as seen in Figure 3.9 & Figure 3.11. The cell structure of legumes consisting of an outer hydrophobic membrane and an inner lipophilic layer has been observed to melt the lipophilic layer when heat is applied (50 ^{o}C) allowing cell separation, hydration and liberation of macronutrients within the cell structure (starch, proteins) (Dhital et al., 2016). The higher insoluble fibre content of GWL is attributed to the higher water absorption value after ball milling in comparison to the RSL.

PM flour WA was unaffected by ball milling, in agreement with previously described data that demonstrated the absence of change in the structure of the starch granules after ball milling (Figure 3.11 & Figure 3.12).

Solubility in food systems is considered as the property of the food substances to dissolve in liquid. Proteins and soluble fibres are major contributors to flour solubility as other components such as starch and fibres are insoluble in water (Godswill et al., 2019). The water solubility of the control and ball-milled GWL, RSL and PM is presented in Figure 3.17.

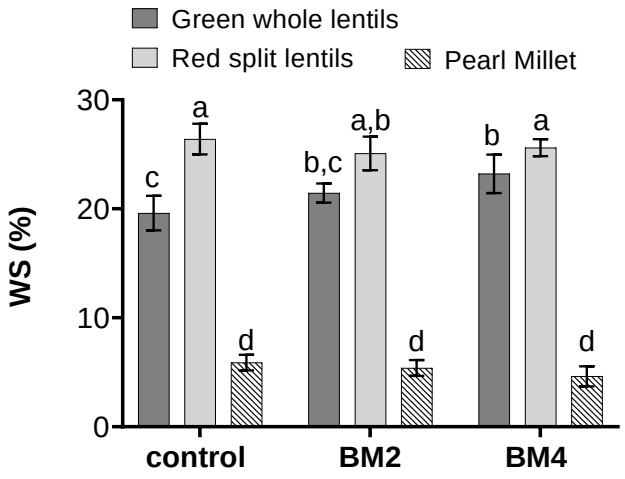


Figure 3.17 Water solubility (%) of control and ball-milled lentils and the pearl millet flours in 1:20 w/v at 25^{o}C. BM2; 2 hours ball milling, BM4; 4 hours ball milling. Different letters represent significant differences at $p<0.05$.

Water solubility was higher for control RSL in comparison to GWL. This difference could be attributed to the pre-treatment of the RSL grain exposed a greater surface area of the components to the milling forces resulting in flour particles with lower particle size and promoting the separation of components within the flour as seen in Figure 3.7b & Figure 3.9b, thus increasing the access of soluble particles within the flour.

The PM had the lowest water solubility which is in line with the low composition of water-soluble proteins and the presence of large particles as seen in Figure 3.7c & Figure 3.9c.

Ball milling increased the water solubility of the GWL but did not change the water solubility of the RSL and PM. Solubility has been associated with damage in the starch granules' structure and solubilisation of degraded and small molecular weight amylopectin molecules which increases solubility (Li et al., 2014). Although in this thesis the disruption

of the crystalline regions was observed for both lentils after ball milling there was not any information indicating the degree of degradation of the starch molecules thus a direct comparison could not be made.

Any change in the water solubility of the lentils would also be affected by the amount of soluble cell wall material, the amount of albumins and the amount of hydrophobic amino acids. GWL had a higher composition of fibres and lower hydrophobicity than the RSL which could account for the higher water solubility compared to the RSL after ball milling.

Ball milling did not impact the solubility of PM flour which is in agreement with previously presented data showing a lack of change in the structure of starch granules in PM, presented in 3.2.3.4 and 3.2.3.5.

Overall, ball milling changed the structure of starch and protein fractions affecting the hydration properties of the lentil flours at 25 ^{o}C.

3.2.3.9 Thermal properties using differential scanning calorimetry (DSC)

The arrangement of amylopectin and amylose in starch granules creates a barrier to water penetration at ambient temperature. However, in the presence of heat and a sufficient amount of water starch granules undergo an order to disorder phase transition (gelatinisation). The transformation of the starch from a polycrystalline state to a non-crystalline and gelatinised state is reflected by an endothermic peak, measured using DSC during heating. The starch gelatinisation onset, peak and conclusion temperatures (T_o, T_p, T_c) correspond to the distribution of the short chains of the amylopectin rather than the

proportion of crystallinity and ratio of amylose and amylopectin (Wani et al., 2016).

Protein denaturation can also be observed using a DSC as an endothermic peak. The rupture of the intermolecular bonds in the native structure of the proteins due to heating is responsible for the appearance of this endothermic peak (Ricci et al., 2018).

Starch gelatinisation and protein denaturation of control and ball-milled GWL, RSL and PM were investigated using DSC and the results are displayed in Figure 3.18 & Table 3.4.

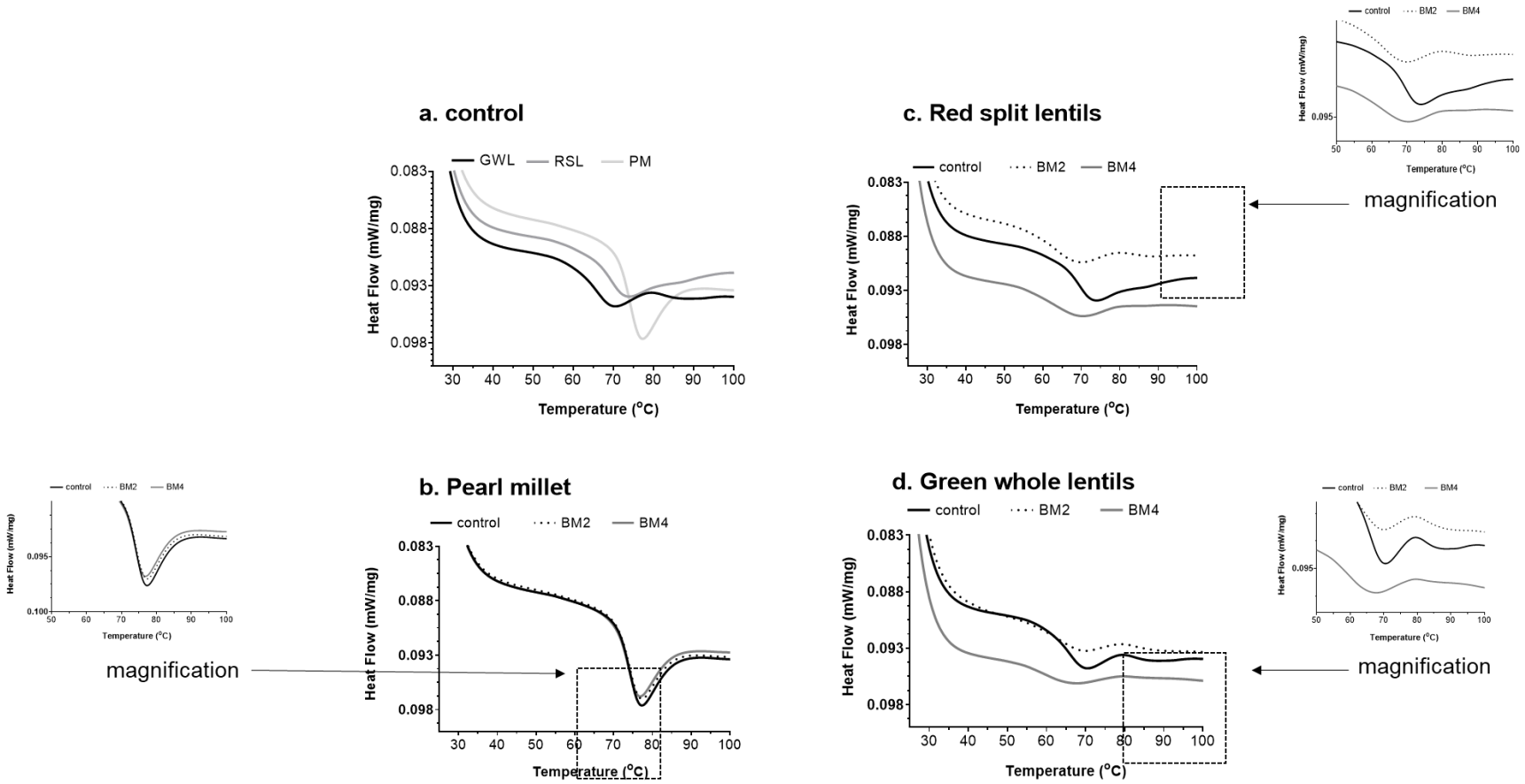


Figure 3.18 Differential scanning calorimetry (DSC) thermograms of the control and ball-milled lentils and the pearl millet flour from 25 to 100 °C. BM2; 2 hours ball milling, BM4; 4 hours ball milling.

Table 3.4 The impact of ball milling on the thermal parameters of starch gelatinisation; first peak and protein denaturation/ amylose-lipid complexes; second peak. BM; 2 hours ball-milled and BM4; 4 hours ball-milled GWL; green whole lentil, RSL; red split lentil and PM; pearl millet, Δh enthalpy, To; start temperature, Tp; peak temperature and Tc; end temperature. One-way ANOVA was conducted to compare GWL, RWL and PM with different letters in the same column representing a difference at $p<0.05$.

	First peak			Second peak		
	T_o (ºC)	T_p (ºC)	T_c (ºC)	T_o (ºC)	T_p (ºC)	T_c (ºC)
GWL						
control	61.20±0.30^{c}	69.60±0.20^{c}	78.60±0.10^{d}	80.90±0.07^{c}	87.30±0.20^{b}	97.00±0.20^{b}
BM2	56.60±1.30^{d}	68.60±0.30^{c}	78.50±0.10^{d}	80.70±0.30^{c}	88.30±0.06^{b}	97.70±0.20^{b}
BM4	53.00±0.30^{e}	66.30±0.60^{d}	78.70±0.10^{d}	80.80±0.30^{c}	85.20±0.30^{b}	92.70±1.30^{c}
RSL						
control	63.90±0.20^{b}	73.50±0.10^{b}	81.40±1.75^{b}	84.30±0.70^{b}	87.30±0.20^{b}	91.90±0.30^{c}
BM2	57.40±1.00^{d}	68.70±0.30^{c}	79.10±0.05^{d}	81.60±0.20^{c}	88.00±0.20^{b}	96.10±0.30^{b}
BM4	55.05±0.40^{d}	69.50±0.20^{c}	83.90±0.10^{c}	83.00±0.60^{c}	86.80±0.20^{b}	90.40±0.70^{c}
PM						
control	71.40±0.08^{a}	77.00±0.03^{a}	87.00±20	95.10±0.50^{a}	99.75±0.18^{a}	104.70±0.55^{a}
BM2	70.90±0.30^{a}	77.10±0.20^{a}	88.30±0.45^{a}	94.10±0.90^{a}	99.00±0.10^{a}	104.50±0.60^{a}
BM4	70.90±0.30^{a}	76.50±0.10^{a}	86.40±0.10^{a}	94.20±1.20^{a}	99.20±0.30^{a}	104.30±0.70^{a}

The thermograms of control GWL and RSL showed an endothermic peak between 60-80 ^{o}C and PM between 70-85 ^{o}C, attributed to starch gelatinisation in line with the literature (Arendt & Zannini, 2013b; Edwards et al., 2020; Zhu, 2014). The absence of a sharp starch gelatinisation peak in the lentils' thermogram has been attributed to starch (amylose)-lipid complexes, starch-protein complexes or to lower protein denaturation temperatures occurring roughly at the same time as starch gelatinisation. For example, the denaturation temperature of globulin proteins in soy protein isolate was found at 68-85 ^{o}C for 7S but 85-100 ^{o}C for 11S (Wang et al., 2021) thus an overlap of the starch gelatinisation peak and protein denaturation peak could occur. However, in lentils the 7S (4%) content has been reported to be lower than 11S (45%) (Gravel & Doyen, 2023) and therefore the first endothermic peak can be mainly attributed to starch gelatinisation. Amylose-lipid complexes were not considered as a factor contributing to the absence of a sharp peak in lentils due to the low lipid composition (>1%).

Moreover, a small shoulder after starch gelatinisation between 80-100 ^{o}C was found only for GWL and attributed to protein denaturation (Ladjal-Ettoumi et al., 2016; Ladjal-Ettoumi & Chibane, 2015) however, such a peak was not observed for the RSL. In literature, protein denaturation of millet was previously found to be at 82-88 ^{o}C (Akharume et al., 2020) and therefore it could occur at the same temperature as starch gelatinisation explaining the absence of a protein denaturation peak.

The control GWL had lower starch gelatinisation onset, peak and conclusion temperatures (T_o, T_p, T_c) compared to RSL. Li, Yeh & Fan,

(2007) observed an increase in the transition temperature of starch in starch/soy protein composites when the protein concentration increased. Therefore, the higher transition temperature for RSL could be attributed to higher protein content in comparison to GWL, reducing water availability for the starch granules.

The higher starch gelatinisation transition temperatures of PM over the lentils can be attributed to differences in the crystallinity degree confirmed by the X-RD data in Figure 3.12. Starch granules with a higher crystallinity degree are more resistant to gelatinisation (Liu et al., 2020).

Ball milling decreased the onset starch gelatinisation (T_o) temperature in lentils from 61 to 53 ^oC for GWL and from 63 to 55 ^oC for RSL but did not change the T_o of PM. Lower transition temperatures have been linked with a loss of starch granule structure followed by faster hydration and gelatinisation (Palavecino et al., 2019; Tian, Wang, Wang, Ma, et al., 2022). Ball milling decreased the birefringence as seen in Figure 3.11 and crystallinity as seen in Figure 3.12 as well as increased the water absorption as seen in Figure 3.16, of GWL and RSL. Those changes explain the change in the onset gelatinisation temperature of starch because the loose and amorphous structure of the ball-milled starches is more sensitive to heat and easily accessible to water in comparison to the rigid structure of the native starch granules allowing gelatinisation to occur at lower temperatures (Bangar et al., 2023).

Ball milling did not change the properties of PM starch in any analysis presented explaining the non-significant differences between the endothermic values of the control and ball-milled PM.

After ball milling the small shoulder observed in the control GWL disappeared. However, a small shoulder appeared for the RSL at around 80 ^{o}C after ball milling. As it was previously discussed, after ball milling multiple phenomena were observed including a decrease in the particle size, starch birefringence and crystallinity. In the control lentil flours, the starch granules are embedded within a protein matrix. However, due to the decrease in the particle size after ball milling starch and protein separation could occur. The starch-protein separation could contribute to the formation of a more concentrated protein phase and explain the sharpening of the protein denaturation peak. A higher efficiency in starch-protein separation in the RSL could be attributed to the absence of a seed coat in the RSL and separation of the cotyledons increasing the available surface area for the ball milling forces to act.

3.2.4 Pasting properties of the control and ball-milled legumes and cereals

In the previous section 3.2.3, the impact of ball milling on the microstructure and physiochemical properties of two types of lentils (GWL, RSL) and millet (PM) was evaluated. It was concluded that ball milling affected the lentil flours by disrupting the crystalline structure of the starch granules and inducing protein denaturation and aggregation, but the same observations were not made for PM.

The pasting properties of various legumes and cereals before ball milling (control) were evaluated in section 3.2.2. Differences in the pasting behaviour between the legumes and the cereals were found to be linked with the native microstructure. Therefore, the impact of ball milling on the microstructure was hypothesised to change the pasting properties. To ensure repeatability of the results different types of legumes and cereals were also included.

3.2.4.1 Pasting properties of ball-milled flours

The impact of ball milling on the pasting and pasting parameters of legumes (GWL, RWL, RSL, YP) can be observed in Figure 3.19 and Table 3.5. A faster increase in viscosity was observed after ball milling within the first couple of seconds after the start of the RVA heating cycle in all legumes, as seen in the magnified sections in Figure 3.19. The loss in starch granules' crystallinity allowed for increased hydration and decreased initial gelatinisation temperature as discussed in the previous section 3.2.3.

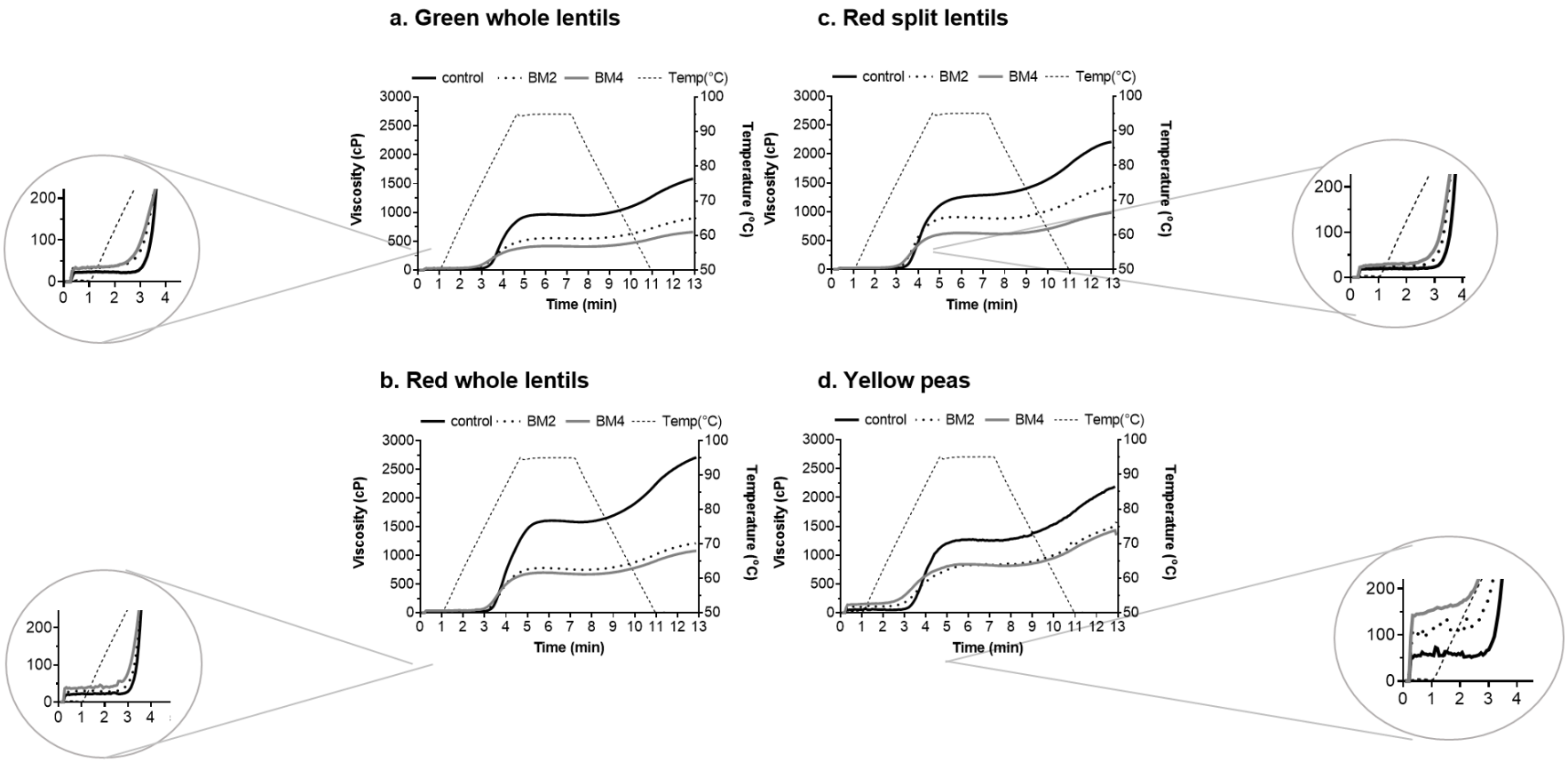


Figure 3.19 Rapid visco analysis (RVA) profile showing the pasting properties of control and ball-milled (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils, and (d) Yellow peas when heating and cooling for thirteen minutes in excess of water and continuous shear. BM2; 2 hours ball milling, BM4; 4 hours ball milling.

Table 3.5 The impact of ball milling on the pasting parameters of control and ball-milled legume flours. GWL; green whole lentil, RSL; red split lentil; YP; yellow pea, BM2; 2 hours ball milling, BM4; 4 hours ball milling. PT; peak temperature, Pt; peak time, PV; peak viscosity, BD; break down, TV; trough viscosity, SB; set-back, FV; final viscosity. One-way ANOVA was conducted across the legumes and different letters represent a significant difference ($p<0.05$) in each column.

	PT (°C)	Pt (min)	PV (cP)	BD (cP)	TV (cP)	SB (cP)	FV (cP)
GWL							
control	79.20±0.05^{a,b}	6.00±0.08^{c}	966.70±9.90^{d}	16.70±4.50^{c,d}	950.00±5.70^{d}	634.30±4.10^{d}	1584.30±6.60^{d}
BM2	77.80±0.70^{b}	6.20±0.08^{c}	557.30±4.20^{g}	9.30±1.25^{e}	548.00±2.90^{g}	350.30±5.25^{h}	898.30±6.20^{f}
BM4	80.40±0.30^{a}	6.10±0.09^{c}	418.70±5.80^{h}	9.30±0.90^{e}	409.30±4.90^{h}	252.30±4.50^{j}	661.70±9.10^{g}
RWL							
control	79.60±0.75^{a,b}	6.20±0.03^{c}	1609.00±15.50^{a}	27.00±7.90^{a,b}	1582.00±17.90^{a}	1129.00±6.20^{a}	2711.00±14.30^{a}
BM2	78.50±0.40^{a,b}	5.70±0.08^{d}	785.30±23.75^{e}	37.30±1.25^{a}	748.00±23.28^{e}	467.70±6.65^{f}	1215.70±29.45^{f}
BM4	76.95±1.50^{b}	5.80±0.20^{d}	704.00±60.90^{f}	32.70±8.90^{a}	671.30±52.09^{f}	410.70±23.90^{g}	1082.00±75.80^{e}
RSL							
control	80.80±0.05^{a}	7.00±0.00^{a}	1287.70±8.60^{c}	26.00±3.70^{b}	1261.70±8.06^{c}	946.30±16.80^{b}	2208.00±11.40^{b}
BM2	78.30±0.00^{a,b}	5.60±0.09^{d}	908.70±4.50^{d}	26.70±6.85^{b}	882.00±11.30^{d}	556.70±10.30^{e}	1438.70±17.00^{d}
BM4	77.80±0.70^{b}	5.90±0.20^{b}	634.30±14.40^{g,f}	18.70±2.05^{c}	615.70±13.80^{f}	367.70±5.40^{h}	983.30±19.10^{f,e}
YP							
control	75.90±0.04^{b}	5.30±0.03^{d}	1484.30±7.85^{b}	14.70±0.40^{d}	1337.70±7.40^{b}	728.70±3.70^{c}	2066.30±10.90^{c}
BM2	75.35±0.70^{b}	6.70±0.10^{b}	671.70±7.70^{f}	12.70±7.30^{d}	659.00±14.90^{f}	409.30±0.50^{g}	1068.30±15.10^{e}
BM4	74.60±1.00^{c}	6.10±0.08^{c}	530.70±20.80^{g}	9.70±4.50^{d,e}	521.00±17.40^{g}	311.30±11.15^{i}	832.30±26.40^{f}

Ball milling decreased the peak viscosity of all legumes indicating a decrease in water absorption and swelling capacity of the starch granules under heat and shear (Table 3.5). The breakdown decreased showing a higher paste stability and a lower setback and final viscosity indicating a lower ability of the starch granules to retrograde and form a viscous paste when temperature decreases (Table 3.5).

Differences in the effect of the ball milling on the cereals were also observed and are presented in Figure 3.20. The pasting temperature and peak time were not affected significantly by the ball milling for any of the cereals (Table 3.5). Ball milling did not affect the peak viscosity, breakdown, trough viscosity, setback and final viscosity of M and PM, however, ball milling decreased those parameters for the W.

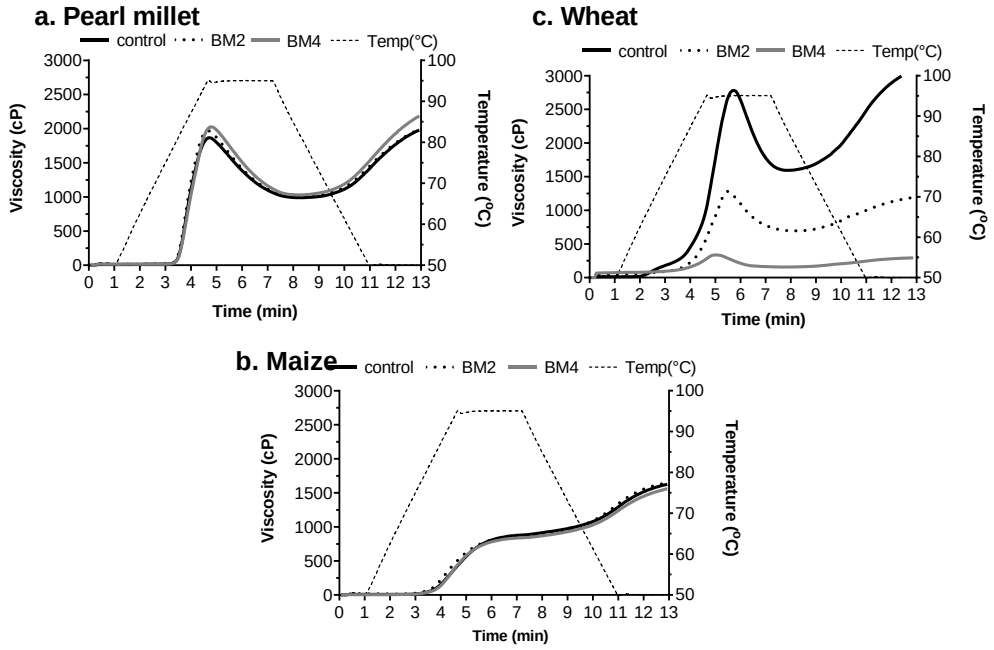


Figure 3.20 Rapid visco analysis (RVA) profile showing the pasting properties (10.6% solids) of control and ball-milled (a) Pearl millet, (b) Maize, (c) Wheat when heating and cooling for thirteen minutes in excess of water and continuous shear. BM2; 2 hours ball milling, BM4; 4 hours ball milling.

Table 3.6 The impact of ball milling on the pasting properties of control cereal flours. PM; pearl millet, M; maize, W; wheat, BM2; 2 hours of ball milling, BM4; 4 hours of ball milling. PT; peak temperature, Pt; peak time, PV; peak viscosity, BD; break down, TV; trough viscosity, SB; set-back, FV; final viscosity. One-way ANOVA was conducted across the cereals and different letters represent a significant difference ($p<0.05$) in each column.

	PT (ºC)	Pt (min)	PV (cP)	BD (cP)	TV (cP)	SB (cP)	FV (cP)
PM							
control	79.10±0.04^{c}	4.70±0.03^{c}	1867.70±17.20^{b}	879.00±10.70^{c}	988.70±6.80^{b}	991.00±15.50^{c}	1979.67±16.90^{b}
BM2	79.10±0.04^{c}	4.70±0.00^{c}	1976.00±85.80^{b}	968.00±68.20^{b}	1008.00±19.25^{b}	991.00±11.40^{c}	1999.00±8.20^{b}
BM4	79.70±0.80^{c}	4.80±0.03^{c}	2027.00±7.80^{b}	998.00±13.60^{b}	1029.00±5.90^{b}	1157.30±17.80^{b}	2186.33±18.80^{b}
M							
control	85.07±1.40^{b}	7.00±0.00^{a}	875.30±139.90^{d}	53.70±2.05^{f}	821.70±138.00^{c}	804.30±146.30$^{c, d}$	1626.00±284.20^{c}
BM2	84.00±1.00^{b}	7.00±0.00^{a}	854.70±10.70^{d}	40.70±3.90^{f}	814.00±11.80^{c}	816.30±17.00^{d}	1630.33±27.45^{c}
BM4	85.55±0.00^{b}	7.00±0.00^{a}	835.70±14.70^{d}	44.70±1.25^{f}	791.00±14.00^{c}	768.00±22.70^{d}	1559.00±36.60^{c}
W							
control	83.20±0.02^{b}	5.80±0.03^{b}	2782.70±21.00^{a}	1189.00±12.00^{a}	1593.70±14.30^{a}	1488.00±3.00^{a}	3081.67±14.00^{a}
BM2	86.90±0.70^{b}	5.50±0.03^{b}	1286.70±17.60^{c}	593.00±12.20^{d}	693.70±5.70^{d}	497.00±1.60^{e}	1190.67±5.25^{d}
BM4	94.70±0.75^{a}	5.10±0.03^{b}	337.00±11.30^{e}	180.00±9.10^{e}	157.00±4.30^{e}	136.70±5.40^{f}	293.67±6.85^{e}

Bangar et al., (2023) reported that damaging isolated starch granules enhances starch granules' hydration and swelling, thus increasing peak viscosity. Enhanced hydration and swelling of the starch granules after milling, attributed to the fragmentation of starch granules into smaller fragments that weakened the entanglement between the amorphous and crystalline growth rings allowing more space for the amorphous growth rings to form hydrogen bonds with the water molecules (Li et al., 2014).

However, other authors showed that extensive damage to isolated rice and potato starch granule structures by extrusion (Liu et al. 2017) and ball milling (Juarez-Arellano et al., 2021) decreases viscosity. Extensive damage or starch degradation was evidenced by an absence of birefringence under the microscope, crystallinity and loss of the typical granular form, all of which decreased thermal stability and swelling ability which explains the lower viscosity.

The retention of granular form in lentil flour after ball milling was observed under fluorescent and polarised microscopy in Figure 3.9 & Figure 3.10, although reduced and there was evidence of crystallinity in Figure 3.12. Therefore, it is hypothesised that starch granules in the legume flours were not extensively damaged, and the starch retained the ability to hydrate, swell and increase the viscosity of the flour in lentils, as seen in Figure 3.19.

Ball milling damages starch granule structure differently depending on amylose and amylopectin content and crystalline structure. Starches

with higher amylose content were impacted less in comparison to the waxy starches (high in amylopectin). The glycosidic linkages in the amylopectin molecules' double helices have shown a greater susceptibility to cleavage by ball milling forces (Li et al., 2014) whilst amylose is affected less by milling and can provide a cushioning effect (acting as mechanical plasticiser) that limits the damage of amylopectin (Lu et al., 2018). For example, starches from three different botanical sources varying in amylose content including mung bean; 47.50%, potato; 29.00%, corn; 26.20% and waxy corn; 4.90% were ball-milled for 0-1 hour at 300 rpm. Waxy corn starch showed the highest degree of damaged starch and mung bean starch the lowest confirming that starches high in amylopectin are more susceptible to the milling forces (Liu et al., 2020).

Ball milling was also reported to affect starch granules depending on their crystallinity type. A-type starch granules have shown a monoclinic structure and contain a-1,6 branched linkages to both the amorphous and crystalline regions. The a-1,6 linkages in the crystalline regions have been characterised as the weak point of the A-type making the lamella weaker and prone to ball mill damage. B-type starch granules have shown a more open structure with thirty-six water molecules in every hexagonal crystal unit that contribute to a strong hydrogen bonding network resistant to ball milling forces (Bangar et al., 2023; Tan et al., 2015).

Therefore, the type of crystallites and amylose-amylopectin content of the starch granules are factors affecting starch degradation by ball

milling. Taking into consideration the higher amylose content of legumes (≈35%) in comparison to cereals (>35%), it would be hypothesised that ball milling would impact legumes to a lower degree because the higher amylose content would cushion the ball milling forces, protecting amylopectin from degradation. However, the pasting results showed a decrease in the viscosity of the legume samples.

For this reason, three different types of isolated starch were ball-milled using the same conditions as used for the flours to investigate the impact of ball milling depending on the type of crystallinity and amylose-amylopectin content.

3.2.4.2 Investigating the impact of ball milling on the pasting properties of three types of starches

Three starches from different botanical origins (maize, potato, pea) and crystallinity (A-, B- and C-type) were ball-milled under the same conditions applied for the legume and cereal flours. The impact of ball milling on the birefringence and morphology of all starches was observed using a polarised and fluorescent light microscope, as shown in Figure 3.21.

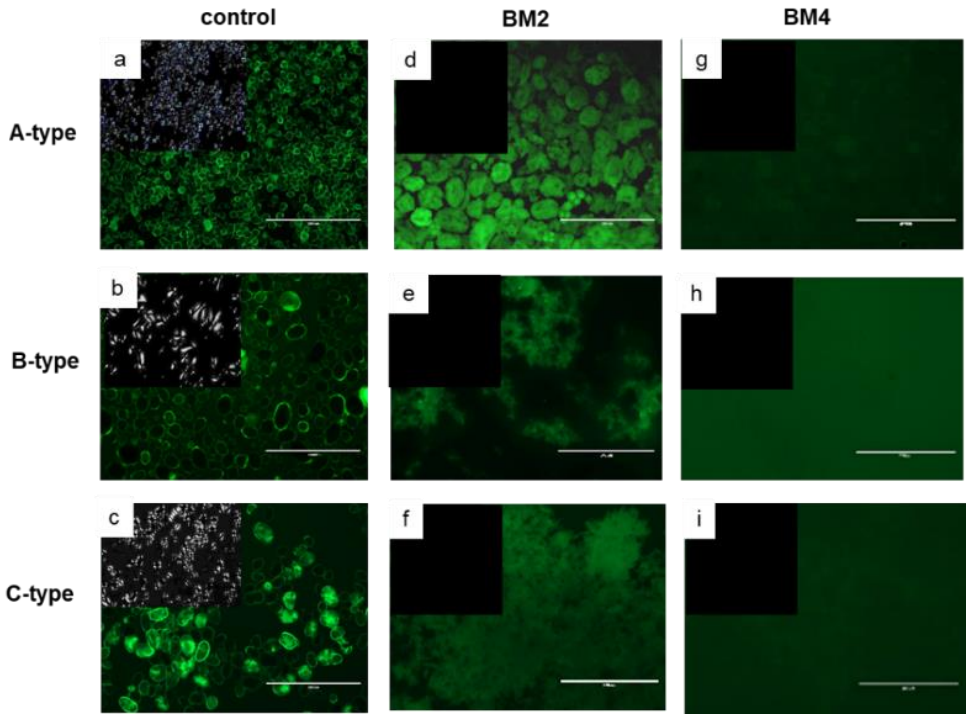


Figure 3.21 The micrographs of control (a-c) and ball-milled isolated starch granules(d-i), from maize (A-type), potato (B-type) and pea (C-type) under polarised and fluorescent light. Scale bar; 200 µm. BM2; 2 hours ball milling, BM4; 4 hours ball milling.

The control starches from different botanical origins were observed under polarised light (Figure 3.21a, b, c). The characteristic pattern of birefringence was observed in all native starches. The fluorescent light of control starch granules showed the typical starch granular shape characteristic of each botanical origin. The shape of the A-type of starch as can be seen in Figure 3.21a, was mostly polygonal and small (>30 µm) but oval and larger (<30 µm) for the B- and C- types of starches as can be seen in Figure 3.21b, c.

All types of starch granules lost birefringence after ball milling indicating severe damage to their structure. The morphology of all starch types under fluorescent light also changed after ball milling. The A-type of starch showed an increase in the size of granules after 2 hours of ball milling however, the B- and C-type only showed aggregates with no

specific shape or size. After 4 hours of ball milling only a green mass was observed for all starches under fluorescent light and there was no evidence of granular structure. Ball milling damaged the double helical crystalline structure of the starch granules increasing the availability of hydroxyl groups to form hydrogen bonds with the water molecules. This behaviour was not observed under polarised or fluorescent light for lentils or the PM flours after ball milling.

The pasting properties of control and ball-milled starches from different botanical origins can be found in Figure 3.22 & Table 3.7.

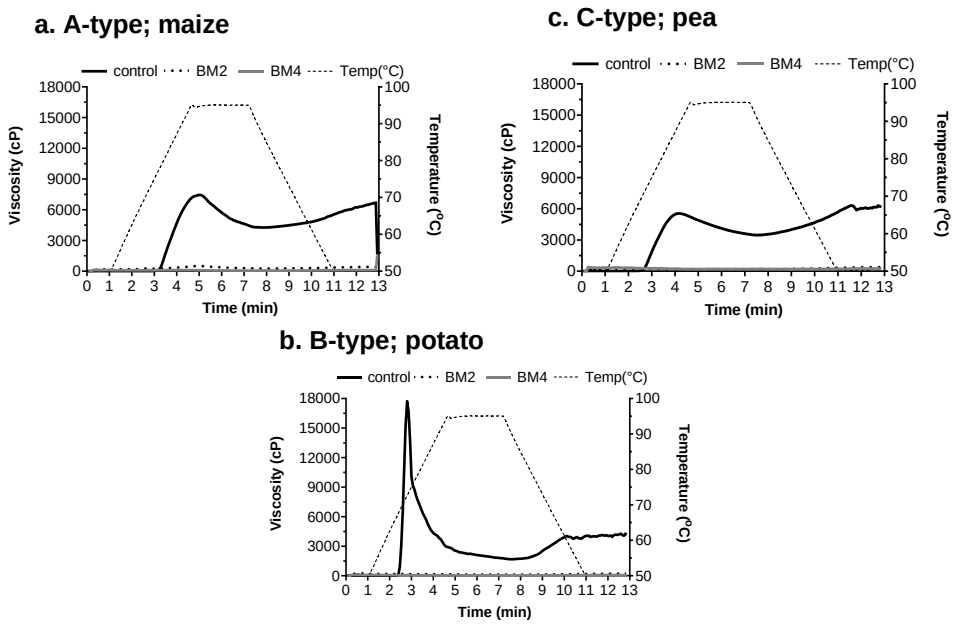


Figure 3.22 Rapid visco analysis (RVA) profile showing the pasting properties of control and ball-milled starches from different botanical origins (a) maize (A-type), (b) potato (B-type), (c) pea (C-type). BM2; 2 hours ball milling, BM4; 4 hours ball milling.

All control starch granule types showed a typical starch viscosity peak, see Figure 3.22. Differences in the viscosity values between the A-type maize starch, B-type potato starch and C-type pea starch could be attributed to differences in botanical source, crystallinity type, amylose/

amylopectin ratios and molecular structure and conformation (Balet et al., 2019).

Table 3.7 The impact of ball milling on the pasting properties of starches from different botanical origins (AS; maize, BS-, potato, CS; pea). PT; peak temperature, Pt; peak time, PV; peak viscosity, BD; break down, TV; trough viscosity, SB; set-back, FV; final viscosity. One-way ANOVA was conducted for all starch and different letters represent a significant difference ($p<0.05$) in each column.

	PT (°C)	Pt (min)	PV (cP)	BD (cP)	TV (cP)	SB (cP)	FV (cP)
A-S							
control	76.70±0.04^{a}	5.10±0.02^{a}	7456.90±64.40^{b}	3195.70±15.60^{a}	4261.20±49.04^{a}	2414.00±7.10^{a}	6675.20±44.20^{a}
BM2	48.10±10.50^{b}	5.00±0.06^{a}	502.00±47.60^{c}	238.30±22.45^{c}	263.70±26.10^{c}	155.70±8.20^{b}	419.30±33.70^{c}
BM4	33.70±0.30^{d}	2.20±1.60^{c}	123.00±13.40^{e}	65.30±20.90^{d}	57.70±7.85^{d}	48.70±9.00^{c}	106.30±9.50^{e}
B-S							
control	68.70±0.04^{b}	2.90±0.03^{c}	17786.30±345.30^{a}	16121.30±321.90^{d}	1665.00±119.80^{b}	2520.70±929.50^{a}	4185.70±839.10^{b}
BM2	51.60±1.60^{c}	1.20±0.20^{c}	203.30±51.80^{d}	82.00±38.10^{d}	121.30±13.90^{c}	104.30±34.40^{b}	225.70±47.40^{d}
BM4	50.00±0.05^{c}	1.20±0.10^{c}	77.00±4.30^{f}	37.70±8.50^{e}	39.30±4.20^{d}	27.00±2.45^{c}	66.30±3.10^{f}
C-S							
control	69.80±0.80^{b}	4.30±0.03^{b}	5548.70±815.30^{b}	2079.30±365.90^{b}	3469.30±449.80^{a}	2732.30±664.50^{a}	6201.70±225.30^{a}
BM2	50.35±0.30^{c}	4.00±1.00^{b}	235.70±30.70^{d}	102.70±50.90^{d}	166.30±18.90^{c}	306.70±68.20^{b}	416.30±92.30^{c}
BM4	53.10±3.60^{c}	1.55±0.40^{c}	306.00±64.75^{d}	135.70±81.20^{d}	70.30±16.00^{d}	29.00±7.80^{c}	85.30±5.25^{e}

The C-type pea showed a higher PT in comparison to A-type maize and B-type potato starch and B-type potato starch showed a higher PV viscosity followed by A-type maize and C-type pea starch. Moreover, FV was lowest for B-type potato starch (Table 3.7).

The type of crystallinity has previously been linked to the amylose content reporting a higher amylose content for C-type pea starch (30-60%) (Sun & Xiong, 2014), followed by A-type maize starch (25-30%) (Arendt & Zannini, 2013a) and B-type potato starch (16%) (Villanueva et al., 2018). Lower PT and higher PV have been attributed to lower amylopectin content in the starch because amylose retards starch disintegration under heat (Berski & Ziobro, 2018), explaining the lower PT and PV of the C-type starch in comparison to A- and B-types.

Amylose retrogradation is considered as a short-term process starting during the cooling cycle of the RVA thus the higher content of amylose accounts for the higher FV in the A- and C-types of starches. The recrystallisation of amylopectin is considered a long-term process and thus does not contribute to the FV (Bangar et al., 2021) which also explains the lower value of FV in B-type starch.

Also, the greater size of B-type potato starch granules would impact viscosity occupying a relatively larger volume in the canister in comparison to smaller A-type maize and C-type pea starches.

The viscosity of all starches decreased, and a flat line appeared on the bottom of the pasting graphs of all starches, after ball milling. This observation was not in line with the hypothesis that ball milling affects

starch granules differently depending on the amylose content and type of crystallinity. Therefore, the conditions used for ball milling processing in the research presented should impact starch granules of legumes and cereals in the same way. The previous results therefore demonstrate that starch granules within legume and cereal flours are not affected by ball milling in the same manner as isolated starch granules. It is again hypothesised that the microstructure and other non-starch components are instrumental in understanding how whole flours change with physical modification.

Other components such as fibres and proteins must protect starch granules from ball milling forces and therefore starch granules experience a lower degree of damage (Enpeng, 2014).

3.2.4.3 Investigating the effect of protein composition on pasting properties after ball milling

Proteins in cereals can also be found on the surface of the cavities of the starch granules. Tian et al., (2022) observed that only when ball milling separated the surface proteins from starch granules did viscosity decrease indicating that the presence of proteins protects the starch granules from the ball milling impact. Previous observations that the lower viscosity of W in comparison to PM and M flours could be attributed to differences either in the type of the protein or differences in the location of the proteins within the microstructure of the flours.

PM and M contain a higher amount of prolamins (alcohol-soluble) in comparison to legumes which are high in globulins (salt-soluble) and

albumins (water-soluble). Cereals contain distinct types of prolamins, called zein, gliadin and kafirin, with differences in the amino acid composition, structure, and functionality (Table 3.8).

Table 3.8 Types of prolamins depending on the type of cereal.

Cereal name	**Type of protein**				**Bibliography**
	Albumin	Globulin	Glutenin	Prolamin	
Wheat	20-25%		35-50%	30-40%	(Žilić et al., 2011)
Pearl millet	32%		25%	43%	(Taylor & Taylor, 2017)
Maize	7.80%	na*	38.20%	50%	(Anderson & Lamsa, 2011)

*na; not available

Various ball milling times have been used to damage the secondary structure of cereal proteins. For example, ball milling damaged the secondary structure and increased the solubility of gliadin (wheat protein) after 40 minutes using a high speed (400 rpm) (Liu et al., 2021). Similarly, ball milling was used for 4 hours to damage the structure of oat bran protein flour at 200 and 800 rpm (Liu et al., 2017). Therefore, depending on the protein type different ball milling times might need to be used.

The lentil proteins showed evidence of aggregation which have been found to impact the functionality of starch in a mixed system. Specifically, the formation of a continuous network from protein aggregates has been found to affect water mobility, and hence, starch swelling (Scott & Awika, 2023).

Milk protein aggregates have also been found to attach to the surface of starch granules thus limiting starch granules hydration, swelling and pasting. In another study, an increase in ionic strength at pH 7.0

increased the solubility and denaturation of pea protein isolates at 95 ^{o}C. The proteins were not able to aggregate and form a strong network under these conditions because a lower number of protein molecules were exposed and protein-protein interactions decreased (Tanger et al., 2021).

Therefore, it was hypothesised that increasing ionic strength would increase protein solubility, decrease the tendency to aggregate and increase starch-water interactions. This hypothesis was based on data presented in Figure 3.4 showing that increasing ionic strength increased the PV of RSL before ball milling. The impact of NaCl on the viscosity of lentil flours after ball milling was investigated and the results are displayed in Figure 3.23.

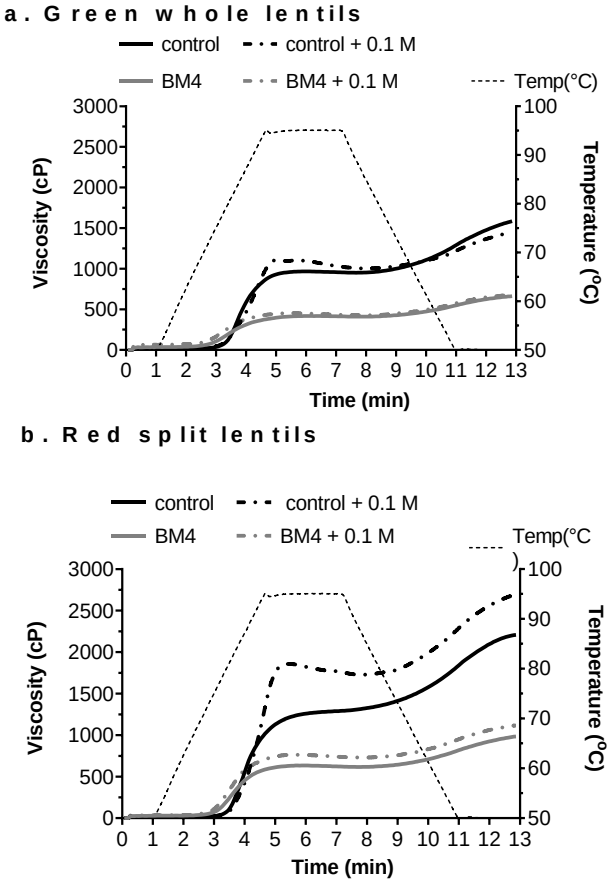


Figure 3.23 Rapid visco analysis (RVA) profile showing the impact of NaCl (0.10 M) addition on the paste forming properties of control and ball-milled (a) Green whole lentils and (b) Red split lentils under heating and cooling for thirteen minutes in excess of water and continuous shear.

The NaCl was expected to bind with the proteins leaving a higher proportion of water molecules to interact with the starch molecules. However, the addition of 0.10 M NaCl did not change the viscosity of ball-milled GWL and RSL. The results indicated that aggregated proteins did not solubilise with NaCl addition thus proteins were still able to prevent starch swelling.

3.3 Conclusion

Ball milling was used as an alternative way of physical processing. The physiochemical and pasting properties of flours from legumes and cereals were evaluated after ball milling.

The impact of ball milling on the physiochemical properties of two types of lentils with (GWL) or without hull (RSL) and a type of millet (PM) were evaluated. Polarised light micrographs of ball-milled flours of both lentils showed a decrease in the birefringence indicating a disruption in the crystalline structure of the starch granules by the ball mill. The decrease in the crystalline diffraction peaks using X-RD and lower initial endothermic gelatinisation peak (T_o) confirmed this hypothesis for the lentils. However, ball milling did not change the birefringence, crystallinity or gelatinisation temperature of PM's starch granules. Ball milling also promoted the formation of protein aggregates in both lentil flours as observed under the fluorescent microscope. The formation of insoluble protein aggregates decreased the proteins' solubility and hydrophobicity in lentils. However, ball milling did not affect the proteins in PM flour.

The pasting properties of ball-milled GWL, RSL and PM were compared to other legumes (YP, RWL) and cereals (W, M). Ball milling decreased the pasting properties of all legume flours. The starch granules were damaged by the ball mill and an increase in viscosity was expected however, the non-soluble protein aggregates could form layers impairing the legume's starch granule's ability to further hydrate, swell and form a

paste. Cereals did not show the same pasting behaviour after ball milling and only the pasting properties of W decreased after ball milling.

4 Chapter: Ball milling as a tool to modify the gel-forming properties of lentil flours

4.1 Introduction

In the past years, there has been a great focus on the use of plant-based ingredients. One example is plant-based foods that mimic animal-based products like yoghurt and cheese. The formulation of these products is based on the formulation of a gel. Hydrogels are viscoelastic three-dimensional networks trapping a vast amount of water (i.e., around 80% for yoghurt). The gel structure of composite food-based products is formed by biopolymers such as polysaccharides and proteins, cross-linking via weak molecular interactions (hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic interactions) (Banerjee & Bhattacharya, 2012; Joshi et al., 2014; Nazir et al., 2017).

In a composite starch-protein mixture such as the legume flours used in this thesis, the ratio of starch and proteins impacts the microstructure and properties of the gel. For example, in fava bean starch-protein composites when the starch concentration was higher (>70%) a tightly packed and strong network of swollen starch granules with the protein filling the space of the network was formed (Nilsson et al., 2023). The composition of the lentil flours in this thesis showed a higher starch content than protein thus a strong network was expected to be formed by the starch granules with the proteins filling the spaces within the starch network.

However, ball milling in this thesis modified the functional properties of the lentils' flour by reducing peak viscosity. According to the literature, ball milling (up to 4 min) of a soy protein isolated material, (Liu et al., 2017) increased the protein solubility thus the gel strength and water holding capacity and vice versa when protein loses solubility (after 10 min of ball milling). Similar research has been conducted on damaged isolated starch material by Liu et al., (2019) that showed an improved texture for dough prepared using damaged cassava starch granules due to higher starch-water interactions. However, there is no known literature evaluating the impact of ball milling on the gelation of a composite lentil system. In the lentil flour composite system to be evaluated, it is hypothesised that the protein aggregation caused by ball milling would limit protein-water interactions and increase water mobility in the starch gel thus, leading to a higher starch retrogradation, hardness and syneresis.

This experimental chapter aimed to address the impact of ball milling on gel formation and gel properties of the lentil flours. To address this aim research was conducted on the following objectives.

- Evaluating the impact of ball milling on gel formation during cooling using a rheometer.
- Evaluating the impact of ball milling on the gel microstructure after storage overnight at 4^{o}C.
- Evaluating the impact of ball milling on the water holding and textural properties of the formed gels after storage at 4^{o}C overnight.

4.2 Pre-phase

In Chapter 3, ball milling of lentil flours for 2 hours (BM2) revealed an intermediate level of changes between the control; pre-ball-milled, and 4 hours ball-milled (BM4) lentils. Thus, it was expected that using the BM2 would result in a gel structure and properties close to both the control and the BM4. It was decided to focus only on the extremes and not the intermediate samples in this chapter. The gelation properties of the control and 4 hours ball-milled (BM4) lentil flours are compared.

Three types of lentils with differences in composition namely green whole lentils; GWL (grain with hull; fibre content of 10.70%), red split lentils; RSL (de-hulled grain; fibre content of 2.70%) and red whole lentils; RWL (grain with hull; fibre content 2.40%) were investigated. Moreover, to elucidate the role of the starch and proteins in the gel formation of the lentils, starch and protein fractions were extracted from the RSL as described in Section 2.3.10.3.

Gel preparation started with the dispersion of the GWL, RWL, RSL flours or the starch and protein RSL fractions in water and heating up to 95 ^{o}C using a rapid visco analyser (RVA) to create a paste. The pasting properties of the control and ball-milled GWL, RWL, RSL flours and RSL fractions of starch and protein can be seen in Figure 4.1.

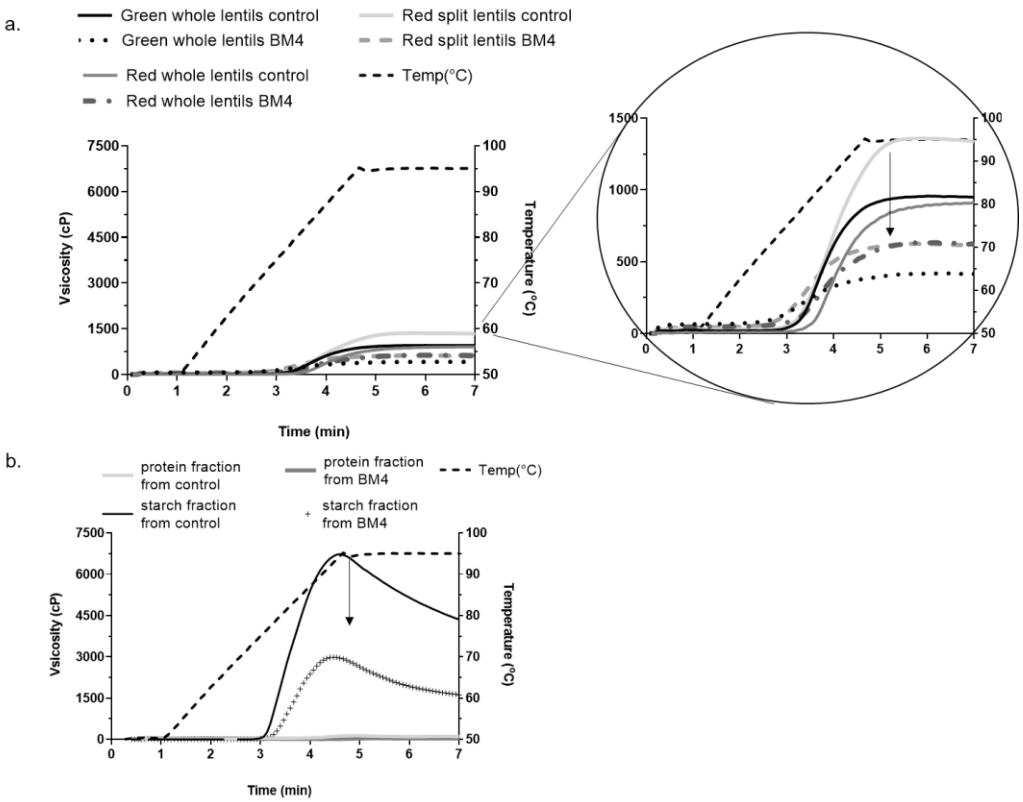


Figure 4.1 Pasting of the (a) control and ball-milled lentil flours and (b) fractionated starch and protein extracted from the control and ball-milled RSL using a rapid visco analyser (RVA) from 50-95 ^{o}C. BM4; 4 hours ball milling.

A decrease in peak viscosity was observed for the ball-milled GWL, RWL RSL flours and the starch extracted after ball milling from the RSL as seen in Figure 4.1a, b. The lower pasting was attributed to protein aggregates which formed a barrier to starch hydration and impaired further increase in peak viscosity. The protein extracted after ball milling from the RSL was not able to form a viscous paste and was observed as a flat line at the bottom of the RVA graph as seen in Figure 4.1b, similarly to the protein extracted from the control RSL.

Gel formation followed the pasting of the lentil flours the properties of which were measured by the G' (elastic modulus) and G'' (storage modulus) using a rheometer as described in Section 2.4.2. The role of

the protein and starch on the gel formation of the lentil flours was also evaluated by using the RSL starch and protein fractions to form gels.

4.3 Gels prepared from lentil flours (control)

4.3.1.1 Small-deformation rheology of the gels prepared with the control lentils and RSL fractions

At the end of the RVA cycle (approximately 95 ^{o}C), the gels were placed in a water bath for three minutes as described in Section 2.4.2.1 prior to measuring the rheological properties of the gels during cooling (temperature was reduced from 70-25 ^{o}C) at a low strain (0.1%) and frequency (10 rad/s). When the gels reached 25 ^{o}C the strain was increased gradually to 100% to measure the linear viscoelastic region (LVR).

LVR refers to the strain region within which the gel shows constant elastic and viscous moduli and indicates the network does not experience structural breakdown. In Figure 4.2 the G’ and G’’ values of the amplitude sweep and the linear viscoelastic region for the gels prepared with the GWL, RSL, RWL flours and the gels prepared using the RSL fractions of starch and protein can be seen.

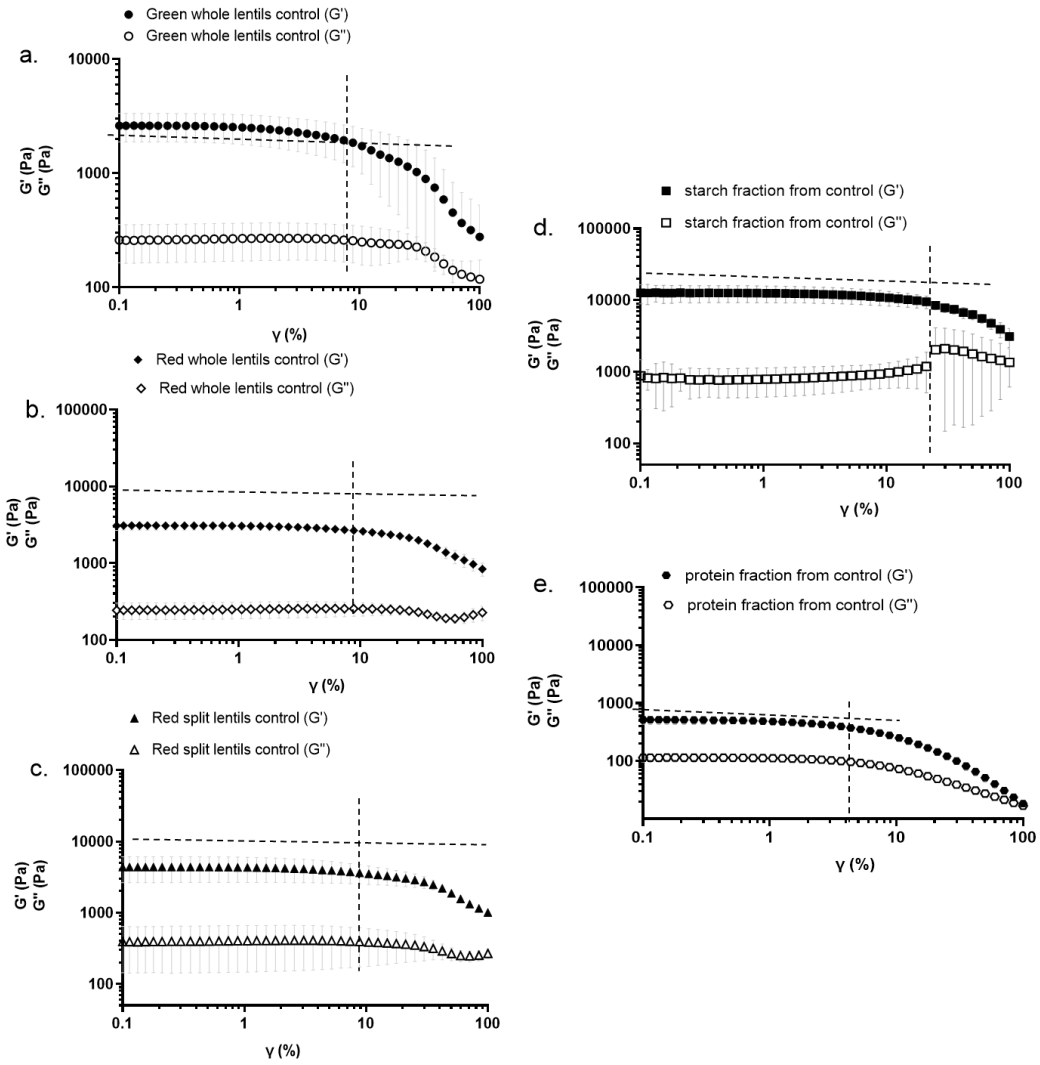


Figure 4.2: Amplitude sweeps between 0.1 to 100% strain, showing the linear viscoelastic region (LVR) of the gels prepared with the control (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils flours as well as the gels prepared with the (d) starch and (e) protein fractions extracted from RSL. In each panel, the LVR limit is shown by the dotted line. The measurements were performed at 25 ^{o}C and with an angular frequency of 10 rad/s.

The GWL, RWL, RSL had a higher G' than G'' indicating a solid-like structure. A solid-like behaviour has been observed on gels formed with legume flours including peas and beans (Acevedo et al., 2013a).

The initial G' value of the RSL was higher followed by the RWL and GWL (4359.0 ± 1538.1 Pa RSL, 3078.9 ± 353.5 Pa RWL, 2616.2 ± 655.3 Pa GWL). The initial G' values of the GWL, RWL, RSL were closer to the G'

values of the starch fraction (12653.7 ± 3391.5 Pa) rather than the G’ of the protein fraction (516.2 ± 77.7 Pa) indicating a more significant role of starch than proteins in the lentil gel network. In general, the lower LVR G’ values of the GWL, RWL, RSL in comparison to the LVR of the starch fraction indicated a lower stiffness, higher tendency to deform and lower resistance to flow.

In composite gels such as the GWL, RWL, RSL in this thesis, the ratio of starch and protein affects the properties of the gel. An increase in the protein content from 0% to 20% in a starch-protein composite decreased the G’ because proteins acted as disrupting agents to the network formation by the starch molecules. Proteins can compete for the available water with starch or adsorb to the starch granules' surface thus reducing starch hydration, swelling and interactions between the starch molecules leading to inhomogeneities in the gel network and a weaker structure (Nilsson et al., 2023).

The length of the LVR region was similar for the GWL, RWL, RSL and appeared as a plateau within 0.1 to almost 10% strain and 0.1% strain was used for the rest of the rheological experiments including frequency sweep and temperature sweep.

The starch fraction showed a wider LVR between 0.1-20% strain and the protein fraction had a shorter LVR between 0.1-2% strain than the GWL, RWL, RSL. A longer LVR shows a lower dependency on strain whereas a shorter LVR shows a higher dependency on strain (Mezger, 2014). Amylose has a linear structure that allows the entanglement between the

starch molecules and the formation of a stable network thus, a longer LVR.

4.3.1.2 Rheological properties of the gels prepared from lentils and RSL fractions upon cooling (temperature sweep)

The rheological properties of the GWL, RWL, RSL during cooling (from 70 ^{o}C to 25 ^{o}C) and the gelation process or transition of the polymers from sol to gel were also evaluated using a temperature sweep test. The strain value (0.1%) was obtained from the results of the amplitude sweep test as seen in Figure 4.2.

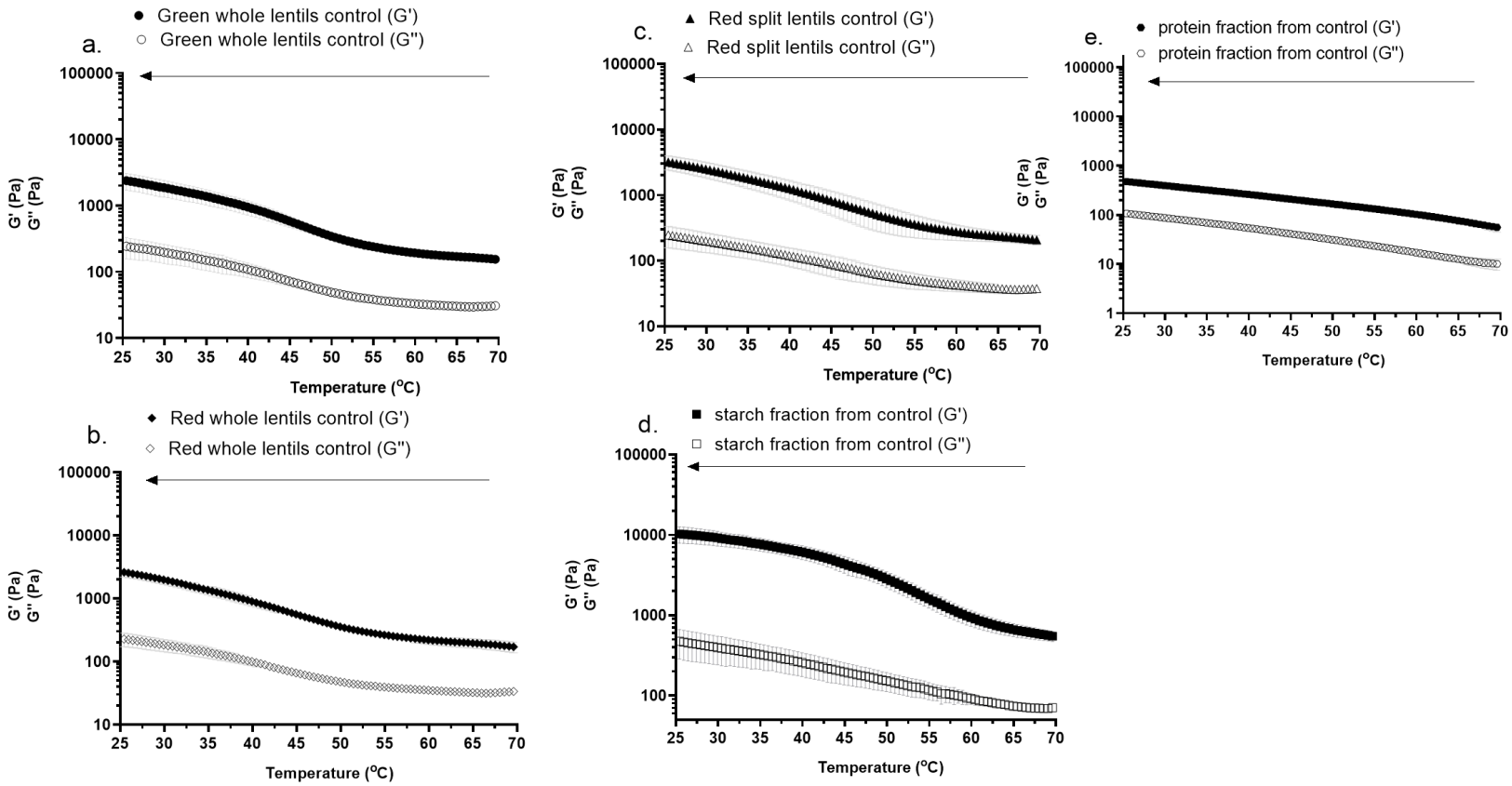


Figure 4.3: Rheological properties of the gels prepared with the control (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils flours and the fractions of (d) starch (e) protein extracted from the RSL during cooling. The measurements were performed from 70-25 °C (0.5 °C/ min) with an angular frequency of 10 rad/s and 0.1% strain.

After heating in the RVA, the GWL, RWL, RSL pastes, and pastes from the starch and protein fractions were placed in a rheometer to investigate their behaviour upon cooling when gelation occurs (Figure 4.3a-e).

The initial G'>G'' at 70^{o}C indicated a solid-like structure thus the transition from a liquid to a solid state (sol-to-gel) occurred at a higher temperature and sooner than the beginning of the rheological test. The continuous increase in G' and G'' upon cooling has been attributed to protein-protein, protein-starch and starch-starch interactions involving the formation of disulfide bonds and hydrophobic or hydrogen interactions. For example, hydrogen bonds are known to be formed between the amylose molecules leading to the formation of junction zones (Scott & Awika, 2023).

The initial G' value of the RSL at the beginning (70 ^{o}C) of the temperature sweep test was 204.2 ± 27.7 Pa, similar to the GWL (152.0 ± 10.0 Pa) and RWL (167.0 ±12.7 Pa). The initial G' values of the starch fraction (536.3 ± 59.8 Pa) were higher and the initial G' values of the protein fraction (51.5 ± 7.4 Pa) were lower than the initial G' of the GWL, RWL, RSL.

As the temperature was decreased from 70 ^{o}C down to 55 ^{o}C, there was an abrupt increase in both moduli for the GWL, RWL, RSL and starch fraction. This abrupt increase has been ascribed to the increased density of the junction zones formed from the hydrogen bonds between the amylose molecules (Nunes et al., 2006). The moduli continued to increase from 55 ^{o}C down to 25 ^{o}C gradually, reflecting to a network

development caused by decreasing the temperature and resulting in the formation of a more solid-like structure. The formation of hydrogen bonds between the amylose molecules stabilises and strengthens the network at lower temperatures (i.e., 25 ^{o}C) and causes a higher G' in comparison to values at higher temperatures (i.e., 90 ^{o}C) (Acevedo et al., 2013a). The lower G' of the GWL, RWL, RSL compared to the starch fraction G' values was attributed to the presence of proteins in the network. Yang et al., (2004) observed the gelation properties of a starch-protein system and found that increasing the protein content from 0% to 15-30% decreased the G' of the starch gel. Protein could act as an inactive filler which occupies more space within the gel network and weakens the overall strength of the starch network. Also, the presence of proteins decreased starch concentration thus, the concentration of the amylose molecules which are responsible for increasing the G' of the gel by forming hydrogen bonds (Yang et al., 2004).

The final G' values at 25 ^{o}C were not different between the GWL, RWL, RSL (GWL; 2449.2 ± 614.0 Pa, RWL; 2664.0 ± 387.8 Pa, RSL; 3247.2 ± 707.0 Pa) but were lower compared to the final G' value of the starch fraction (10359.0 ± 1998.7 Pa) and higher than the final G' value of the protein fraction (500.5 ± 43.4 Pa).

Tan δ was the ratio of G''/G' and was used to get a better insight into gel strength evolution upon cooling. Tan δ>1 suggests a viscous-like behaviour but a tan δ closer to zero shows a more elastic gel (Tabilo-Munizaga & Barbosa-Cánovas, 2005). A comparison between the tan δ of the gels prepared with control flours from the GWL, RWL, RSL was

conducted. A comparison between the tan δ of the GWL, RWL RSL and starch, protein fractions was also conducted.

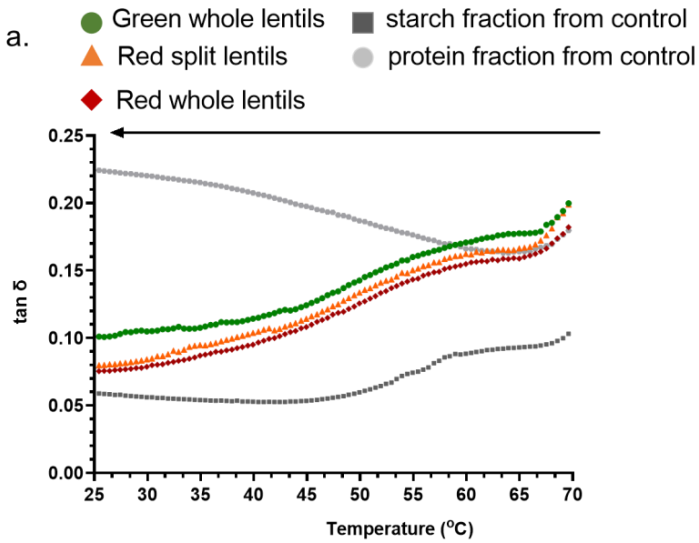


Figure 4.4 Tan δ of control gels prepared with lentils and lentil starch and protein fractions upon cooling (70-25 °C). Green whole lentils (round shape in green colour), Red whole lentils (diamond shape in red colour), Red split lentils (triangle in orange colour), starch gel (square in dark grey), protein gel (round in light grey).

Tan δ of the GWL, RWL, RSL showed a continuous decrease as the temperature was reduced. The starch fraction showed a plateau from 70 °C to 60 °C, followed by a decrease down to 45 °C and a plateau from 45 °C until the end of the test at 25 °C. The tan δ of the protein fraction showed a decrease from 70 °C to 60 °C followed by an increase from 60 °C until the end of the test at 25 °C.

The tan δ values of the GWL, RWL, RSL at 70 °C were close to the tan δ values of the protein fraction indicating a more viscous gel structure in comparison to the starch fraction. However, tan δ values from RWL (0.07 ± 0.01), RSL (0.08 ± 0.01) and GWL (0.10 ± 0.01) at 25 °C were close to the starch fraction (0.06 ± 0.01) indicating the formation of more elastic gels in comparison to the protein gel. To better understand the behaviour

of the gels under cooling and the role of the proteins and starch in the microstructure of the gels, microscopic images of the gels were taken.

4.3.1.3 Microstructure of the lentil pastes and gels

At the end of the heating cycle, the lentil pastes were placed under fluorescent microscopy and their microstructure was observed. The gels formed after the end of the cooling step were also observed under the microscope. The micrographs can be seen in Figure 4.5.

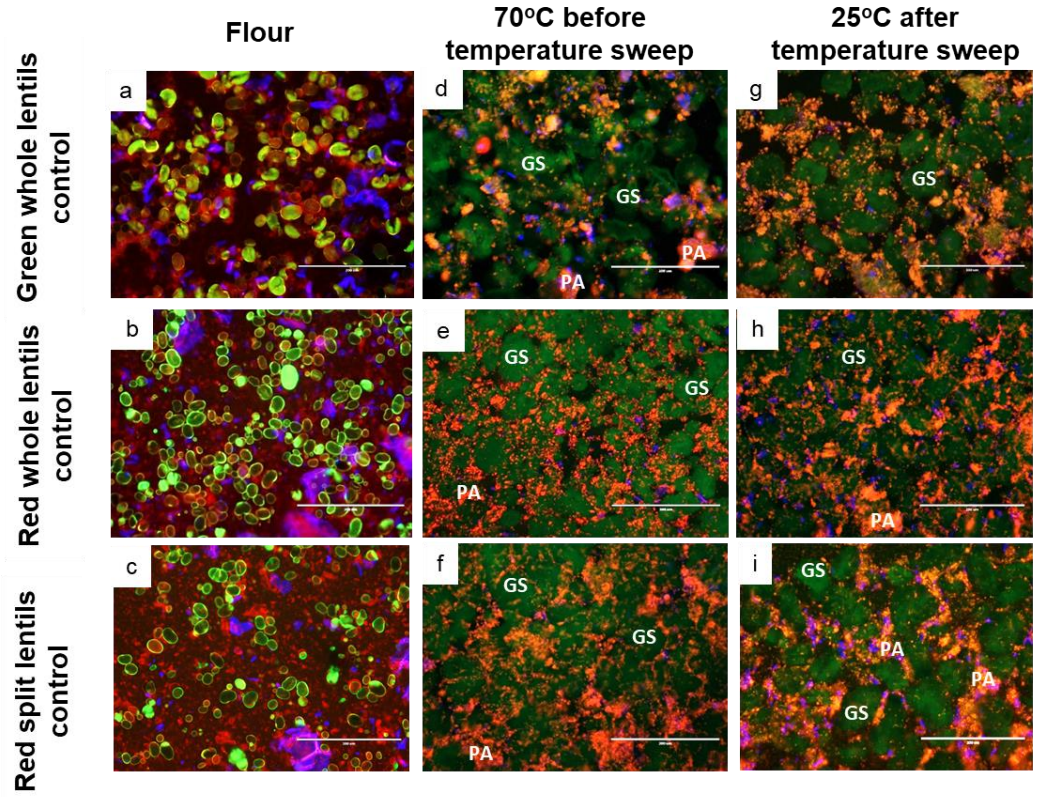


Figure 4.5 Fluorescent microscopy of the control Green whole lentils, Red whole lentils, Red split lentils (a-c) flours, (d-f) pastes at 70 °C before temperature sweep and (g-i) gels at 25 °C after the temperature sweep. Scale bar; 200 µm. GS; gelatinised starch, PA, protein aggregate, starch; green colour (stained with fluorescein isothiocyanate), proteins; red colour (stained with rhodamine B), fibres; blue colour (stained with calcofluor white).

The fluorescent microscopy of the control flours (Figure 4.5a-c) showed oval-shaped starch granules (green colour), protein bodies (red colour) and fibres (blue colour) in line with other findings (Dalgetty & Baik, 2003b; Jennings & Foster, 2020; Joshi et al., 2013b).

The microstructure of the GWL, RWL, RSL pastes at 70 ^{o}C before the temperature sweep showed gelatinised starch with no specific granular shape and size. Proteins formed small aggregates at the same temperature (70^{o}C) and fibres were observed as small fragments but their role as active or inactive fillers was not clear from the micrographs. The presence of larger areas of red-stained components in RWL and RSL was attributed to higher protein content (25.70% and 30.00% respectively) in comparison to GWL (23.80%). The black areas in the fluorescent micrographs that are mostly observed in the GWL, represent little or no fluorescence indicating that there is no or very low presence of the labelled target components (starch, proteins and fibres) and could be due to a lack of fluorophore binding. For example, the presence of specific amino acids impacts the binding of rhodamine B because it interacts with the positively-charged amino acids in the proteins, the hydrogen bond donors/ acceptors on the protein surface i.e., side chains of amino acids and with non-polar amino acids.

Further interactions between the proteins increased the size of the protein aggregates but in general decreasing the temperature from 75 ^{o}C to 25 ^{o}C did not reveal major differences in the microstructure of the GWL, RWL, RSL. There were no differences between the microstructure

of the GWL, RWL, RSL in line with the rheological data showing a similar final G' for these gels as seen in Figure 4.3.

It has been suggested that the overall rheology of a mixed system is highly dependent on the continuous phase (Chung et al., 2013) and the GWL, RWL, RSL gels showed a similar behaviour to the gel from the starch fraction. However, the role of the proteins was not clear by observing the micrographs.

Further evaluation using a frequency sweep test was conducted to collect information about the microstructure and stability of the GWL, RWL, RSL gels at 25 ^{o}C when the temperature sweep test and cooling cycle were over.

4.3.1.4 Frequency sweep of the lentils and RSL fractions

After the end of the cooling cycle using the rheometer from 70 ^{o}C down to 25 ^{o}C the GWL, RWL, RSL gels and gels prepared with the starch and protein fraction of the RSL were evaluated using a frequency sweep test conducted at 25 ^{o}C and 0.1% strain within 0.1-10.0 rad/s and the results can be seen in Figure 4.6.

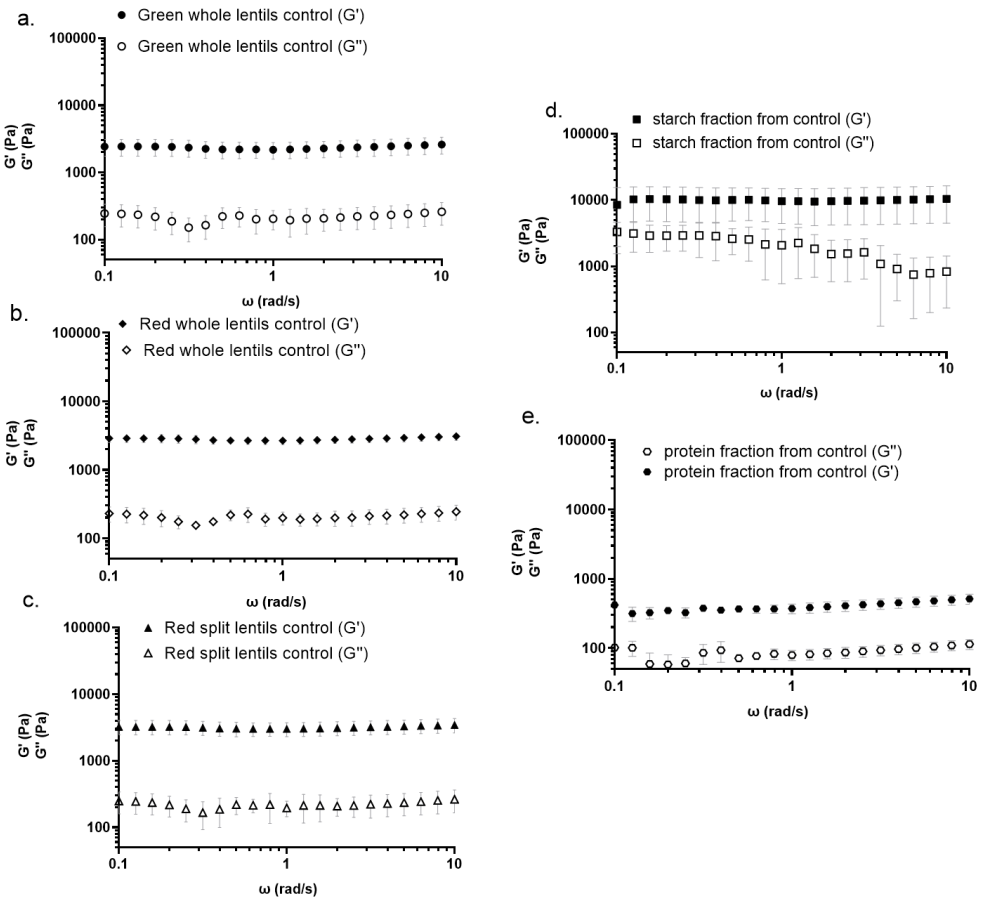


Figure 4.6 Frequency sweep between 0.1-10 rad/s at 25 ^{o}C and 0.1% strain of the gels prepared with the (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils flour and the (d) starch and (e) protein fractions extracted from Red split lentils.

Values of G'>G'' were observed for all GWL, RWL, RSL gels within the frequency range used indicated a solid-like structure at 25 ^{o}C. and was constant within 0.10-10.00 rad/s indicating frequency independence. Frequency independence indicates that the gel's consistency and behaviour or performance do not vary with different rates of shear or oscillation. This property shows that the gels will maintain their stability, structure and performance across a wide range of oscillation frequencies.

The gel from the starch fraction also showed frequency independence indicating the formation of a strong network. However, the gel from the

protein fraction showed a slight increase with increasing frequency thus indicating a frequency dependence and a weaker network in comparison to the GWL, RWL, RSL gels and the gels from the starch fraction.

In general, weak gels have been described as networks that break easily under stress and true gels as networks that are more difficult to break and thus independent of frequency (Rao, 2014). Thus, the GWL, RWL, RSL gels and gels from the starch fraction could be characterised as true gels but the gels from the protein fraction as weak gels. Also, the GWL, RWL and RSL gels showed a more similar rheological behaviour to the gel from the starch fraction indicating a stronger role of the starch in the gel network. Literature shows that starch-based gels form a stronger network and have a lower dependency on frequency (Joshi et al., 2014) compared to protein gels, which show a greater dependency on frequency and the formation of a weaker gel upon cooling (Nilsson et al., 2023).

To further understand the GWL, RWL, RSL gel's properties a texture analysis was conducted on a new set of gels because during the rheological measurement deformation was applied (frequency). The gels were allowed to cool down in plastic tubes in a water bath and not in the rheometer and then left in storage at 4 ^{o}C overnight as described in Section 2.4.1.

4.3.1.5 Visual observation of the lentil gels' macrostructure

The GWL, RWL and RSL gels were removed from the plastic tubes the next day, with all samples presenting a self-standing ability as can be seen in Figure 4.7.

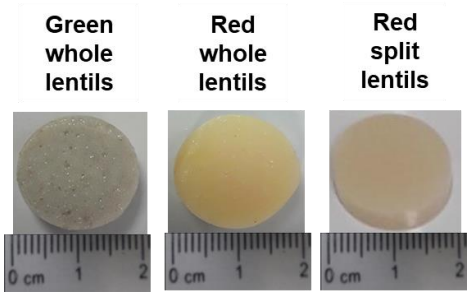


Figure 4.7 The appearance of the heat-set gels formed by the Green whole lentils, Red whole lentils, Red split lentils control flours in plastic tubes after being stored overnight at 4 ^{o}C.

The photos were taken before conducting any uniaxial compression test or microstructural analysis. The differences in the colours of the GWL, RSL, RWL gels were naturally derived from the flours and were representative of the colour of each grain. GWL showed a green colour, RSL an orange colour and RWL a yellow colour.

The self-standing ability of the gels was attributed to the short-term reassociation of the amylose molecules as amylopectin molecules have been described as requiring a long-term reassociation (Wang et al., 2022). The microstructure of a gel network can be observed using fluorescent microscopy by labelling the components (starch, protein, fibres) of interest in the gel with specific dyes.

4.3.1.6 Microstructure of the lentil gels

The microstructure of control lentil gels, after storage in the fridge (4 ^{o}C) overnight, was observed using a fluorescent microscope as seen in Figure 4.8.

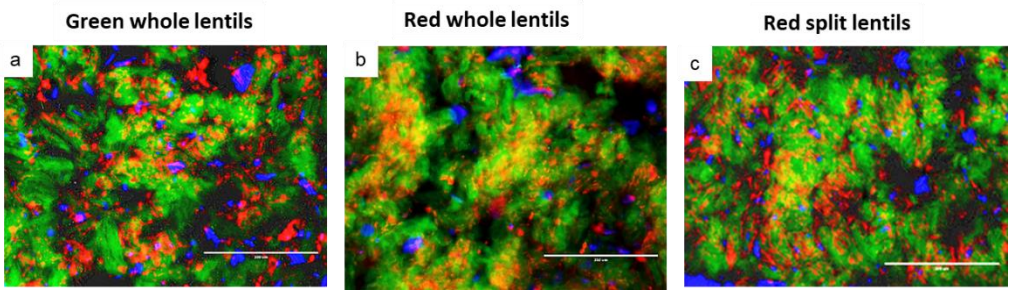


Figure 4.8 Fluorescent micrographs of gels prepared with the control flours of (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils. Scale bar; 200 µm. starch; green colour (fluorescein isothiocyanate), proteins; red colour (rhodamine B), fibres; blue colour (calcofluor white).

The green mass represents gelatinised starch forming a network with internally dispersed fibres and protein aggregates. Starch-based networks have been described in the literature as continuous networks of swollen and fragmented starch granules (Ribotta et al., 2007) The starch seemed to form a continuous network, but it was not clear from the micrographs whether the proteins and the fibres acted as active or inactive fillers. According to Johansson et al., (2022) particles in gels can be categorised as active fillers when contributing to the functionality or performance of the gels and tend to increase the strength. In this thesis, the oscillatory tests showed that proteins weakened the gel network of the GWL, RWL RSL by decreasing the moduli (G,' G'') in comparison to the gel from the starch fraction thus acting as inactive fillers. Inactive fillers do not contribute to the primary functionality of the gel but could modify its physicochemical properties such as water retention and tend

to decrease the gel strength (Johansson et al., 2022). For this reason, the water retention properties and the textural properties of the GWL, RWL, RSL gels were investigated.

4.3.1.7 Water holding of the lentil gels

The water-holding of the gel refers to the water retained by the gels after centrifugal forces were applied (5000 rpm for 30 min), which is presented in Figure 4.9 for the GWL, RWL, RSL gels. The water loss of the gel during centrifugation is termed syneresis and is also a parameter to consider.

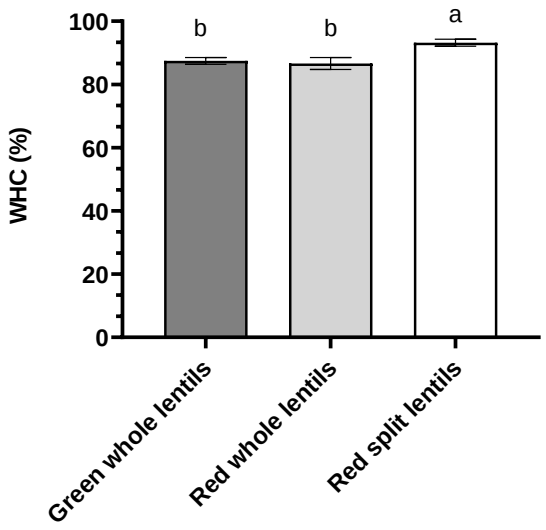


Figure 4.9 Water holding capacity (WHC) of the gels prepared with the control flours from Green whole lentils, Red whole lentils, Red split lentils. Different letters represent a significant difference at $p<0.05$.

All gels were able to retain 80% (water holding) of the initial gel volume, with 20% lost as water (syneresis). GWL and RWL lost more water compared to RSL. Water is usually expelled by the gel network due to amylose and amylopectin retrogradation during cooling which depends

on several factors including the presence of non-starch components like proteins or fibres (Scott & Awika, 2023).

An increase in starch concentration of the continuous phase of a gel when other components are present leads to water immobilisation, preventing starch reassociation and decreasing water expulsion. For example, water-soluble proteins such as albumins in legumes have hydrophilic properties due to their amino acid composition. When these proteins are dispersed in a gel network can interact with the water molecules forming hydrogen bonds (Scott & Awika, 2023). The hydrophilic amino acid composition between the lentils used in this thesis could impact the water holding of the network by reducing the 'free' water in the system and molecular mobility preventing starch recrystallisation thus water expulsion.

Non-starch and water-soluble polysaccharides in the system act in the same manner as the proteins by interacting with water and reducing both the available water molecules and water mobility in the system (Scott & Awika, 2023). According to the composition of the three lentils (Table 3.1), the total amount of protein and fibres in the RSL (28%) was lower in comparison to GWL (34.50%) and RWL (35.40%) and had a higher carbohydrate content (59.13%) in comparison to GWL (51.88%) and RWL (54.22%). The higher protein-fibre fraction but lower carbohydrate fraction in GWL and RWL compared to the RSL indicated a dilution effect on starch composition thus, lower starch-water interactions and the formation of a less compact network in comparison to the RSL that cannot hold the entrapped water when centrifugal force is applied.

Water holding has been linked to the textural properties of the gels, thus in the next Section 4.3.1.8 the textural properties of the lentil gels are discussed.

4.3.1.8 Textural properties of the lentil gels

Hardness and Cohesiveness of control lentil gels (25 replicates; 5 per batch) were obtained at a non-destructive strain (25%) using a texture analyser at a compression speed of 1 mm/ sec and are presented in Figure 4.10a, b.

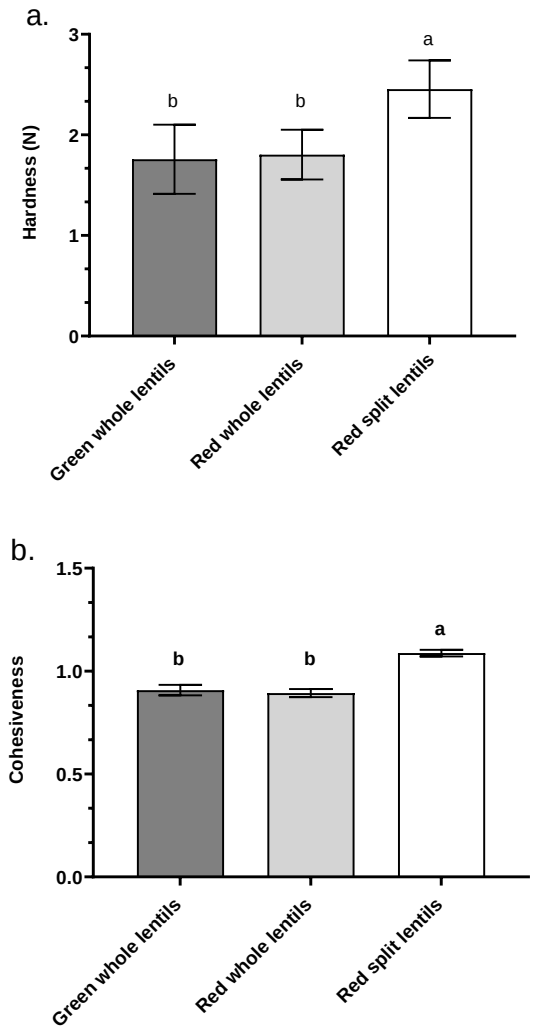


Figure 4.10: Hardness (a) and cohesiveness (b) of control heat-set lentil gels at 25% strain. Different letters represent a significant difference at $p<0.05$.

The hardness of the gels is the maximum force obtained on the first deformation cycle and represents the resistance of a sample to deformation (Pons & Fiszman, 1996).

The RSL gel was harder in comparison to GWL and RSL. Upon heating, as previously discussed in Figure 4.1, RSL showed a greater viscosity attributed to increased starch-water interactions and starch hydration, swelling and viscosity due to pre-processing involving the de-hulling and splitting of the RSL grain. Although the temperature oscillatory tests from 70-25 ^{o}C (Figure 4.3) and microstructural analysis at 25 ^{o}C (Figure 4.8) did not show differences between the gels formed from control lentil flours, it has been suggested in the literature that the continuous retrogradation of amylose molecules in a more ordered structure after cooling and for a short storage time (typically, between 12 to 24 hours) causes an increase in the hardness of the gels (Ribotta et al., 2007).

The higher hardness of the RSL could be attributed to composition. The RSL showed a higher carbohydrate content (59.2%) than the GWL (51%) and RWL (54%). As fibre composition was calculated separately from the carbohydrate content in the compositional analysis in this thesis, it can be assumed that most of the carbohydrates were starch and a higher proportion of starch in the flour would increase the amount of amylose and thus hardness which could explain the result.

The amylose content between the red and green lentils depends on the variety thus a direct comparison between the amylose contents of the red and green lentils could not be done in this thesis.

Water-holding capacity data presented in Figure 4.9 also correlates with the greater RSL gel hardness, with RSL gel showing a higher water-holding capacity confirming that starch formed a strong network with water entrapped within it. In contrast, GWL and RWL gels had a lower water-holding capacity which was linked to the composition and role of the non-starch components in the gel network. The higher fibre in GWL and higher protein in RWL could either interfere with the ability of the starch molecules to form a strong network (inactive fillers) or occupy space within the gel network thus diluting the proportion of starch and reducing the hardness of the gel network. For example, proteins have been found to act as inactive fillers by interacting with the amylose chains and disrupting the interactions between the amylose molecules, i.e., the formation of junction zones, leading to a weakened structure (Ribotta et al., 2007).

The hardness of the gels is linked to another textural property, cohesiveness because a higher resistance to deformation could be due to the strong network of molecules in a cohesive gel (Pons & Fiszman, 1996). Cohesiveness is calculated by the ratio of the area that occurs in the second compression divided by the area that occurs in the first compression. To assess the gel's structural integrity and stability the cohesiveness is conducted to a non-destructive (25%) strain where the gel is able to withstand deformation (Tabilo-Munizaga & Barbosa-Cánovas, 2004). Cohesiveness was evaluated at a non-destructive deformation at 25% strain and the results can be seen in Figure 4.10b.

It was observed that the cohesiveness of the gels was close to 1. Values close to 1 indicate a highly cohesive gel that will experience an almost full recovery at the second compression at the given strain (Hoon Moon et al., 2017). The higher cohesiveness of RSL than the GWL and RSL could be related to the formation of a harder network indicating well-structured gels. Generally, starch-based networks have a higher degree of cohesion compared to the protein-based networks and the addition of non-starch components in the starch network could influence the degree of the network's cohesion. Differences in the composition between the lentils could account for these results with the fibres in GWL and proteins in RSL influencing the cohesion of the resulting gel network.

To further examine the textural characteristics of the lentil gels, texture analysis experiments were conducted on a new set of lentil gels using a higher deformation strain.

The mechanical resistance or the ability of the gels to withstand break/ rupture/ fail under high deformation and the lentil gels compressed to 80% strain. The results can be seen in Figure 4.11.

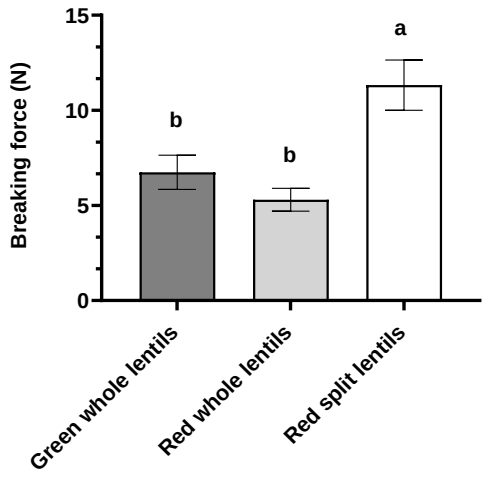


Figure 4.11: Breaking force or the ability of the control lentil gels to withstand rupture when compressed to 80% strain. Different letters represent a significant difference at ($p<0.05$).

The fracture of a gel is the ability to fail when force is applied. The gels were compressed to 80% strain and the force needed to break the gels and therefore the ability of each structure to withstand rupture was measured. The fracture in gels has been suggested to occur by cracks at the surface of the gel or represent structural defects (Bourne, 2002).

A higher force was needed to break the control RSL gel and a lower force to break GWL and RWL. The mechanical properties of composite gels depend on the type of gel formed i.e., filled, or interpenetrated. For example, in filled gels, a component forms the continuous phase and the other the dispersed phase. Particles acting as active fillers in the dispersed phase strengthen the network and induce the ability of a gel to withstand rupture but inactive fillers increase the structural defects in the network and lead to a gel with a lower ability to withstand rupture (Lyu et al., 2022). In the previous section (Section 4.3.1.7), it was discussed that the protein-fibre fraction of the GWL and RWL acted as

inactive fillers reducing the gels' water retention ability. Similarly, the same fractions weaken the GWL and RWL structure leading to a lower ability to withstand fracture compared to the RSL.

The first part of this chapter discussed the rheological, microstructural, textural properties and the water-holding ability of the GWL, RWL and RSL gels prepared with the control flour showing that:

- The GWL, RWL and RSL had a G'>G'' indicating a solid-like structure.
- The GWL, RWL and RSL gels had similar rheological properties to the gel prepared with the starch RSL fraction indicating a higher role of the starch in the gel network than the protein.
- The RSL gel formed a harder, more cohesive, stronger network with a higher ability to retain water upon centrifugation than the GWL and RWL due to lower composition in fibres and proteins.

The sections provided a fundamental understanding of the gels prepared with the control flours. In the second part of this chapter (4), a comparison between the properties of the gels prepared using the control and ball-milled GWL, RWL and RSL flours will be conducted. The aim is to understand the impact of ball milling on the gel-forming properties of the lentil gels. The starch and protein fractions extracted from the ball-milled RSL were also used to better understand their role in the lentils' gel network.

4.3.2 The impact of ball-milled lentil flours on gel formation and gel properties

4.3.2.1 Small-deformation rheology of the gels prepared with the ball-milled lentils and RSL fractions

The pastes prepared using ball-milled GWL, RWL, RSL, starch and protein fractions from ball-milled RSL were cooled down using a rheometer from 70 ^{o}C down to 25 ^{o}C at strain 0.1% and frequency of 10 rad/sec. At 25 ^{o}C the strain increased to 100% and the impact of ball milling on the linear viscoelastic region (LVR) can be seen in Figure 4.12.

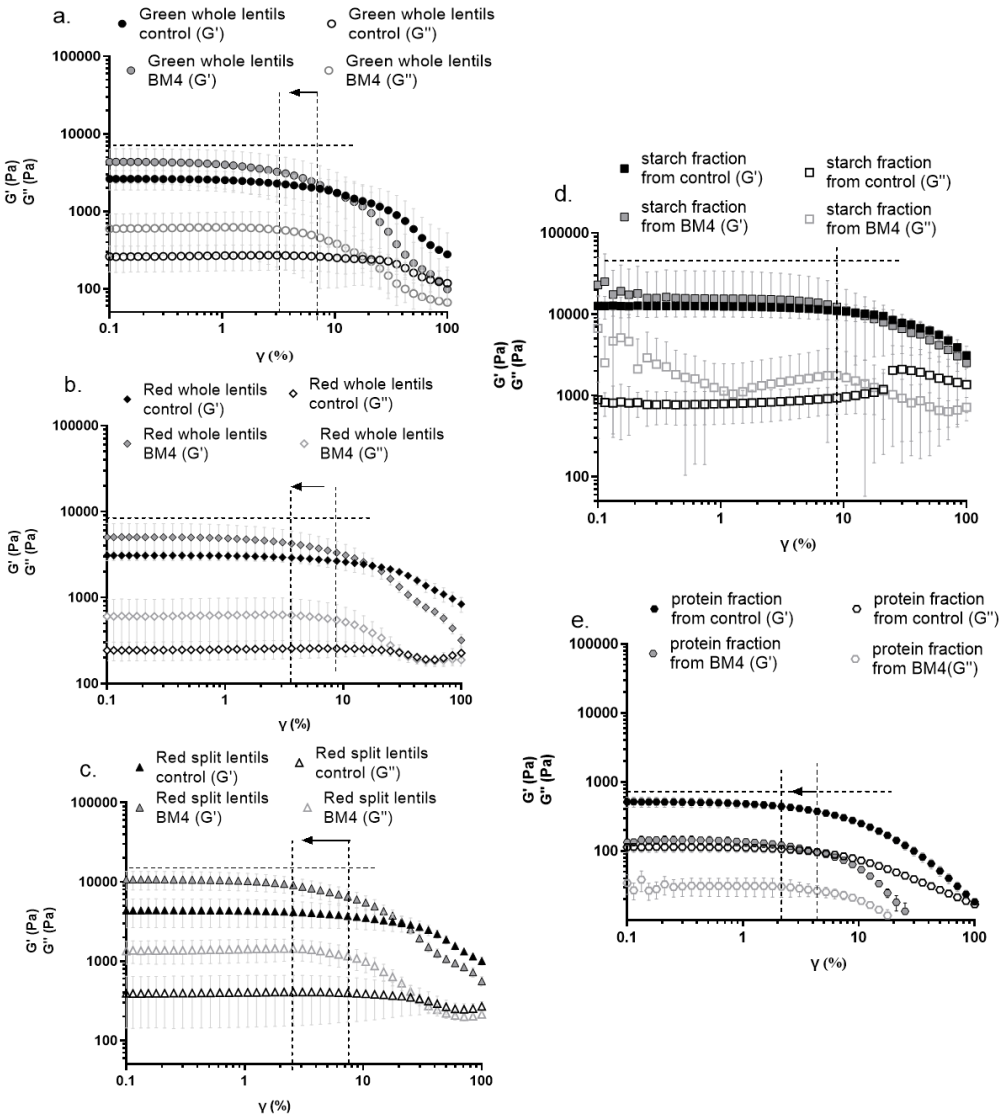


Figure 4.12 Amplitude sweeps between 0.1 to 100% strain, showing the linear viscoelastic region (LVR) of the gels prepared with the control and ball-milled (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils flours as well as the gels prepared with the (d) starch and (e) protein fractions extracted from control and ball-milled RSL. In each panel, the impact of ball milling on the LVR can be seen by the direction of the arrow. The measurements were performed at 25 ^{o}C and with an angular frequency of 10 rad/s.

Differences in the impact of ball milling on the LVR length between the GWL, RWL, RSL gels and the RSL fractions were observed. Ball milling decreased the length of the LVR for the GWL, RWL, RSL and a plateau was observed between 0.1 to roughly 3% strain indicating a structure more dependent on strain after ball milling. A decrease in the length of the LVR has been related to inhomogeneities in the gel structure created

by the starch, proteins, fibres, or a combination of them (Nilsson et al., 2023). Ball milling did not change the LVR length of the starch fraction indicating that ball milling did not impact the resistance of the gel to deformation. Ball milling decreased the LVR length of the protein gels (from 0.1 to 4% to 0.1 to 2%) indicating a reduction in resistance to deformation.

Ball milling did not impact the initial G' values from GWL and RWL (2616.2 ± 655.3 Pa to 4336.1 ± 1846.4 Pa for the GWL and from 3078.9 ± 353.5 Pa to 5043.8 ± 1990.4 Pa for the RWL). However, the ball milling increased the initial G' values of the RSL gel from 4359.8 ± 1538.1 Pa to 10732.5 ± 2538.2 Pa. Also, ball milling did not change the initial G' values of the starch fraction but decreased the initial G' values of the protein fraction. Cross-over of the G' and G'' moduli was only clearly observed for the ball-milled GWL at the end of the test representing the point of phase transformation from solid-like to liquid-like and indicating a slightly weaker network in comparison to the RWL and RSL.

Ball milling decreased the final G' values of the GWL, RWL, RSL gels indicating a decrease in the stiffness. Between the control and ball-milled final G' values, there was a decrease from 325.1 ± 166.3 Pa to 209.7 ± 74.9 Pa for the GWL, from 836.9 ± 145.1 Pa to 318.8 ± 47.1 Pa for the RWL and from 10006.8 ± 103.6 Pa to 559 ± 67.2 Pa for the RSL. Ball milling did not change the final values of the starch fraction but decreased the final G' values of the protein fraction.

The lower G' and G'' of the protein fraction after ball milling were attributed to the insoluble protein aggregates formed during ball milling. Similarly, in soy protein gels a higher proportion of insoluble aggregates decreased the G,' weakened the gel structure and gel stability. This happened because ball milling induced the formation of non-soluble protein aggregates leading to a coarse network thus explaining the lower moduli and length of the LVR (Klost et al., 2020).

Although this might be true for the protein lentil gel, in composite systems such as the lentil gels, the protein-protein and protein-starch interactions upon gel formation should be taken into consideration before drawing any conclusions. The rheological properties of the gels prepared from the ball-milled GWL, RWL, RSL and RSL fractions were measured using a temperature sweep test.

4.3.2.2 Rheological properties of the gels prepared with the ball-milled lentils and RSL fractions upon cooling (temperature sweep)

At the end of the RVA analysis, the pastes from the ball-milled GWL, RWL, RSL and the fractions extracted from the ball-milled RSL were placed in a rheometer to monitor the gelation process or transition of the polymers from sol to gel, during cooling from 70 ^{o}C down to 25 ^{o}C. The strain value (0.1%) used in this experiment was obtained from the results of the amplitude sweep test as seen in Figure 4.12. The results of the temperature sweep were compared with the gels prepared with the control GWL, RWL, RSL and RSL fractions as seen in Figure 4.13.

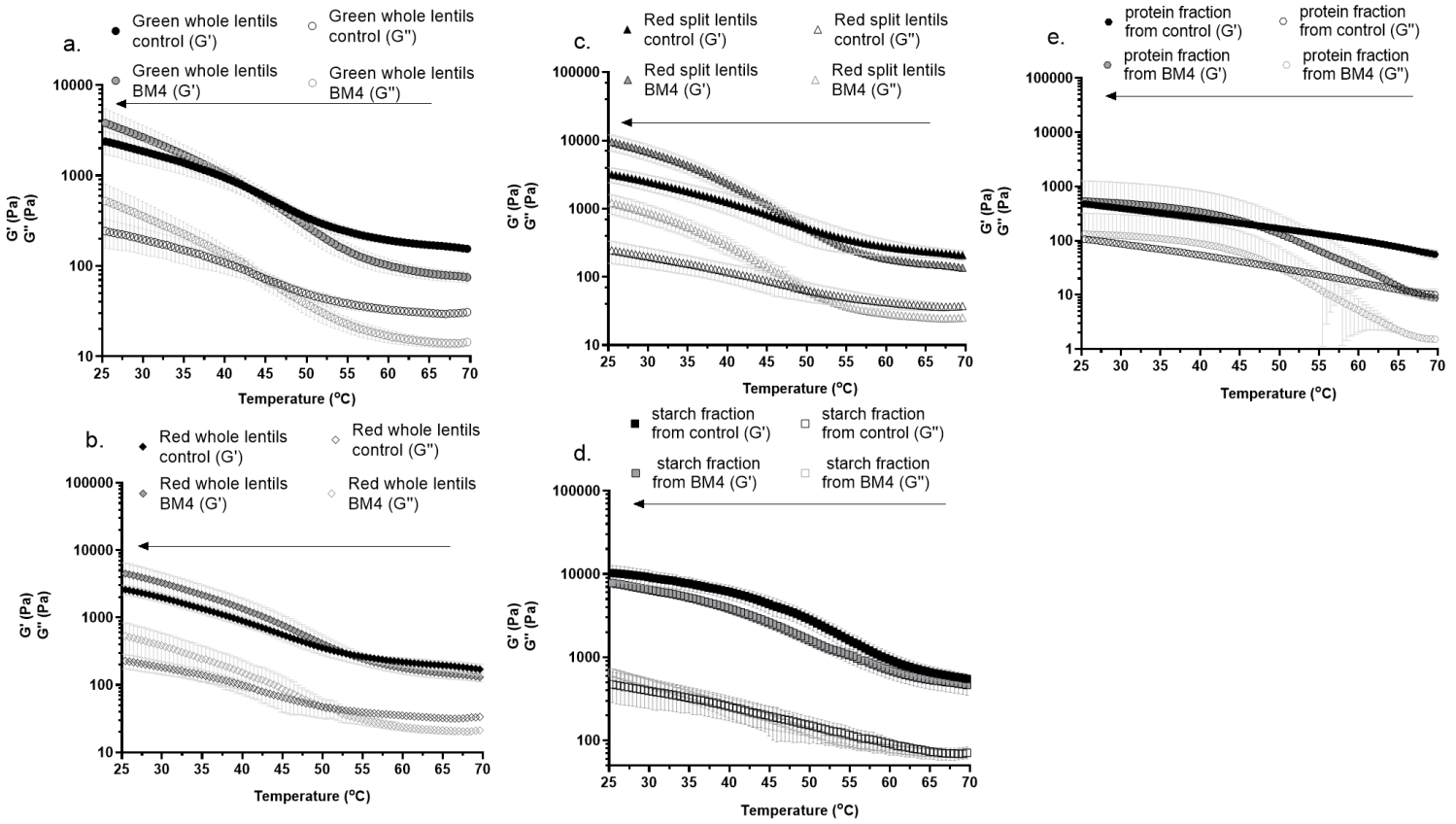


Figure 4.13 Comparison between the rheological properties of the control and ball-milled (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils, (d) starch and (e) protein fractions extracted from the control and ball-milled RSL during cooling. The measurements were performed from 70 °C down to 25 °C (0.5°C/ minute) with an angular frequency of 10 rad/s and 0.1% strain. BM4; 4 hours ball milling.

After heating, a cooling cycle from 70 ^{o}C to 25 ^{o}C followed using a rheometer. The GWL, RWL, RSL gels as seen in Figure 4.13a, b, c and the gels from the starch and protein fractions as seen in Figure 4.13d, e showed a solid-like structure after ball milling with G' being higher than the G'' similarly to the control. The initial G' value (at 70 ^{o}C) of the RSL, RWL and starch fraction did not change after ball milling. However, the initial G' value of the GWL and the protein fraction decreased after ball milling.

The final G' value at the end of the temperature sweep test (25 ^{o}C) was not different between control and ball-milled GWL (from 2449.2 ± 614.0 Pa to 3950.4 ± 1582.4 Pa), control and ball-milled RWL (from 3247.2 ± 707.0 Pa to 4653.6 ± 1812.6 Pa). However, the final G' was higher for the ball-milled RSL compared to the control RSL (from 3259.5 ± 711.2 Pa to 9897.0 ± 2332.0 Pa). The final G' values of the starch and protein fractions did not change after ball milling. Comparing the final G' values between the GWL, RWL, RSL and the starch, protein fractions showed that GWL, RWL, RSL had closer G' values to the starch fraction indicating a higher role of the starch in the gel network than the proteins.

To further investigate the behaviour of the gels prepared with the ball-milled GWL, RWL, RSL and the starch, protein fractions extracted from the ball-milled RSL, the tan δ data were compared as seen in Figure 4.14.

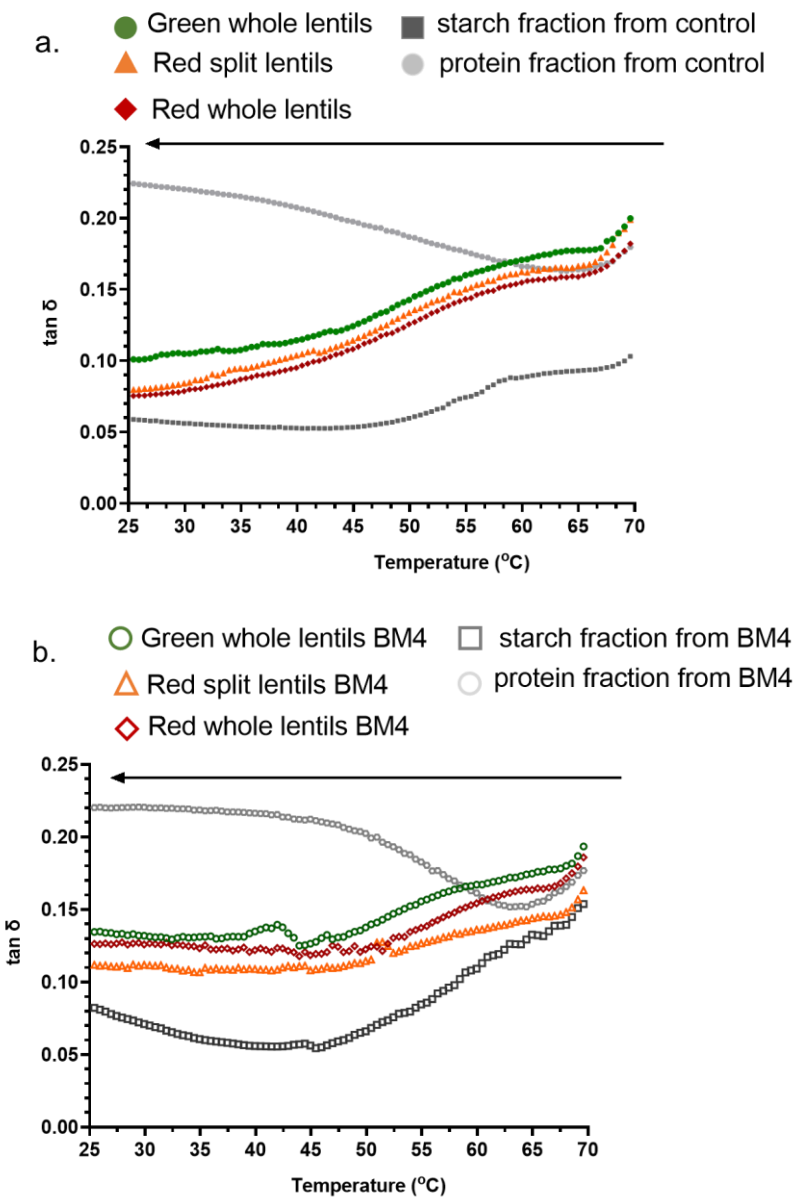


Figure 4.14 A comparison between the tan δ of the (a) control and (b) ball-milled GWL, RWL, RSL, starch and protein fractions extracted from the control and ball-milled RSL as a function of temperature from 70°C to 25°C. Green whole lentils (green circles), Red whole lentils (red diamond), Red split lentils (orange triangle), starch gels (grey square) and protein gels (light grey circle). Filled shapes represent the control and empty shapes of the 4 hours ball-milled gels; BM4.

The tan δ of the control lentil gels showed a continuous decrease with decreasing temperature, indicating the formation of a more solid network. However, after ball milling the tan δ of GWL and RWL showed a decrease between 40-45 °C and then remained at the same value until the end of the cooling cycle at 25 °C indicating the gelation point at

around 40-45 ^{o}C and the formation of a softer network. Similarly, ball-milled RSL showed a decrease in tan δ down to 50 ^{o}C and a plateau for the rest of the cooling cycle.

The tan δ of the protein fraction did not change after ball milling, but the tan δ of the starch fraction increased after ball milling and was attributed to the purity of the sample. The aggregation of the proteins in the RSL was observed under the fluorescent microscope in Figure 3.9 after ball milling and protein solubility also decreased as it was shown in Figure 3.14. The method used for the separation of the starch and proteins from the RSL was achieved by selectively solubilising the proteins at pH=8.0 and the starch sediment as it was not soluble in such conditions. The non-soluble protein aggregates formed during ball milling could sediment along with the starch, interrupt the formation of the starch network during gelation thus increasing the softness of the gel prepared with the starch fraction from the ball-milled RSL.

According to the literature, increasing the proportion of protein aggregates in a composite starch-protein gel could result in a softer network in comparison to the pure starch gel. The result was attributed to the protein aggregates interfering with the formation of junction zones between the amylose molecules (Min et al., 2022) and could also explain the softer structure observed after ball milling for GWL and RWL gels.

Also, the formation of protein networks with starch granules acting as fillers was reported to decrease the tan δ (Lavoisier & Aguilera, 2019) but, this observation was made on composite systems with a low

proportion of starch and thus it could not be applied to the data of this thesis. Starch and proteins have been described as thermodynamically incompatible and form separate networks. However, a synergistic effect between those two networks has been found to lead to an interpenetrating network and the strengthening of the starch-protein system (Fan et al., 2017b) which could also explain the slightly lower tan δ of the RSL.

4.3.2.3 Microstructure of the pastes and gels prepared with ball-milled lentils

The changes in the structure of the ball-milled GWL, RWL, RSL gels in comparison to the control GWL, RWL, RSL gels upon cooling were further evaluated using a fluorescent microscope on the GWL, RWL, RSL pastes at 70 ^{o}C before the beginning of the rheological measurements and on the lentil gels after the end of the rheological measurements at 25 ^{o}C as seen in Figure 4.15.

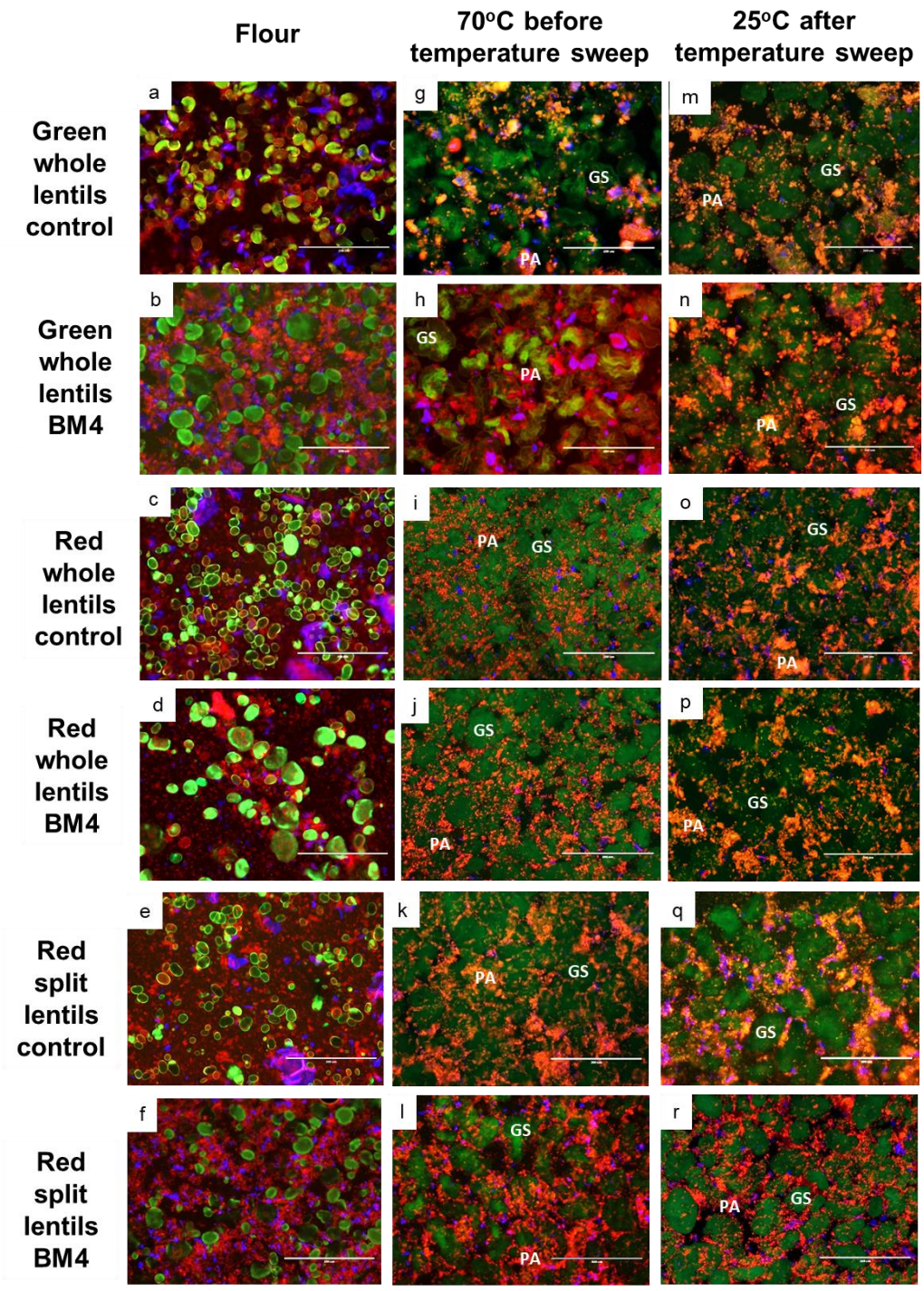


Figure 4.15 Fluorescent micrographs of the control and ball-milled lentil (a-f) flours, (g-i) before the temperature sweep at 70°C and (m-r) after the temperature sweep at 25°C. Scale bar; 200 µm. starch granules; green colour (stained with fluorescein isothiocyanate), proteins; red colour (stained with rhodamine B), fibres; blue colour (stained with calcofluor white). GS; gelatinised starch, PA, protein aggregate.

Oval-shaped starch granules (green colour), protein bodies (red colour) and fibres (blue colour) of many sizes were observed for all the control

lentil flours. The fluorescent micrographs of all lentil flours showed an increase in the particle size of the proteins after ball milling due to aggregation. Ball milling also affected the crystalline structure of the starch granules and increased cold water absorption which explains the observed increased size. The size of the fibres decreased after ball milling.

At 70 ^{o}C in both control, as seen in Figure 4.18g, I, k and ball-milled, as seen in Figure 4.18h, j, l, the GWL, RWL and RSL gels, swollen starch granules that had lost their granular integrity were observed. Protein was observed in the form of aggregates in both control and ball-milled GWL, RSL and RWL. The size of the protein aggregates was increased after ball milling supporting the observation of lower measured viscosity for all lentils (Figure 4.1a).

At 25 ^{o}C the ball-milled GWL and RWL showed similar networks under the microscope with the starch comprising the continuous phase of the gel network. The protein aggregates functioned as inactive fillers that disrupted the continuous starch network.

However, RSL showed a different microstructure after ball milling explaining the difference in the moduli in comparison to the GWL and RWL. The protein aggregates in the ball-milled RSL formed a protein network with starch showing a filler-like rather than a structural role. However, the oscillatory deformation tests showed that the moduli of the RSL were similar to the starch gel after ball milling indicating that the

starch comprised the continuous network and that an interpenetrating network was formed between the starch and proteins.

Further evaluation of the structure of the GWL, RWL, RSL gels after ball milling was conducted using a frequency sweep test.

4.3.2.4 Frequency sweep of the gels prepared with ball-milled lentils and RSL fractions

The pastes from the ball-milled GWL, RWL, RSL, starch and protein fractions extracted from the ball-milled RSL were cooled from 70 ^{o}C down to 25 ^{o}C using a rheometer. At 25 ^{o}C a frequency sweep test of 0.1% strain and within the range of 0.1 to 10 rad/s was applied to these gels and the results can be seen in Figure 4.16.

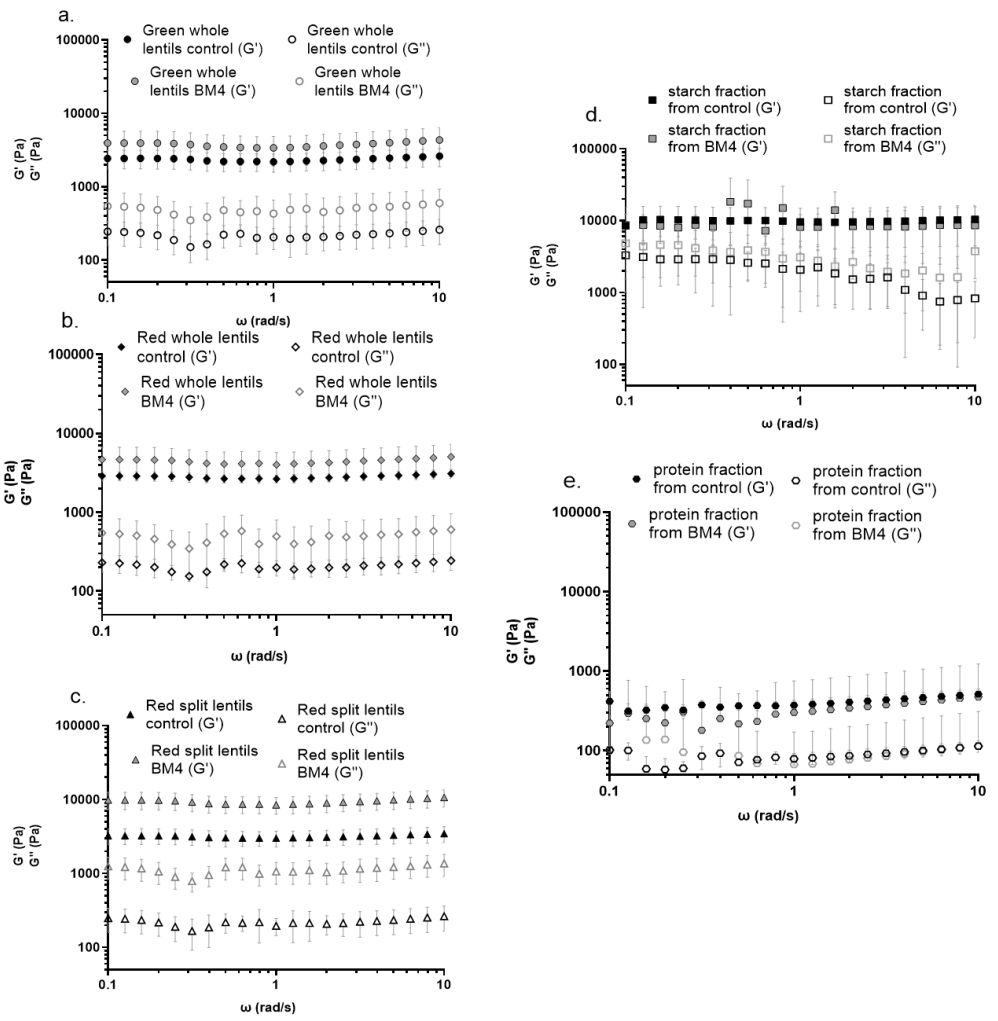


Figure 4.16 The impact of ball milling on the frequency sweep test of (a) Green whole lentils, (b) Red whole lentils, (c) Red split lentils, (d) starch and (e) protein fractions extracted from the control and ball-milled RSL at 25^{o}C and 0.1% strain within the range of 0.1 to 10 rad/s.

Ball milling did not change the solid-like nature (higher G' than G'') of the gels from GWL, RWL, RSL, and the RSL starch and protein fractions. Both moduli remained relatively constant after ball milling for the GWL, RWL, RSL gels between 0.1 to 10 rad/s indicating independence on frequency and the formation of true gels as it was observed for the gels prepared with the control GWL, RWL, RSL. The same result was observed for the moduli of the gel prepared from the starch fraction extracted from the control and ball-milled RSL. However, the gels

prepared with the protein fraction extracted from the ball-milled RSL showed a slight increase in G' and G'' with increasing frequency, indicating frequency dependence and the formation of weak gels.

Ball milling did not change the initial value of G' for GWL (from 2434.8 ± 614.4 Pa to 3938.2 ± 1584.4 Pa) and RWL (from 2876.7 ± 333.8 Pa to 4645.2 ± 1815.4 Pa) gels. However, ball milling changed the initial G' values of the RSL gel which were higher in comparison to the initial G' of the control RSL gel and increased from 3243.5 ± 709.9 Pa to 9897.1 ± 2335.1 Pa. Ball milling did not change the final G' value of the GWL and RWL but increased the final G' values of the RSL from 3458.3 ± 749.2 Pa to 10709.5 ± 2519.8 Pa in comparison to the control gels.

4.3.2.5 Visual observation of the gels prepared with ball-milled lentil flour

A new set of gels was prepared from the ball-milled GWL, RWL, RSL using a water bath for two hours after heating in the RVA and not the rheometer and stored in the fridge overnight (4^{o}C). Ball milling did not change the self-standing ability of the gels as can be seen from Figure 4.17.

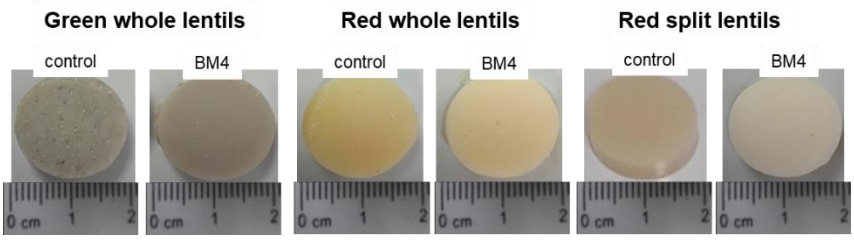


Figure 4.17 The appearance (macrostructure) of the gels prepared with the control and ball-milled Green whole lentils, Red whole lentils, and Red split lentils. The gels were prepared using a rapid visco analyser (RVA) and stored at 4 ^{o}C overnight before taking the pictures.

The photos were taken before conducting any textural or microscopical analysis. The self-standing ability of the gels was attributed to the short-term retrogradation of the amylose as amylopectin has been described as a long-term retrogradation.

The differences in the colours of the lentil gels (GWL, RSL, RWL) were naturally derived from the flours and were representative of the colour of each grain. GWL showed a green colour, RSL an orange colour and RWL a yellow colour.

4.3.2.6 Microstructural differences between the control and ball-milled lentil gels

The macrostructural observation of the gels did not show any differences in the shape or ability of the gels to stand after ball milling thus fluorescent microscopy was used to evaluate the microstructural appearance of the gels as seen in Figure 4.18.

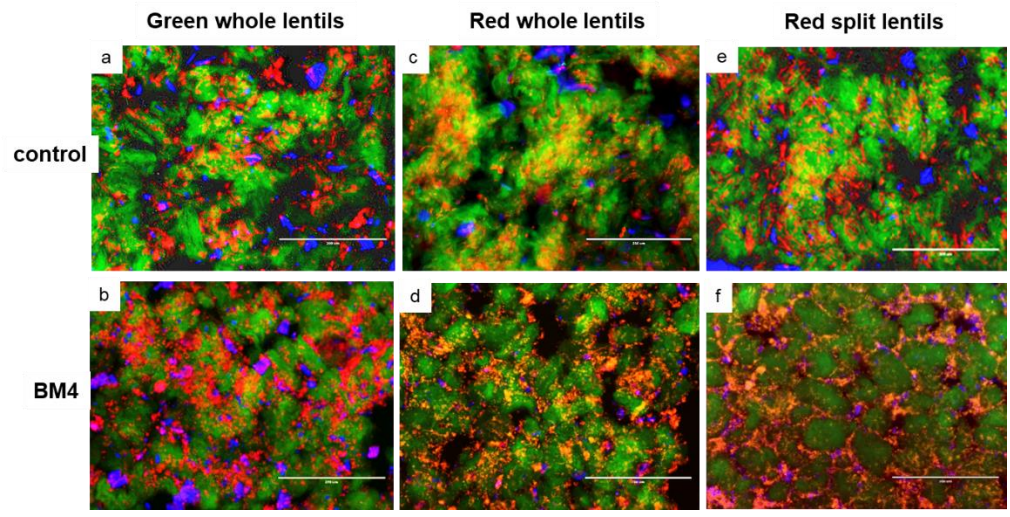


Figure 4.18 Fluorescent microscopy showing the microstructure of the gels prepared with the control and ball-milled (a, b) Green whole lentils, (c, d) Red whole lentils, (e, f) Red split lentils. Scale bar; 200 µm. starch granules; green colour (stained with fluorescein isothiocyanate), proteins; red colour (stained with rhodamine B), fibres; blue colour (stained with calcofluor white).

The gels prepared with control GWL, RWL, RSL displayed a green mass, identified as the gelatinised starch granules, which dominated the gel microstructure. Fibres and proteins did not form a continuous dominant network but functioned as a filler in the spaces within the starch network.

Ball milling did not change the microstructure of the gel prepared with the GWL showing a homogeneous starch network with proteins and fibres dispersed within the starch network. This observation was in line with the oscillatory tests showing no significant differences in the moduli of the gels prepared with the control and ball-milled GWL. The same observation was made for the gels prepared with the control and ball-milled RWL.

Ball milling slightly changed the microstructure of RWL gels indicating a higher interaction between the protein molecules in comparison to the control and showed a slightly less homogeneous and less continuous starch network.

Ball milling changed the starch-based network of the RSL gel and induced the formation of an interpenetrating network where proteins and starch acted synergistically which was in line with the higher moduli. These differences in the microstructure of the gels and the higher proportion of protein aggregates in the lentil flour after ball milling could impact the water-holding capacity and textural properties of the lentil gels.

4.3.2.7 Water holding of the gels prepared with ball-milled lentils

The impact of ball milling on the ability of the gels to retain water after centrifugal forces (5000 rpm for 30 min) is presented in Figure 4.19 for the GWL, RWL, RSL gels. The water loss of the gel during centrifugation is termed syneresis and is also a parameter to consider.

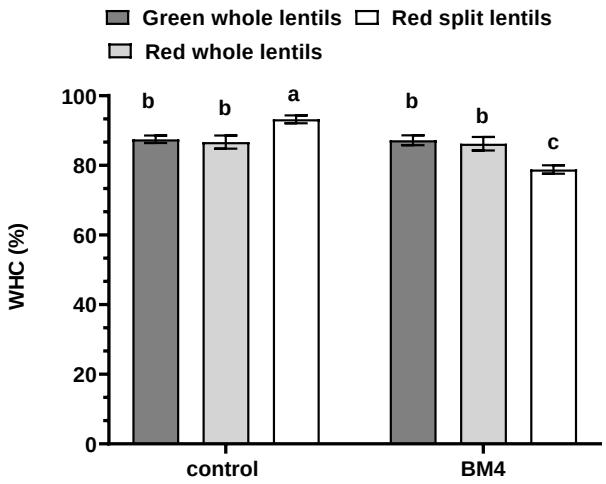


Figure 4.19 Water holding capacity (WHC) of the gels prepared with the control and ball-milled Green whole lentils, Red whole lentils, Red split lentils. Different letters represent a significant difference at $p<0.05$. BM4; four hours ball milling.

Ball milling did not change the ability of the GWL and RWL to retain more than 80% (water holding) of the initial gel volume, with 20% lost as water (syneresis) but, significantly decreased the ability of the RSL gel to retain water.

Water can be found in gels either bound to starch, proteins and fibres or entrapped within the gel network. Microstructure has been related to changes in the water holding of the gels (Lan et al., 2021). For example, water-holding capacity is linked to syneresis which occurs due to starch retrogradation in starch-based systems. However, in composite

systems, the proteins have been found to impair starch retrogradation and increase water holding (Joshi et al., 2014).

Ball milling enhanced the formation of protein aggregates in the lentil flours, however, did not change the microstructure of the GWL and RWL. Starch granules in the gels prepared with the ball-milled GWL and RWL were equally likely to retrograde in comparison to the control explaining the non-significant changes in the water-holding capacity of the control and ball-milled lentil gels.

However, protein aggregates in the RSL gel formed a network after ball milling indicating an increase in protein-protein interactions and a decrease in protein-water interactions. An increase in protein-protein interactions could decrease the proportion of proteins impairing starch retrogradation and thus increasing water expulsion explaining the decrease in the water retention ability of the gels prepared with the ball-milled RSL.

4.3.2.8 Textural properties of the gels prepared with the ball-milled lentils

A new set of gels was formed and the impact of the ball milling on the hardness and cohesiveness of the lentil gels was conducted at 25% strain, the value of which was selected so as not to significantly disrupt the lentil gel macrostructure (Figure 4.20).

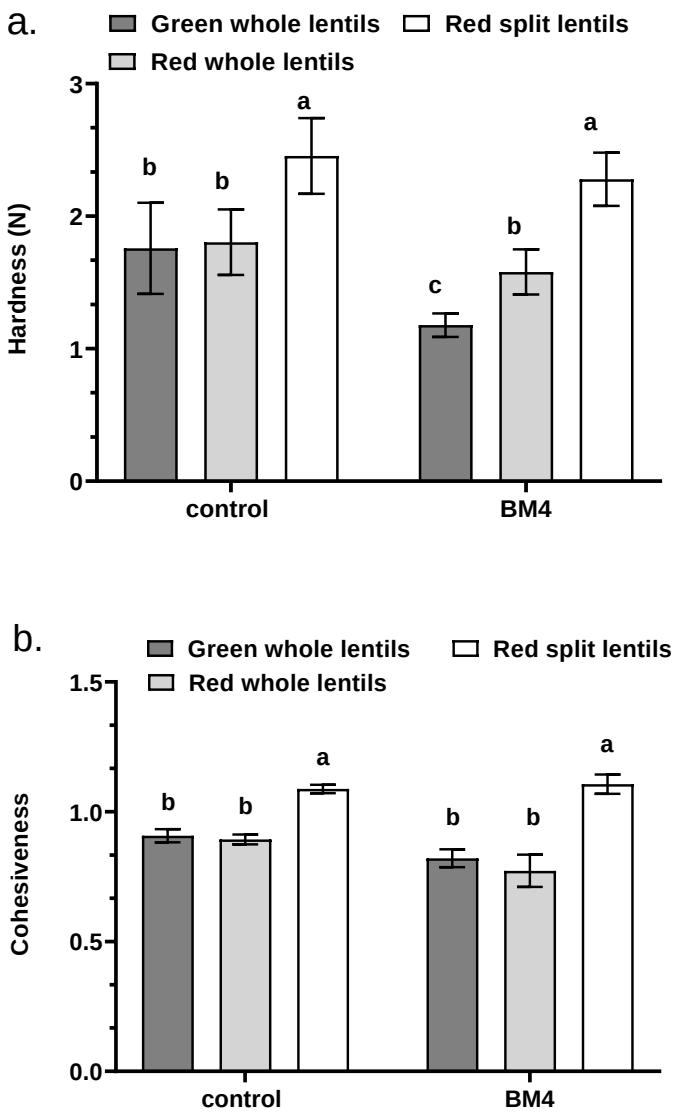


Figure 4.20: The impact of ball milling on the (a) hardness and (b) cohesiveness of the Green whole lentils, Red whole lentils and Red split lentils gels. All tests were conducted at 25% strain and 25^{o}C. Different letters represent a significant difference at $p<0.05$. BM4; 4 hours ball milling.

Ball milling did not change the hardness of the RWL and RSL gels as seen in Figure 4.20a however, decreased the hardness of the GWL gels.

The decrease in the hardness of GWL gel after ball milling could be attributed to the higher fibre content in comparison to the other two lentils. Ball milling decreased the particle size of the fibres in the lentil flour thus exposing polar groups and increasing the surface area and

water-binding sites of the fibres with the water thus increasing the water absorption of the GWL. Fibres do not gelatinise or aggregate after cooling but impact the proportion of starch and protein in the gel network. Moreover, the competition between fibres, starch and proteins for water molecules may lead to insufficient gelatinisation or denaturation and the formation of a weak gel network (Zheng et al., 2019).

Regarding the RWL and RSL, it was hypothesised that the strain used for this experiment was the reason for non-significant differences between the controls and the ball-milled gels and a higher strain was needed to be used to evaluate the textural properties of the gels.

The cohesiveness of the gels and their ability to retain their shape after the first cycle was also conducted to evaluate further the intermolecular interactions holding together the components in the gels after ball milling as seen in Figure 4.20b.

Ball milling did not change the cohesiveness of the lentil gels. The cohesiveness of all gels was found close to 1 indicating highly cohesive gels that experience an almost full recovery which was attributed to the non-destructive strain (25%) applied at the first compression.

The hardness and cohesiveness tests did not show any differences between the RSL and RWL thus to further evaluate the textural properties of the gels a fracture test was performed using a high deformation test by compressing at 80% strain where gels showed fracture as it can be seen in Figure 4.21.

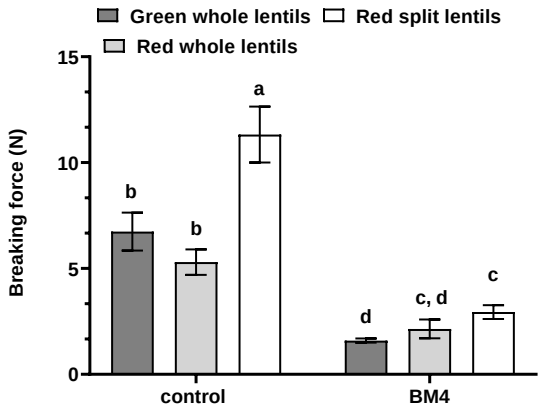


Figure 4.21 The impact of ball milling on the breaking force of the Green whole lentils, Red whole lentils, Red split lentils gels when gels were compressed at 80% of their height at 25 ^{o}C. Different letters represent a significant difference at $p<0.05$.; 4 hours ball milling.

Ball milling decreased the breaking force of the gels indicating a decrease in their ability to withstand rupture and the formation of a less ordered structure in comparison to the control. Between the lentils, RSL needed a high force to break followed by the RWL and last the GWL.

The ability of lentil gels to break under lower uniaxial force after ball milling was attributed to the non-starch components in the gels. Both fibres and proteins impair starch retrogradation decreasing the ability of the amylose molecules to re-associate and form strong gels. For example, proteins impair the network formation of starches by either interacting with the amylose and amylopectin or by acting as a barrier to their retrogradation (Joshi et al., 2014; Ribotta & Rosell, 2010).

4.4 Conclusion

The impact of ball milling on the gelation-forming properties of three types of lentils (green whole lentils; GWL, red whole lentils; RWL, red split lentils; RSL) was investigated. Both control and ball-milled lentils were subjected to heat-induced gelation under controlled conditions

using a rapid visco analyser. The impact of the ball milling on starch and protein gels extracted from the control and ball-milled RSL was also evaluated to further understand the role of the starch and protein on the lentil gels' microstructure, rheological and textural properties.

The pasting properties of all lentil flours decreased after ball milling due to the formation of protein aggregates acting as a barrier to further swelling of the starch granules.

During cooling from 70 ^{o}C down to 25 ^{o}C ball milling did not affect the viscoelastic properties of the GWL and RWL gels but increased the viscoelasticity of the RSL. The increased viscoelasticity of the RSL was attributed to the formation of an interpenetrating network supported by both protein and starch.

Fluorescent microscopy observations revealed a starch-based network after ball milling for all lentils but the role of the proteins in the RSL gel network was more prominent. Although the similarities in the microstructure of the control and ball-milled lentil gels, tan δ increased after ball milling indicating that protein-protein interactions reduced starch retrogradation and the formation of a less compact gel.

Ball milling did not affect the water-holding capacity of the GWL or RWL gels but decreased the water-holding capacity of the RSL gel. The more prominent role of proteins in the ball-milled RSL gels indicated that protein-protein interactions reduced protein-starch interactions thus leading to higher starch retrogradation and water expulsion from the gel network.

Textural properties showed that only the hardness of the GWL was decreased by ball milling due to starch dilution. A lower breaking force was needed to break the ball-milled gels confirming a reduced starch retrogradation in comparison to the control gels due to the disruption effect of the protein aggregates formed by the ball milling.

5 Chapter: Conclusion and Future Work

5.1 General conclusions

Lately, there has been a big interest in ‘clean-label’ ingredients for food formulation aiming at a healthier food, planet and people. Specifically, the market value and sales of plant-based foods increased compared to animal-based products due to a shift in the health-related habits of consumers. However, plant-based foods are often produced using refined ingredients and complex processing methods raising concerns about human health. Therefore, there is a need to shift to less complex food processes and the application of unfractionated ingredients in plant-based formulations.

This research investigated the minimal processing of unfractionated plant-based flours. Ball milling has recently attracted attention as a method of physical modification. In line with the literature most research has been focused on ball milling of fractionated food ingredients, such as starch and proteins but not on unfractionated flours. Two types of grains considered environmentally friendly (i.e., nitrogen fixation), and healthy (i.e., protein composition >10%) but, underutilised by the food industry named the lentils (RSL, GWL) and pearl millet (PM) were selected.

Microscopy, X-ray diffraction and differential scanning calorimetry revealed changes in the structure of the lentil starch granules. Ball milling reduced the rigidity of the amorphous rings and entanglement with the crystalline regions. Therefore, decreasing birefringence, the crystalline

diffraction peaks and leading to a greater hydrogen bonding between the hydroxyl groups of the amorphous regions and water molecules that decreased the initial gelatinisation temperature of the lentils' starch granules. Ball milling also induced changes in the lentils' protein behaviour. The water-soluble proteins decreased leading to the formation of protein aggregates observed under a fluorescent microscope and leading to lower hydrophobicity. However, the physical and microstructural properties of the PM did not show any significant impact by ball milling indicating a structure resistant to ball milling conditions used in this research. From these data, it is evident that ball milling is a promising method that can be used to modify the physicochemical properties of unfractionated lentil flours.

Having identified the impact of ball milling on lentils and observing the resistance of the PM, the impact of ball milling on the pasting properties of these flours in comparison to other legumes (YP) and cereals; Wheat (W) and Maize (M), was conducted. All legumes and W showed a lower viscosity after ball milling however, the pasting behaviour of the PM and M remained unchanged. Amylopectin is more susceptible to cleavage than amylose and the A-type crystallites have weak points (the a-1,6 linkages) that are highly affected by the ball milling forces. Considering the differences between the legume and cereal starches, three types of isolated starch material (A-, B, C-, type of crystallinity) were evaluated after ball milling. However, all different starches could not form a paste after ball milling and viscosity was seen at the bottom of the pasting graph.

The presence of other components (proteins, fibres) could have a protective effect on the level of damage to the starch granules caused by ball milling and breakage by heat and shear. The impact of proteins on the pasting of the legumes after ball milling was also evaluated by adding NaCl which solubilises the major class of proteins in lentils (salt-soluble globulins). However, NaCl addition did not change the viscosity of the lentils indicating that the aggregation of these proteins by the ball milling reduced the interactions with the NaCl molecules. Protein aggregates could form a layer inhibiting starch hydration, swelling and pasting.

This ability of the ball mill to alter the properties of unfractionated lentil flours indicated a change in the gelation mechanism. Many plant-based products such as plant-based yoghurts and cheeses are gels. These gels are produced using fractionated materials (mainly starch). The uniformity of fractionated material offers more control over the desired properties (i.e., thickening) of the final product in comparison to unfractionated flours, however, lacks nutritional value and sustainability. Therefore, the impact of ball milling on the lentil's gel formation was investigated using three types of lentils, red split lentils; RSL, higher carbohydrate content, red whole lentils; RWL, higher protein content and green whole lentils; GWL, higher fibre content. The lentil flours were dispersed in water and heated to 95 ^{o}C in an RVA to induce heat gelation.

The rheological properties of the lentils during cooling were compared to fractionated starch and proteins extracted from RSL to understand the role of these components in gel formation. The textural, water-holding,

and microstructural properties of the self-standing gels after overnight storage in the fridge were also evaluated.

The microstructural data during cooling indicated the formation of a starch network prior to the formation of a protein network in the control gels. This result was in line with the thermal properties. Starch gelatinisation (≈70^{o}C) occurred first, and protein denaturation (≈87^{o}C) followed. The G' and G'' values of the control lentil gels were closer to the G' and G'' of the fractionated lentil starch indicating a higher role of the starch on the gel network compared to the proteins.

The control RSL formed a more viscoelastic and strong gel in comparison to the other lentils. The higher protein content in RWL and higher fibre content in GWL in comparison to RSL could destabilise the junction zones of the starch molecules. Therefore, the composition could be used to predict the texture of the gels produced from control flours.

Ball milling initiated the formation of two gel microstructures, a starch-based and an interpenetrating network. The ball-milled GWL and RWL formed starch-based gels with larger protein aggregates compared to the control. Ball milling did not change the viscoelasticity of the RWL or GWL samples. However, the tan δ curve, showed a plateau after 50 ^{o}C indicating an impact of the protein aggregates to gelation by restricting further the amylose molecules retrogradation.

The viscoelasticity of RSL increased after ball milling indicating the formation of a stronger gel. Comparing the rheological data of the ball-milled RSL with the fractionated ingredients revealed starch rather than

protein domination. The impact of proteins on ball-milled RSL was evidenced by tan δ as a plateau after 50 ^{o}C. It was concluded that proteins formed a network at the same time as the gelatinised starch granules. An interpenetrating or two-phase network dominated by starch was formed.

The formation of such networks was linked to the water-holding capacity. Both control and ball-milled GWL and RWL formed starch-based networks with the same water-holding capacity. However, the interpenetrating network formed after ball milling of the RSL decreased the water-holding capacity of the RSL gel network. In this case, the protein-protein interactions increased and protein-water interactions decreased thus increasing starch retrogradation and water expulsion.

Ball milling did not change the hardness of RSL and RWL gels due to the low strain (25%) used in this experiment but, decreased the hardness of the GWL in line with the lower viscosity and tendency to retrograde after ball milling. Ball milling decreased the value of the force needed to break the structure of all lentil gels at higher strain (80%). This evidence was in line with a higher tan δ caused by the disruption of the starch-based network by the protein aggregates.

To conclude, the minimal processing of unfractionated flours using ball milling is promising in terms of ingredient preparation. Ball milling could be used as an alternative method to minimise time and energy-consuming processes such as ingredient fractionation. Moreover, differences in the gel microstructure and gel texture between the ball-

milled GWL, RWL, RSL could impact the mouthfeel allowing for more customisation in product development. In general, the ball-milled lentil could be used as a more nutritious ingredient in product development eliminating the consumers' chemical exposure when consuming plant-based products.

5.2 Future work

Further investigations on the gels produced with lentil flours need to be conducted including:

- Gels contain substantial amounts of fluids (i.e., water) that influence their stability, appearance and texture. For example, the fluid separation in yoghurt is unappealing. Texture evolution through storage should be conducted to identify optimal storage conditions and minimize undesirable changes.
- The traditional animal-based products have distinct sensory characteristics. Consumers are increasingly seeking out plant-based products but do not want to compromise on taste, texture and nutrition. For example, the beany flavour of legumes constrains their consumer acceptance thus, further research should be conducted on reducing or masking any undesirable aroma, flavour and mouthfeel.
- The properties (molecular, chemical, and physical) of plant-based ingredients differ from those of animal-based ones. As an example, caseins found in milk have anionic phosphate groups that can be linked by calcium anions playing an important role in their ability to form gels (i.e., yoghurt, cheese). It is critical to

further understand the fundamental gelation properties (gelation mechanism and types of interactions) of the lentil flours so they can be assembled into structures resembling those found in animal products.

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